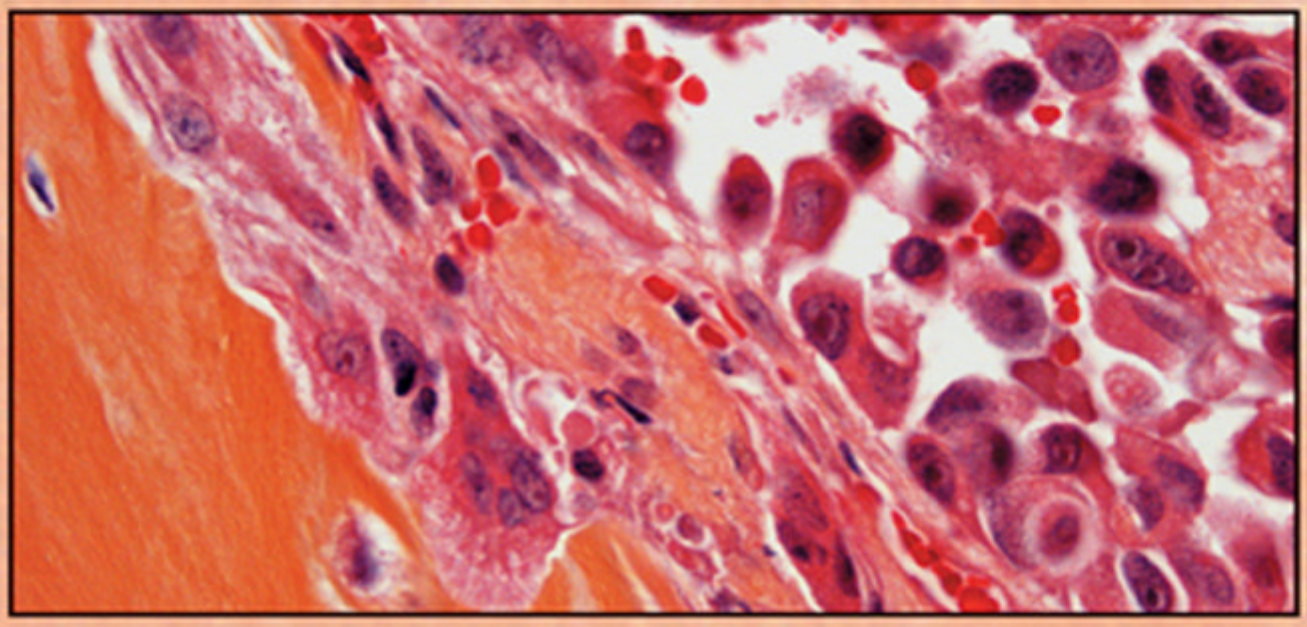
CANCER METASTASIS

Biologic Basis and Therapeutics



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CANCER METASTASIS

Metastasis is responsible for a large burden of morbidity and mortality among cancer patients; currently, however, few therapies specifically target metastatic disease. Further scientific dissection of the underlying pathways is required to pave the way for new therapeutic targets. This groundbreaking new text comprehensively covers the processes underlying cancer metastasis and the clinical treatment of metastatic disease. Whereas previous volumes have been compendia of laboratory research articles, the internationally renowned authors of this volume have summarized the state-of-the-art research in the metastasis field. A major section covers the cellular and molecular pathways of metastasis and experimental techniques and the systems and models applied in this field. Subsequently, the clinical aspects of the major cancer types are considered, focusing on disease-specific research and therapeutic approaches to metastatic disease. The focus is on novel pathophysiological insights and emerging therapies; future directions for research and unmet clinical needs are also discussed.

David Lyden is the Stavros S. Niarchos Chair and Associate Professor of Pediatrics and Cell and Developmental Biology at Weill Cornell Medical Center in New York, a pediatric neurooncologist at Memorial Sloan-Kettering Cancer Center, and faculty member of the MD/PhD program at the Tri-Institute (Rockefeller University/Weill Cornell Medical College/Memorial Sloan-Kettering Cancer Center). Dr. Lyden was recently elected to be an investigator for the Champalimaud Metastasis Center, the first of its kind for research, prevention, and treatment of metastatic disease. His honors and awards include the Princess Takamatsu Lectureship Award (2006) and the Leonard Weill Memorial Lecturer Award (2007). In 2007, Dr. Lyden received the Presidential Medical Distinction Award from President Cavaco Silva of Portugal.

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Bethan Psaila is a Research Fellow at the Imperial College of Medicine in London and Weill Cornell Medical College in New York. Dr. Psaila is the recipient of a Fulbright Scholarship in Cancer Research and a Kay Kendall Leukaemia Fund Travelling Fellowship.

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This book is dedicated to all advanced cancer patients and their families with the hope of inspiring a brighter future with improved treatment of metastatic disease.

– David Lyden, Danny R. Welch, and Bethan Psaila

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Overview: Biology Is the Foundation of Therapy

After a diagnosis of primary cancer is established, the urgent question is whether the cancer is localized or if it has already spread to the regional lymph nodes and distant organs. Despite improvements in surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer still result from the progressive growth of metastases that are resistant to conventional therapies.

The process of tumor metastasis is highly selective and consists of a series of sequential, interrelated steps, all of which must be completed by metastatic cells to produce clinically relevant lesions. After the initial transformation and growth of cells, vascularization must occur if a tumor mass is to exceed 1 mm in diameter. Next, local invasion of the host stroma occurs as a consequence of the enhanced expression of a series of enzymes such as collagenase. After the invading cells penetrate the lymphatic or vascular channels, they may grow there, or a single cell or clumps of cells may detach and be transported within the circulatory system. The tumor emboli must survive immune and nonimmune defenses and the turbulence of the circulation, then lodge in the capillary bed of receptive organs, extravasate into the organ parenchyma, proliferate, and establish a micrometastasis. Finally, the growth of these microscopic lesions requires development of a vascular supply and evasion of host defense cells.

The search for factors that regulate metastasis began in 1889 when Paget analyzed postmortem data of women who died of cancer and noticed the high frequency of metastasis to the ovaries and the different incidence of skeletal metastases associated with different primary tumors. Paget concluded that the organ distribution of metastases is not a matter of chance and suggested that metastases develop only when certain tumor cells are compatible with specific organs. These findings contradicted the prevailing theory proposed by Virchow that metastasis can be explained merely by the lodgment of tumor cell emboli in the vasculature. Paget concluded that "remote organs cannot be altogether passive or indifferent regarding embolism" and provided the everlasting "seed-and-soil" principle. In 1929, James Ewing challenged Paget's theory and proposed that metastatic dissemination occurs by purely mechanical factors that are determined by the anatomical structure of the vascular system. Ewing's viewpoint prevailed for decades.

In the 1970s, the selective nature of metastasis was demonstrated by Fidler and the biologic heterogeneity of neoplasms was reported by Fidler and Kripke. In the 1980s, Hart and Fidler provided the definitive proof of Paget's hypothesis by demonstrating that although tumor cells reached the vasculature of all organs, metastases developed only in compatible organs. Collectively, these studies established three key principles. First, neoplasms are biologically heterogeneous. Second, the process of metastasis is highly selective, favoring the survival and growth of a small subpopulation of cells that preexist in the heterogeneous parent neoplasm. Third, the outcome of metastatic growth depends on multiple interactions of metastatic cells with host homeostatic mechanisms that the tumor cells can usurp.

The following chapters provide the reader with an intelligent and detailed analysis of the various steps in the pathogenesis of metastasis. It is most encouraging to discover that the process can now be studied on the systemic, cellular, and molecular levels. Understanding the mechanisms that regulate metastasis must continue to be a primary goal of cancer research. Only from a better understanding can we design better approaches to therapy of this fatal phase of cancer. Succinctly stated: *Biology is the foundation of therapy.*

Isaiah J. Fidler, DVM, PhD

Introduction to Basic Research

Harold L. Moses

Since Stephen Paget's "seed-and-soil" theory was published in 1889, a wealth of research has focused on the cascade of events involved in the spread of cancer cells from the primary tumor to secondary organs. As Paget highlighted, in addition to the intrinsic properties of metastatic cancer cells, features of the microenvironment in target organs of metastasis are also critical for successful tumor dissemination. Over the past century, metastasis research has focused predominantly on the genetic and phenotypic properties that confer the "seed" with a migratory and invasive phenotype. More recently, the contributions of cells, the extracellular matrix, and secreted factors in the metastatic microenvironment have gathered attention. In addition, although it was traditionally thought that metastasis occurred as a late event during tumor growth, there are now several lines of evidence to suggest that the onset of metastatic progression occurs early during carcinogenesis. The contributors to this book have made seminal contributions toward furthering our understanding of the molecular and cellular pathways in tumor dissemination. As outlined here, their chapters highlight the key scientific advances as well as the modern models and tools for studying metastasis.

The first four chapters focus on the state-of-the-art models and systems employed in metastasis research. In Chapter 1, Janet E. Price describes animal models of metastasis. Such in vivo approaches have distinct advantages over in vitro assays, allowing real-time study of the multistep processes of metastasis in its physiological context. Even so, there are limitations to the application of animal models, such as the relatively low number of tumor cell lines that are available. In addition, animal models often use immunodeficient hosts, thereby eliminating important immune cell and stromal cell contributions to the metastatic process. The development of improved models of metastasis using both cell lines and genetically engineered models is required. In Chapter 2, Elisa C. Woodhouse and Kathleen Kelly discuss the advantages of studying metastasis with genetic models in *Drosophila* and zebrafish. The advantages of these models are to rapidly generate mutations in vivo and specifically examine their effects on the metastatic process. In Chapter 3, Wayne S. Kendal provides an alternative approach to studying metastasis, describing how mathematical models executed by computer may be used to simulate complex biological systems. This approach enables predictions of system behavior, testing of hypotheses, the understanding of complex data, and the development of new hypotheses. In Chapter 4, Cristina Hidalgo-Carcedo and Eric Sahai describe the use of intravital imaging, the high-resolution optical sectioning of live tissue that provides unique, real-time insights into events occurring within tumors.

Genetic studies remain the predominant focus of cancer research; comparing the genetic characteristics of metastatic tumor cells with those of primary tumor cells remains a relatively new field of study. Devanand Sarkar and Paul B. Fisher explore these studies in Chapter 5. The targeting of specific genetic pathways identified by these studies may prevent seminal steps in the metastatic process, including extravasation, survival in the bloodstream, intravasation, and/or growth at a new organ site. In Chapter 6, Brunilde Gril and colleagues discuss metastasis suppressor genes, which prevent spontaneous metastasis without affecting primary tumor growth. A common trait of highly metastatic tumors is their ability to adapt the topology of local and distant microenvironments to better aid their progression. In Chapter 7, Bedrich L. Eckhardt and coworkers review the role of the stroma during metastatic progression and highlight that the propensity to metastasize to certain organs requires homing mechanisms that involve specific ligand/receptor interactions. They discuss the use of phage-display technology to discover novel endothelial markers that may be used to disrupt tumor progression and metastasis. In Chapter 8, Amaia

Lujambio and Manel Estellar discuss epigenetic mechanisms, including DNA hypo- and hypermethylation and aberrant histone modifications, that lead to metastasis-promoting genes. Adding to the complexity, microRNAs can also be regulated by epigenetic mechanisms and they can simultaneously regulate hundreds of target genes. These studies suggest that epigenetic therapies, such as DNA demethylating agents or histone deacetylase, may be powerful tools in the control and prevention of metastatic disease.

Host factors have considerable impact on metastatic outcome. In Chapter 9, Nigel P. S. Crawford and Kent W. Hunter state that metastatic progression is influenced by host germline variation and describe susceptibility genes facilitating this process. Despite the accumulated somatic mutations within a tumor, the inherent ability of any tumor to disseminate is also influenced by host genetics. In Chapter 10, Futoshi Okada and Hiroshi Kobayashi focus on host age-associated factors and social environment factors that modulate tumor development and metastasis.

Leonard Weiss introduced the concept of metastatic inefficiency, which often involves survival and cell death upon entry and circulation in the lymphatic and hematogenous routes during tumor cell invasion and metastatic progression. In Chapter 11, Lilian Soon and colleagues discuss the steps involved in epithelial-to-mesenchymal transition and the reverse mesenchymal-to-epithelial transition, introducing the concept that hybrid cells equipped for both systems are involved in metastasis. Deportation of tumor cells from the primary tumor mass often results in apoptosis, anoikis, and senescense in the circulation and metastatic microenvironments; in Chapter 12, Wen Liu and Kounosuke Watabe focuses on tumor cell survival and cell death. The study of tumor cell entry into lymph nodes is arguably the least well understood, as research has traditionally been hampered by a lack of markers that distinguish blood vessels from lymphatic ones. However, lymphatic-specific molecular markers and growth factors have now been recognized. In Chapter 13, Ann F. Chambers covers the important topic of metastatic inefficiency and tumor dormancy, suggesting that tumor cells can coexist in a viable state for many years and, in certain cases, go on to progress as late-developing metastatic disease. She also ascertains the idea of the possibility of treating dormant disease.

In 1863, Rudolf Virchow first proposed that inflammation contributes to disease processes, including cancer growth. Despite Virchow's early observation of leukocytes in malignant tissues, the involvement of stromal cells and extracellular matrix constituents in metastatic progression has, until recently, been poorly understood. In Chapter 14, Sunhwa Kim and Michael Karin describe the role of intrinsic and extrinsic mediators, such as toll-like receptors and heat shock proteins, in regulating inflammation and immune responses in tumors. In Chapter 15, Steven Mason and Johanna A. Joyce discuss the multiple roles of proteases at the primary tumor site, during intravasation into the blood or lymphatic circulation and extravasation at secondary sites. In addition to their role in the degradation of the basement membrane and extracellular matrix, proteases are important for cell signaling in both cancer cells and microenvironmental stromal cells. In Chapter 16, Barbara Fingleton specifically reviews the role of matrix metalloproteinases, a family of proteolytic enzymes that act as potent regulators of cell growth, death, and chemotaxis. In Chapter 17, Hector Peinado, Bethan Psaila, and David Lyden discuss the contribution of cell-membrane–derived vesicles in the crosstalk between tumor cells and other cell types. First described in megakaryocytes and platelets, microvesicles are now known to have multifunctional roles in coagulation, immune regulation, intercellular crosstalk, and molecule delivery, potentially supporting tumor invasion and metastasis. In Chapter 18, Marianna Papaspyridonos, David Lyden, and Rosandra Kaplan discuss the cellular and molecular context at the premetastatic niche, describing the development of a receptive microenvironment that is permissive for the engraftment and growth of metastatic tumor cells. They and others have described the contributions of bone-marrow–derived progenitor cells, fibroblasts, and factors including fibronectin, lysyl oxidase, and the S100 proteins to premetastatic and metastatic niches.

Factors and particles secreted by the cells within the primary tumor may have both local and systemic effects. Therefore, the earliest events in target organs of metastatic spread may occur even prior to the arrival of disseminating tumor cells; these events are an important area of investigation for understanding metastatic progression. In Chapter 19, Suzanne A. Eccles provides a comprehensive discussion of how soluble or cell-bound growth factors and their receptors contribute to the process of site-selective metastasis. Because metastasis suppressors generally regulate the rate-limiting steps of metastatic formation, they make attractive targets for molecular therapies. In Chapter 20, Yibin Kang discusses organotropism in metastasis, highlighting the molecular interactions between tumor cells and their microenvironment. In Chapter 21, Julio A. Aguirre-Ghiso, Daniel F. Alonso, and Eduardo F. Farias discuss the protease uPA and its receptor uPAR, which is involved in tissue remodeling, enabling tumor cell dissemination and metastasis development. In Chapter 22, Tara Karnezis and colleagues address the three pathways for tumor cell dissemination, which include direct invasion of surrounding tissues and hematogenous and lymphatic metastasis.

Several aspects of the metastatic process remain mysterious. For example, organotropism and the transportation mechanism remain unclear; questions include why some cancers spread through the lymphatic system, whereas others metastasize by a hematogenous route. The regulation of tumor dormancy is not well understood. The role(s) of the extracellular matrix and its physical properties, such as stiffness, as well as the involvement of inflammatory cells in matrix regulation, need further exploration.

To conclude, basic research in metastatic disease has reached an exciting time. Our knowledge and understanding of long-standing scientific theories, as well as entirely new paradigms, have been expanded using the modern scientific approaches described in these chapters. By encouraging specific emphasis on the process of tumor dissemination, we hope that improved and new approaches may be developed to predict, prevent, and treat metastatic disease.

1 Animal Models of Cancer Metastasis

Janet E. Price

The invasive and metastatic abilities of malignant cells comprise one of the key "hallmarks of cancer" [1]; metastasis is the principal cause of death of the majority of patients diagnosed with invasive cancer [2]. Pathologists have long known that metastasis is not a random process, and that certain cancers have distinct patterns of metastasis to different organs [3]. The predictability of the organ distribution patterns of breast or lung cancers, for example, indicates that the development of distant tumors is a function of interactions between the disseminating cells and the sites of the metastases. This, in essence, is the "seed and soil" hypothesis presented by Stephen Paget in 1889 [4]. More than a century later, researchers continue their efforts to identify molecular mechanisms for the patterns of metastasis that are characteristic of different types of cancer. A common goal of research into the basic mechanisms is to find new insights into ways to prevent or control metastatic disease.

Metastasis can be viewed as the most difficult cancer phenotype to simulate and thereby study using in vitro techniques. Several tissue culture traits have been identified as potential indicators of metastatic potential, notably invasion through a basement membrane [5] and growth in semisolid agarose [6]. Development of three-dimensional tissue bioreactors – for example, with osteoblasts or hepatocytes – allows study of interactions of metastatic cells in bone and liver [7, 8]. However, these and other in vitro assays generally can evaluate a cancer cell's performance of only a single step in the multistep process of metastasis. Thus, animal models have become standard systems for analyzing molecular mechanisms of metastasis and for evaluating antimetastatic therapies. The majority of such studies have used rodents, predominantly mice. Some reasons for this are the availability of inbred and immunodeficient strains, small size and relative affordability (compared with larger species), and the development of genetically engineered mouse (GEM) models.

The transplantation of cell lines established from animal and human tumors into syngeneic or immunodeficient host animals, respectively, is the basis for most experimental studies of cancer metastasis. Established cell lines of human and animal tumors that are commonly used for metastasis studies can provide reliable and reproducible numbers and distribution patterns of metastasis. These models can be used to generate information of the metastatic phenotype that could not be obtained using in vitro techniques – for example, identifying gene expression profiles reflecting the propensity to metastasize to different organs [9–12].

One of the shortcomings of using transplantable tumor models, however, is that there are relatively few cell lines, especially of human cancers, that are reliably metastatic. So much of what has been learned from experimental animal models has come from investigations using a small number of cell lines, which do not reflect the heterogeneity of human cancers. Another limitation of xenograft models with human cell lines is the requirement to use immunodeficient host animals, which lack human stromal elements and immune cells that may contribute to metastatic progression [13].

Transgenic and GEM tumor models can provide alternatives that may overcome some of the shortcomings of transplantable models, notably in providing immunocompetent systems [14, 15]. Not all GEM tumor models are suitable for metastasis research, although the increasing sophistication of the types of genetic modifications being introduced into transformed cells and/or stromal cells will likely increase the use of GEM models for analyses of metastatic progression [16–19]. The use of tissue grafts for studying species-specific tumor–stroma interactions [20, 21] as an approach for overcoming the lack of appropriate stroma interactions is discussed later in this chapter. Continuing efforts designed to develop new models of metastasis, using traditional transplantable cell lines as well as GEM models, will provide additional

TABLE 1.1. Rodent models of cancer metastasis

Cell line	Host strain	Sites of metastasis	References
Mouse tumors			
B16 melanoma	C57BL/6	Lungs, lymph nodes, brain, ovary, liver	[25–28]
CT-26 colon carcinoma	BALB/c	Liver, lungs	[29, 30]
K1735 melanoma	C3H/HeN	Lungs, lymph nodes, heart, brain	[31, 32]
Lewis lung carcinoma 3LL	C57BL/6	Lungs, liver	[33, 34]
Mouse mammary tumor lines 66,67,168, 410.4, and derivatives	BALB/c	Lungs, liver, lymph nodes, bone	[35, 36, 9]
Rat tumors			
Dunning rat prostate lines	Copenhagen	Lung, lymph nodes	[37, 38]
13762NF mammary adenocarcinoma	Fischer 344	Lungs, lymph nodes	[39, 40]

Notes: Some transplantable rodent tumor cell lines that are commonly used for metastasis research. The sites where metastases develop may depend on the route of inoculation of the cells (also see Table 1.3).

resources for research into the molecular mechanisms of metastasis, as well as preclinical models for testing antimetastatic therapies [22, 14].

SYNGENEIC TUMOR MODELS

Inbred strains of rodents have provided the foundation for a large body of cancer research. The development and introduction of inbred mouse strains began in the United States in the early decades of the twentieth century, resulting in well-characterized strains that are used for studying the initiation and progression of autochthonous tumors and as recipients for transplantable tumors [23, 24]. Transplantable cell lines developed from tumors arising in inbred laboratory rodents, or induced by carcinogenic treatments, have proved invaluable for metastasis research. Some examples of cell lines that are widely used by the metastasis research community are shown in Table 1.1. Many of the basic principles of the pathobiology of metastasis have come from experimental studies using these and other well-characterized, transplantable tumor cell lines [25, 26, 33, 35].

The introduction of transgenic GEM models has extended the opportunities for studying the roles of specific genes in tumor initiation and progression [15]. A variety of GEM models that simulate different human cancers has been described, with one advantage over xenograft models of generating tumors in immunocompetent animals. The targeted mouse models of cancer may provide valuable tools for future preclinical screening of new therapeutic strategies [15, 22]. Some, but by no means all, GEM models of cancer show consistent and reproducible progression to metastasis [16, 41–43]. One notable example is the MMTV-polyoma middle T antigen (PyVmt) model, with mice producing multifocal mammary adenocarcinomas with relatively short latency, along with metastasis to lungs and lymph nodes [44]. This model has been used in a number of studies to identify genes that contribute to, or can modify, the metastatic phenotype [45]. For example, crossing the MMTV-PyVmt mice with RhoC-deficient animals demonstrated that RhoC expression was not essential for tumor formation, but was required for efficient metastasis [46]. Breeding the MMTV-PyVmt mice with twenty-seven different inbred strains of mice identified thirteen strains for which the F1 hybrid mice had significantly reduced metastatic burden, suggesting the presence of genetic modifiers of metastasis in these strains [47]. This led to the identification of polymorphisms of *Sipa,* a signal transduction molecule, as a regulator of metastasis [48].

The introduction of inducible or conditional promoters in GEM models can help identify molecular mechanisms of tumor progression and metastasis [16]. A doxycycline-inducible *Wnt1* transgenic model of mouse mammary tumors demonstrated that growth of the tumors and metastases was dependent on continued signaling through the Wnt pathway. Progression of tumors to become Wnt-independent and grow in the absence of doxycycline was facilitated by the loss of one wild-type *p53* allele [49].

Not all GEM models are suited for preclinical testing for a number of reasons, including complexity of breeding schemes, extended or variable tumor latency, and variable times to progression to metastasis. The multifocal nature of tumors may also limit the usefulness of transgenic mice for preclinical testing, or for investigations of the metastatic phenotype, if the mice need to be euthanized as a result of large primary tumor burden before metastases are evident. One approach to overcome this problem is to transplant GEM tumors into syngeneic, nontransgenic mice. This can generate a cohort of age-matched animals with comparable tumor

burdens. With several MMTV-driven mammary tumor GEM models, the incidence of metastasis from transplanted tumors was comparable with that seen in the donor mice [50].

Another limitation of transgenic GEM models of cancer, which is shared with conventional mouse transplantable tumors, is that these models generally do not simulate the metastatic patterns of the equivalent human cancer. For example, mouse mammary tumor models commonly metastasize to lungs and lymph nodes, but metastases to other visceral organs, brain, or bone – all common sites of human breast cancer metastasis – are only rarely reported [45, 51].

XENOGRAFT MODELS

A variety of immunodeficient strains is available for xenograft studies, with the athymic (also known as "nude") and severe combined immunodeficient (SCID) mice used most widely. Additional mutant strains, such as *bg* with reduced natural killer (NK) cell activity, or recombination activation gene-2 (RAG-2)-deficient mice, lacking mature B and T lymphocytes, may be crossed with the nude or SCID background. Some studies also add sublethal X-irradiation, treatment with chemotherapeutic drugs, or antibodies to asialo GM1 to deplete NK cell activity for suppression of residual immune function [10, 52, 53]. Comparisons of tumor growth and metastasis in different strains of immunodeficient mice have shown different results in different strains; some, but not all, studies have found increased tumor growth or metastasis in the more severely immunocompromised animals [54–57]. Obviously, the successful use of immunodeficient mice for human xenograft studies requires the availability of specific pathogen-free barrier facilities and adherence to careful animal husbandry protocols.

Early enthusiasm for injecting human tumors into immunodeficient mice was somewhat dampened by the realization that not all established tumor specimens or cell lines will grow, let alone metastasize, from subcutaneous (sc) injection [58, 59]. One approach that has been demonstrated to increase tumor take rates and frequency of metastasis is the injection or implantation of cells into anatomically appropriate tissues, known as orthotopic injection; the use of orthotopic models for human cancer metastasis will be discussed in more detail in a later section. The success rate of xenografting human tumor samples can also vary depending on the type of cancer. Melanoma, sarcomas, and colon cancers have been reported to engraft with a relatively high frequency, whereas the success rate with breast and prostate cancer specimens may not exceed 10 percent [60]. However, the tumor specimens that do grow, and in some cases metastasize, may represent the more aggressive phenotypes [61, 62].

Another factor that may limit the success of xenografting fresh tumor specimens is that only a small proportion of the cells isolated from the sample, which will be mixtures of tumor and stromal cells, have the ability to grow when implanted into immunodeficient mice. When populations of cells expressing putative tumor stem cell markers, such as $CD133^+$ for colon cancer and $CD44^+CD24^{-/low}$ for breast cancer, were isolated from fresh tumor samples, these selected populations had much greater tumorigenic potential in immunodeficient mice than did nonselected cells [52, 53]. Combining the isolation of $CD133^+$ cells from fresh tumor specimens of glioma and medulloblastoma with orthotopic implantation into mouse cerebellum and cerebrum, respectively, was found to be an effective procedure for preserving the $CD133^+$ tumor subpopulations through repeated in vivo transplantations [63].

STROMAL INTERACTIONS IN REGULATING TUMOR GROWTH AND METASTASIS

One criticism of transplantable tumor models in nonorthotopic sites, especially with human tumor xenografts, is the lack of stromal elements of a tumor microenvironment derived from the appropriate tissue [64]. The addition of reactive stromal cells and carcinoma-associated fibroblasts has been used to enhance the tumor take and growth of human tumor cell lines [65]. Co-injection of tumor cells with Matrigel, a mixture of basement membrane components, can increase tumor take and enhance tumor growth rates [56, 66]. The addition of the stromal cells and matrix proteins may stimulate the local release of cytokines and factors that contribute to improved vascularity, and hence improved growth of the tumors [67]. Bone-marrow–derived mesenchymal stem cells are recruited to the stroma of transplanted tumors [68] and can contribute to the growth and metastatic phenotype of human xenografts. The enhancement of metastasis from human breast cancers growing in SCID mice was dependent on signaling through the CCR5 chemokine receptor on the cancer cells, in response to CCL5 expressed by the co-injected mesenchymal stem cells [69].

The contributions of stromal-derived factors to malignant progression have been elegantly illustrated using GEM models in which the stromal factor has been depleted or removed by gene "knockout" approaches. This strategy was used to demonstrate that host-derived matrix metalloproteinase 9 (MMP-9) plays a significant role in the angiogenesis and tumorigenicity of pancreatic and ovarian cancers [70, 71]. Injecting the same number of the cancer cells into wild-type mice or MMP-9–deficient mice resulted in reduced tumor growth in the deficient host animals. Adoptive transfer of

TABLE 1.2. Orthotopic models of human cancer growth and metastasis

Cancer	Injection site	Sites of metastasis	References
Bladder	Bladder wall	Lymph nodes, lungs	[77]
Breast	Mammary fatpad	Lymph nodes, lungs	[78, 79]
Colon	Cecum wall	Lymph nodes, liver	[80, 81]
Gastric	Stomach wall	Lymph nodes, liver	[82]
Lung	Intrabronchial, or injection into lung	Dissemination in lungs, regional lymph nodes	[83, 84]
Melanoma	Dermis	Lymph nodes, lungs, brain	[76, 85]
Pancreas	Distal pancreas	Liver, lymph nodes	[86, 87]
Prostate	Prostate gland	Regional lymph nodes	[88]
Renal cell	Renal subcapsule	Lungs	[89]
Thyroid	Injection into thyroid	Lungs, invasion of larynx and trachea	[90]

Note: Examples of different routes of injection of human tumor cells into appropriate organ site to generate orthotopic models, and sites where metastases may be found.

wild-type bone marrow into MMP-9–deficient mice partially restored the impaired tumor growth, indicating that the marrow-derived cells contribute to the tumor microenvironment [70]. Mice lacking tissue inhibitor of metalloproteinase 3 (TIMP-3) were found to be more susceptible to metastasis of EL-4 lymphoma and B16 melanoma cells, and more pro-MMP-2 was measured in the organs in which metastases formed, identifying TIMP-3 as a regulator of metastatic dissemination [72]. In another example, the osteolysis resulting from implantation of prostate cancer cells into mouse calvariae was reduced in MMP-7-deficient mice compared with wild-type animals. The study implicated MMP-7 in the activation of RANKL, which is required for osteoclast-mediated bone resorption and driving the "vicious cycle" of bone destruction in lytic bone metastases [73]. Advances in GEM modeling, creating animals with altered tumor and tissue microenvironments that can be combined with transgenic tumor models or traditional tumor transplantation models, are likely to provide further insights into the pathobiology of metastasis.

SCID–HUMAN TISSUE MODELS FOR STUDYING TUMOR–STROMA INTERACTIONS

Another approach taken to overcome the poor rate of tumor growth and metastasis of some human cancers, and also to provide a model of species-specific tissue interactions, is to implant the human target organ tissues into immunodeficient mice [55, 74]. Fragments of human fetal lung and bone marrow were implanted into SCID mice; human small-cell lung cancer cells injected intravenously (iv) into the mice were found to preferentially colonize these tissues, and not the normal mouse lung or bone marrow [20]. Fragments of either fetal or adult bone engrafted into SCID mice were colonized by human prostate cancer cells injected iv, demonstrating organ-tropism of metastasis to one of the preferred sites of prostate cancer spread in humans [21, 75].

Growth of human melanoma cells was compared in human skin grafts in SCID mice and in the mouse skin. Following injection into the human skin grafts, the melanoma cells grew and invaded with characteristic patterns, and some metastasized to distant organs. In contrast, the same cells formed noninvasive tumors in mouse skin [76]. These models can be useful for studying the growth and metastasis of human tumor cells in different human tissue microenvironments.

ORTHOTOPIC IMPLANTATION MODELS

Injecting tumor cells into the equivalent normal organ or tissue of appropriate recipient animals, generally termed *orthotopic injection*, has been successfully used to improve tumor take and growth rates and also increase the likelihood of metastasis. The orthotopic model of injection has been used for a number of different human cancers, with some examples shown in Table 1.2, but the same principles apply to rodent tumors. The basic principle behind the orthotopic implantation approach is that tumor growth and progression can be influenced by autocrine, paracrine, and endocrine pathways mediating interactions between the malignant cells and surrounding host tissues [2]. A common observation from comparing tumors implanted into orthotopic versus ectopic sites is that the former are well-vascularized, or have a characteristic

histological appearance, and are more likely to seed metastases to regional lymph nodes. For human breast cancers and rodent mammary tumors, the appropriate site is the mammary fatpad; there is an extensive literature describing growth-modulating effects of the mammary fatpad on normal, preneoplastic, and malignant epithelial cells [91, 92]. There are numerous examples of using orthotopic models to isolate more aggressive and metastatic variants of human cancers, selecting for the metastatic subpopulations, which are suitable for further analyses of the malignant phenotype and for preclinical therapy studies [46, 79, 85].

The orthotopic transplantation of fragments of tumor tissues, from tumor specimens taken directly from patients or serially passaged tumors from immunodeficient mice, is termed *surgical orthotopic implantation* (SOI). This has resulted in faithful reproduction of the metastatic potential of a variety of different human cancers [93]. One explanation for this is that the stromal structure present in the tissue fragments allows for continued expression of genes essential for growth and metastasis. In contrast, when tumor cells are separated from stroma and propagated in tissue culture, the tumor–stroma interactions are lost and metastasis-promoting gene expression may be reduced or silenced. The concept of tumor–stroma interactions influencing the malignant phenotype can also apply to transplantation of cells into orthotopic versus ectopic sites. Clinical and experimental studies have reported differential chemosensitivity of metastases in different organs [94, 95]. Although this could be a function of heterogeneity of the tumor populations, the influence of the tissue microenvironment cannot be excluded. The sensitivity of mouse mammary tumor cells to different chemotherapeutic agents was evaluated in vivo, comparing responses of sc tumors with cells in bone marrow, spleen, lungs, liver, and brain. In general the sc tumors were sensitive, whereas lesions growing in liver and brain were less sensitive to alkylating agents. Cells growing in the bone marrow showed variable sensitivity to different drugs, and the addition of an antiangiogenic agent enhanced killing of these micrometastases by cyclophosphamide [96]. Thus the tissue microenvironment can influence sensitivity of metastatic cells to chemotherapy, and modulating the angiogenic response to the cancer can also modulate treatment outcomes.

Advances in molecular biology and microanalytical techniques have made investigations of the molecular basis of tumor–stroma interactions possible. Microarray analysis was used to compare gene expression profiles of human glioma cells grown in vitro and in vivo, either as sc tumors or orthotopic, intracerebral tumors in immunodeficient mice. A comparison between two glioma tumor cell lines grown in vitro or as sc tumors revealed disparate gene expression profiles, yet profiles from the orthotopic samples were very similar, demonstrating how the tumor phenotype may be modulated by the microenvironment [97]. The availability of species-specific expression arrays allows for analyses of gene expression in human metastatic tumor cells and mouse stromal elements in the same samples, and can identify reciprocal tumor and host interactions that may contribute to the metastatic process [98].

For some tumor models, either mouse or human, the surgical removal of the "primary" tumor allows more time for metastases to grow; otherwise, the mice may succumb to the local tumor burden before the metastases are readily detected [79, 85]. This experimental design is suitable for preclinical studies testing therapies targeted at micrometastatic disease [99]. This approach is limited to models in which removal of the primary tumor is relatively easy, such as breast cancer or melanoma tumors grown in the mammary fatpad or dermis, respectively, and is not practical with other orthotopic models, such as prostate or lung cancers.

A limitation of many mouse models is that the patterns of metastasis from orthotopic tumors do not always accurately mirror those of the original human cancer. Notably, metastasis to bone and brain from tumors growing in the appropriate primary site are not commonly seen in rodent models, although there are reports of brain metastasis from orthotopic mouse and human melanoma [27, 85, 100]. Different routes of injection of tumor cell suspensions can be used to target cells to specific organs (Table 1.3). For many of these injection routes, the most likely site of metastasis development is the first capillary bed in which cancer cells arrest, and thus this approach can be used for studying metastasis to a specific organ – for example, liver metastasis from portal vein or intrasplenic injection of cells [34, 105].

Whereas orthotopic injection can model primary tumor growth and local invasion and intravasation of cells into the lymphatics and bloodstream, the injection of tumor cells by these different routes can simulate later steps in the metastatic process. For example, direct injection of cells into the internal carotid artery can lead to experimental brain metastases, with patterns of growth that can be characteristic of the original cancer [101]. Injection of cells into the left ventricle of the heart results in dissemination of cells throughout the body; this route has been successfully used to seed bone and brain metastases [106–108]. Direct injection of cells into mouse bones, usually the tibia or femur, is used as a model of tumor–stroma interactions in the bone microenvironment, resulting in the development of progressively growing lesions characteristic of the cancer. Breast cancer and renal cell cancer lines can produce predominantly osteolytic lesions, and prostate cancer lines produce osteoblastic lesions [104, 109].

TABLE 1.3. Different routes of tumor cell injection for experimental metastasis models

Injection route/site	Organ or site of tumor growth	References
Intracarotid artery	Brain	[101, 102]
Intravenous (tail vein)	Lungs, systemic dissemination	[10, 31]
Intraperitoneal	Abdominal dissemination	[103]
Intratibia or -femur	Bone tumors	[100, 104]
Intrasplenic or portal vein	Liver	[34, 105]
Left heart ventricle	Systemic dissemination; sites of metastasis can include bone, brain, adrenals	[106, 107]

IN VIVO IMAGING

In vivo imaging techniques offer significant advantages for metastasis research using rodents. With many of the orthotopic implantation models that have been developed, the use of fluorescent proteins can aid in monitoring local tumor growth, angiogenesis, invasion, and metastasis [110]. Many reports have used stable transfection of a reporter fluorescent protein, such as green fluorescent protein (GFP) into transplantable tumor cell lines, allowing detection of tumors and metastases in a variety of organ sites [102, 108, 111]. Figure 1.1 shows the perivascular growth of GFP-expressing MDA-MB-435 cancer cells in the brain of a nude mouse, twenty-one days after injection of cells into the left heart [111]. In addition to transplantation of fluorescently labeled tumor cells, fluorophores expressed in transgenic tumors, or in different normal cell types of recipient mice, provide powerful systems for imaging tumor–stroma interactions with multiphoton microscopy. Some examples include Tie2-GFP mice with fluorescent endothelial cells and c-fms-GFP mice with fluorescent macrophages and granulocytes [112].

Firefly luciferase is another reporter used to monitor tumor growth and metastasis using transplantable tumors engineered to stably express the bioluminescent gene. Detection of luminescence from the tumors can be used to spot metastases that might not be apparent from visual examination of the animal (Figure 1.2), and monitor tumor growth and responses to therapy [11, 100, 113]. The ability to monitor tumor size noninvasively using bioluminescence adds accuracy and sensitivity to many orthotopic models, such as bladder, prostate, or pancreas cancer [114, 115]. Bioluminescent reporters can be combined with transgenic tumor models – for example, breeding mice expressing luciferase

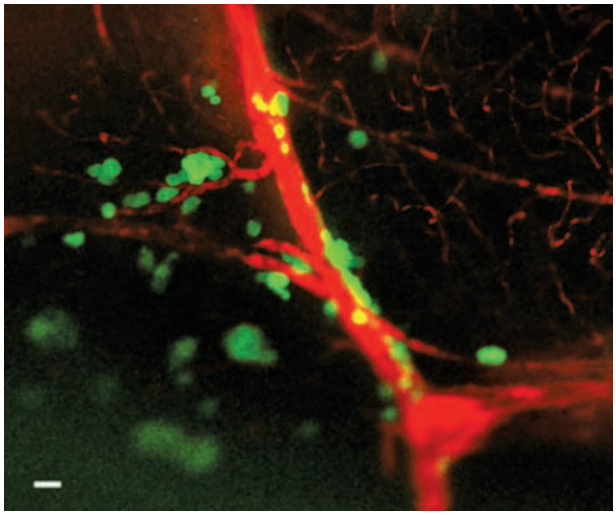

Figure 1.1. Perivascular growth of metastatic cells in the brain of a nude mouse, twenty-one days after left-heart injection of GFP-expressing MDA-MB-435 cells. The mouse was injected with rhodamine-albumin 1 hour before killing and exposing the brain for imaging with a laser scanning confocal microscope. The image was published in Lu et al. 2007 [111] and reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

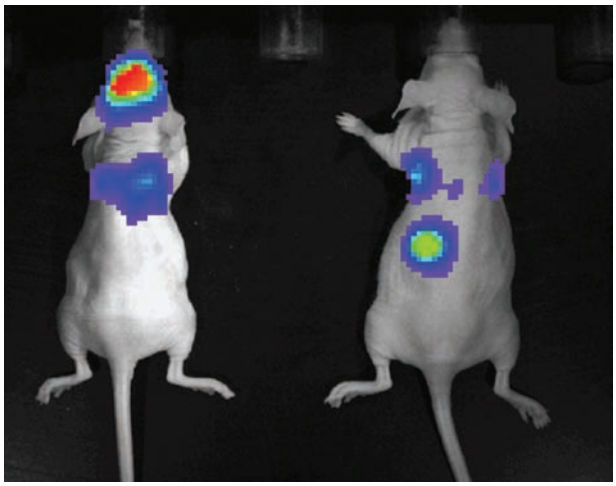

Figure 1.2. Bioluminescent detection of metastases in lungs, adrenal, and brain of nude mice previously injected with luciferase-expressing MDA-MB-435 human cancer cells. The cells were injected into the mammary fatpad, and the tumors removed when 1 cm in diameter. Six weeks later, before obvious signs of metastatic disease, the mice were injected with the substrate luciferin and imaged using Xenogen IVIS, demonstrating the utility of the noninvasive imaging technique.

in the prostate with transgenic adenocarcinoma mouse prostate (TRAMP) mice, which develop tumors and metastases that can be monitored by measuring luminescence [116]. Successful experiments using reporter genes with transplantable tumor lines require stable expression of the reporter. With GFP there are reports of loss of expression from the cells upon transplantation in vivo, possibly owing to unstable integration or transcriptional silencing [102, 117]. Use of reporter genes in tumors transplanted into immunocompetent mice can lead to immune detection and loss of tumorigenicity and metastatic potential [118]. This may depend on the cell system, reporter construct, and strain of mice; there are many reports of success using reporters in immunocompetent animals. However, the retention of tumorigenic and metastatic properties after the introduction of a reporter gene into transplantable tumor cell lines should always be verified.

Different imaging modalities, including magnetic resonance imaging, positron emission tomography, computed tomography, and ultrasound, are being used more frequently as more equipment is adapted for use with small animals [14]. However, the expense and access to the instruments and technical support may limit use of some of these technologies. Availability of the equipment within a barrier facility may be required, especially for studies using immunodeficient animals, or for time-course experiments with repeated imaging of the same animals.

CONCLUSIONS

The pathogenesis of cancer metastasis involves complex interactions between malignant and normal cells. With appropriate design and selection of techniques, animal models of cancer growth and metastasis can provide a wealth of information that cannot be simulated with tissue culture models. Increasing numbers of tumor models are available, and new technologies are being developed for monitoring tumor progression; the choice of which model to use will depend on the hypothesis to be tested. The introduction of GEM models provides the opportunity to directly address the influence of the tissue microenvironment, as well as the role of specific genes on the progression of metastasis. With the increasing development of new therapies targeting the tissue microenvironment and tumor vasculature, valid animal models are important for testing the effectiveness of these agents for controlling or preventing metastatic disease.

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2 *Drosophila* and Zebrafish: Genetic Models for Cancer Metastasis

Elisa C. Woodhouse and Kathleen Kelly

Scientists have long used genetic models – in particular, mouse models – to study tumor metastasis. Other organisms have been useful for the genetic analysis of myriad biological processes, such as development, signal transduction, and cell growth. Recently, it has become evident that genetically tractable, nonmammalian models may significantly contribute to the study of cancer and metastasis. One such organism is the fruit fly, *Drosophila melanogaster*.

Since the 1970s, it has been known that *Drosophila* develop tumors in specific mutant lines. Scientists have gained an understanding of the molecular basis of these tumors, including the specific tumor suppressors mutated in these lines, the defects produced in the cell, and the way in which these disruptions lead to cancer in *Drosophila*. A key advantage of metastasis models using *Drosophila* is the ability to rapidly generate mutations in vivo and assess their effects. The research in this field has now matured from model development to discovery-based investigation. In this chapter, several aspects of the *Drosophila* models will be discussed, including tumor suppressors and their human homologs, the role of cell polarity in tumorigenesis and progression, and current approaches in *Drosophila* that are being used to understand metastasis and migration, along with examples of knowledge that has been gained through studies using each of the models.

Another organism that is amenable to genetic manipulations for which cancer models are being developed is the zebrafish (*Danio rerio*). As a vertebrate, the zebrafish has a more complex physiology and anatomy than *Drosophila*, extending beyond the invertebrate models the potential mechanisms of metastasis that can be addressed. Methods for using zebrafish as well as a description of existing cancer models are discussed later in this chapter. The *Drosophila* and zebrafish genetic models can be used to study discrete aspects of key processes in metastasis, which can then be validated in mouse models and human cancer.

DROSOPHILA CANCER MODELS

Drosophila Tumor Suppressor Mutant Phenotypes

Tumor suppressor genes that are involved in carcinogenesis when inactivated have been identified in *Drosophila*. Although loss of function of a large number of tumor suppressors in *Drosophila* causes hyperplastic growth, a smaller group of tumor suppressors causes true neoplasias. These neoplastic tumor suppressor genes are the most relevant for the study of cancer and metastasis in *Drosophila*. Mutations in these genes cause disruptions in the differentiation and growth of the imaginal discs, sacs of epithelial cells in the larvae that will give rise to specific adult structures such as eyes, legs, and antennae, in addition to disruptions in the larval brain. Neoplastic tumor suppressor genes that have been widely studied include *lethal giant larvae* (*lgl*) (Gateff 1978), *discs large* (*dlg*) (Woods and Bryant 1989), *brain tumor* (*brat*) (Arama et al. 2000), and *scribble* (*scrib*) (Bilder et al. 2000). The mutant phenotypes of these genes are very similar, and are all lethal in the late larval period. Larvae develop overgrown imaginal discs with a disorganized, multilayered structure, as well as overgrown larval brains with a loss of differentiation and an increase in neuroblast number.

Studies over the past several years have revealed that mutations in these *Drosophila* tumor suppressor genes cause neoplasia in the larval brain through a disruption in cell polarity. Some *Drosophila* tumor suppressors have been shown to disrupt the neuroblast lineage, resulting in excessive self-renewal of neuroblasts and tumors with metastatic properties (discussed later). The understanding of the biological principles underlying the neuroblast tumor model system have contributed to the current interest in the hypothesis that self-renewing cancer cells capable of metastasis share properties with self-renewing normal stem cells.

Drosophila Tumor Suppressors in Cell Polarity and Progression

Loss of cell polarity is an important step in tumorigenesis. The study of the roles of the Lgl and Brat proteins in maintaining cell polarity, and the subsequent tumorigenesis that is observed in the absence of either protein, have elucidated some of the molecular mechanisms that control this process. Neuroblasts in the larval brain normally divide asymmetrically to yield two daughter cells, a ganglion mother cell (GMC) derived from the basal side and a larger neuroblast cell from the apical side. The GMC divides once more, giving rise to two terminally differentiated neuronal cells, and the self-renewing neuroblast continues the lineage, producing a GMC and a neuroblast (Figure 2.1). In *Drosophila* neuroblasts, asymmetrical division is maintained through the partitioning of apical and basal components into their respective cortical domains. Neuroblast cell lineage is dependent on the downregulation of neuroblast gene expression in the GMC as well as the segregation of neural determinants to the GMC. Lgl has been found to interact with the PAR complex (Bazooka/Par-6/aPKC, in *Drosophila*) to regulate apical/basal cell polarity (Betschinger et al. 2003; Rolls et al. 2003). Control of Lgl and the PAR complex protein, aPKC, occur through mutual inhibition. Apically localized Lgl is phosphorylated by aPKC, causing its release as a result of a change in conformation (Betschinger et al. 2005), restricting active Lgl to the basal side of the neuroblast (Betschinger et al. 2003). Active Lgl then inhibits aPKC in the basolateral domain. Genetic data indicate that Lgl regulates aPKC, whereas aPKC functions directly to promote self-renewal of the neuroblast through control of neuroblast-specific genes and neuron cell fate determinants (Lee et al. 2006a). Proteins that promote neural cell fate are localized basally. The Miranda protein (Ikeshima-Kataoka et al. 1997; Shen et al. 1997) and its cargo protein, the transcription factor, Prospero (Hirata et al. 1995; Knoblich et al. 1995; Spana and Doe 1995), are key factors among the basally localized neural cell fate determinants. The Brat protein is also targeted basally through its association with the Miranda protein as a cargo protein (Lee et al. 2006b). Brat is required for the basal localization of Prospero, possibly through stabilizing the interactions between Miranda and Prospero. In mutants lacking functional Brat protein, the basal daughter cell expresses the Miranda protein, but Brat and Prospero are absent. The *brat* mutant GMC daughter cell maintains some neuroblast-specific proteins and appears to revert to a neuroblast type at a high frequency, leading to ectopic cell renewal at the expense of differentiation (Figure 2.1) (Lee et al. 2006b), which causes a phenotype similar to that seen in *lgl* mutants. Mutations in other cell polarity determinants have also been shown to cause invasive tumors in *Drosophila*. Larval brain tissue with mutations in *pins*, *miranda*, *numb*, or *prospero* formed secondary tumors at distant sites (i.e., metastases) on transplantation (Caussinus and Gonzalez 2005).

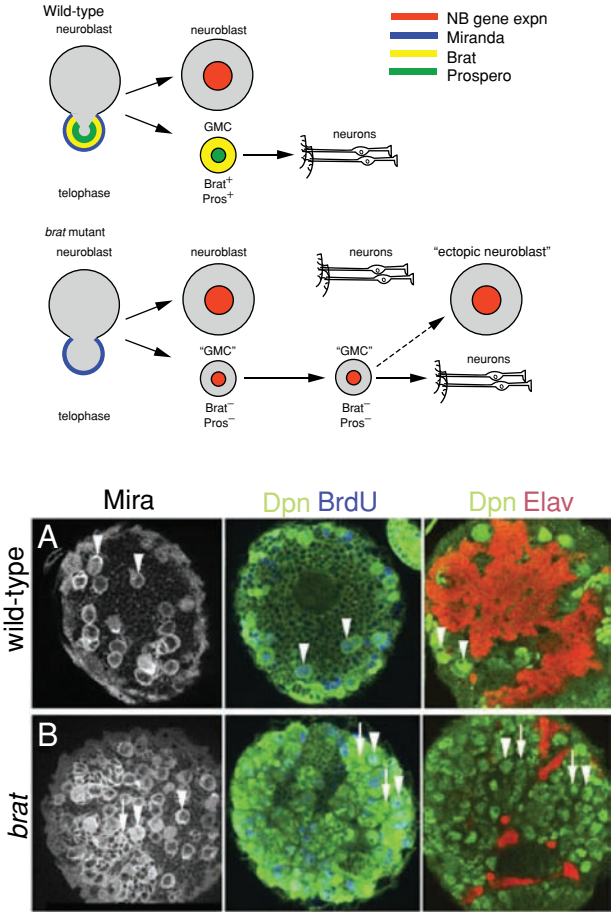

Figure 2.1. (Schematic) Brat inhibits neuroblast self-renewal and promotes GMC differentiation. Top, wild-type neuroblasts partition Miranda, Brat, and Prospero (Pros) into GMCs. In GMCs, Miranda is degraded, Brat is cytoplasmic, Pros is nuclear, and neuroblast genes are downregulated. GMCs differentiate into two postmitotic neurons. Bottom, in *brat* mutants, neuroblasts partition Miranda, but not Brat or Pros, into GMCs. GMCs maintain neuroblast gene expression and show delayed differentiation; some ultimately form neurons, while others appear to enlarge into proliferative neuroblasts. (A, B) *brat* mutants have ectopic neuroblasts. Single brain lobes of wild-type or brat ^{11}/brat^{11} mutants 96 hr after larval hatching stained for the neuroblast markers Miranda (Mira) and Deadpan (Dpn) and the neuronal marker Elav and assayed for proliferation by performing a 4-hr BrdU pulse prior to fixation. Representative neuroblasts, arrowheads; GMCs, arrows (Lee et al. 2006b).

Human Homologs of *Drosophila* Tumor Suppressors and Carcinogenesis

The disruption of apical–basal determinants has also been shown to occur in human tumor progression. Human homologs of aPKC and Lgl, aPKCζ and Hugl-1,

have been associated with a variety of epithelial cancers. Reduced levels of Hugl-1 expression have been associated with tumor formation and progression of colorectal cancer (Schimanski et al. 2005) as well as melanoma (Kuphal et al. 2006). Hugl-1 loss is also associated with lymph node metastasis and poor prognosis in endothelial cancer (Tsuruga 2007). Proper localization of these proteins also appears to be critical to the suppression of tumorigenesis. Mislocalization of the aPKCζ and Hugl-1 proteins was found in mucinous and serous ovarian tumors (Grifoni et al. 2007). In mucinous and serous carcinomas, aPKCζ and Hugl-1 were cytoplasmic, whereas apical specificity of aPKCζ was lost. Increased expression of aPKCι was also observed in ovarian (Eder et al. 2005) and lung (Regala et al. 2005) cancers. Loss of expression of human homologs of two other *Drosophila* tumor suppressors, *discs large* and *scribble*, are also associated with tumor progression in colon cancer (Gardiol et al. 2006). Decreases in the levels of both proteins, hDlg and hScrib, were associated with loss of cell polarity and tissue architecture. Previous studies linked these proteins to human cancer by showing that Dlg and Scribble are targets of HPV E6 degradation (Kiyono et al. 1997; Nakagawa et al. 2000). A recent study examining global gene expression in multiple tumor cell lines compared with nontumorigenic parental lines found that a mammalian homolog of a protein known to regulate polarity in *Drosophila*, Crumbs, was dramatically downregulated in the tumor lines. When *crumbs3* expression was repressed in mouse kidney epithelial cells, apical–basal polarity and contact-inhibited growth were disrupted. Restoration of gene expression suppressed these effects and inhibited migration and metastasis of the tumor lines (Karp et al. 2008). Taken together, the evidence indicates that the mechanisms that control polarity, maintain tissue integrity and architecture, and suppress metastasis in *Drosophila* are conserved in mammalian cancers. Control of these processes may prove to be useful in the treatment of human cancers.

Metastasis of *Drosophila* Neoplastic Tumors

Models have been developed in *Drosophila* to visualize and manipulate tumor growth at secondary sites to better understand fundamental underlying molecular events important for metastasis. We refer to tumor growth at a distance from the primary tumor as metastasis, with the understanding that invasion, migration, and colonization are the properties being analyzed. Essentially, two types of models exist for metastasis using the *Drosophila* neoplastic tumor suppressors. One method involves the transplantation of tissue from a tumor suppressor mutant into a wild-type host; the second method examines metastasis in situ.

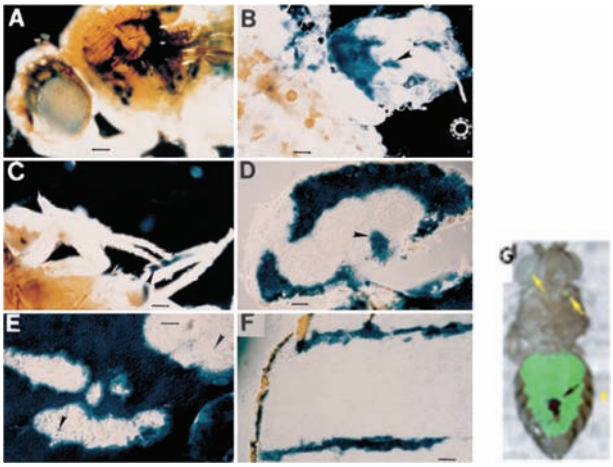

Figure 2.2. Secondary tumors from transplanted *lethal giant larvae*, *brain tumor*, and *discs large*; mutant brain fragments or brain fragments carrying mutant *miranda* clones. (A) *brain tumor*, secondary tumors in the head and thorax; whole mount. (B) *discs large*, secondary tumors in ovary (arrowhead); whole mount. (C) *discs large*, secondary tumor in leg (arrowhead); whole mount. (D) *lethal giant larvae*, secondary tumors in head (arrowhead indicates tumor in brain); section. (E) *lethal giant larvae*, arrowheads indicate tumor cells within lumen of gut; section. (F) *discs large*, secondary tumors in the thorax; section (bars A–C 100 μm; D–F 50 μm). (G) GFP-labeled fragments of larval brain fragments carrying *mira*^{ZZ17} clones grew to many times the original size of the implant in two weeks. A large mass of implanted tissue (green) filled the host, and small tumor colonies (yellow arrows) were scattered at a considerable distance from the point of implantation (black arrow) (Woodhouse et al. 1998; Caussinus et al 2005).

Transplantation Metastasis Model

The transplantation model was applied in the initial studies that identified *lethal (2) giant larvae* as tumor suppressor mutants (Gateff and Schneiderman, 1974), although subsequently the method was modified to introduce a β-galactosidase tumor cell marker that allowed the quantitation of tumor cell growth and metastasis (Woodhouse et al. 1998). The method involves isolation of donor tissue from the mutant larva and injection into the abdomen of a wild-type adult host fly. After a culture period during which the tumor grows and undergoes metastasis, the adult host is stained for β-galactosidase activity. Tumor-derived cells can be identified at sites distant from the injection site (Figure 2.2). Using this method, it was found that the majority of *lgl* (87%) and *brat* (84%) brain tissue fragments and a substantial number of *dlg* (22%) brain tissue fragments were metastatic. In addition, imaginal disc tumors from *lgl* (43%) and *dlg* (53%) were metastatic. The analysis of growth dynamics of the tumors after transplantation showed that the tumors arose from a small population of cells that represent only 1 to 2 percent of the cells in the transplanted tumor tissue.

The transplantation method using injection of marked *Drosophila* tumor tissue into adult hosts has

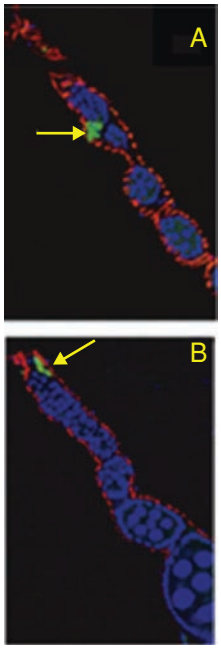

Figure 2.3. *Drosophila* brain tumor cells form micrometastases within host ovarioles. Confocal sections through a host ovariole containing *brat* (A) *or lgl* (B) mutant micrometastasis (green, arrow) that have crossed the muscle layer of the epithelial sheath (red) (Beaucher et al. 2007a).

been used to identify genes required for the metastasis of this tissue (Woodhouse et al. 2003). Using random P element mutagenesis, specific mutations were isolated that disrupted metastasis of *lgl* cells, and the affected genes were identified. The *semaphorin 5c* gene was found to be required for tumorigenesis and metastasis of *lgl* tumors. Semaphorin 5c is a member of the Semaphorin family, which are molecules important in axon guidance (Nakamura et al. 2000). Another mutation affecting the *apontic* gene specifically affected metastasis. The *apontic* gene has been shown to act as a transcription factor necessary for migration in embryogenesis (Eulenberg et al. 1997). A third mutation activating the *pointed* gene, an Ets-like transcription factor necessary for tracheal cell migration and other aspects of development (Klambt 1993), decreased the survival times of hosts injected with these tumors in comparison with wild-type.

As the *Drosophila* host has an open circulatory system, it is possible for cells to move from the injection site by migrating without crossing tissue barriers, so the host transplanted with neoplastic tissue may have a mixture of passively and actively migrating cells. The transplantation metastasis assay was therefore further modified to exclusively examine cells with invasive behavior (Beaucher et al. 2007a), looking at the micrometastases in the host ovarioles (Figure 2.3). This method was used to show that metastatic tumors from *lgl* and *brat* tissue had different properties. For example, the frequency of ovariole invasion by *lgl* tumor cells increased with in vivo culture time, whereas micrometastases from *brat* tumors did not. Also, by looking at the expression of neuronal cell markers, the investigators discovered that the metastatic cells that arise from *lgl* and *brat* tumors are different. Metastatic cells derived from *lgl* tissues coexpressed neuronal and glial cell markers, whereas most of the *brat* metastatic cells expressed neither marker. This shows that although the Lgl and Brat proteins both disrupt the neuroblast cell division by shifting the balance toward excessive cell renewal, the molecular properties of the metastatic tumors are, indeed, quite distinct. Another modification to the transplantation assay was made (Caussinus and Gonzalez, 2005) by the marking of the tumor cells with green fluorescent protein (GFP) to distinguish tumor cells in the adult host (Figure 2.2G). As described earlier, mutations in the polarity determinants Pins or Miranda led to tumorigenesis. These tumors metastasized, as visualized by the presence of fluorescent clones sites distant from the injection site. Advantages of using GFP-labeled tumors are that the tumors can be visualized in live hosts, live GFP-positive cells can be recovered, and importantly, biomolecules can be isolated from the tumor cells without undergoing a staining procedure, which would compromise their integrity.

In Situ Tumor Metastasis Model

Another approach for modeling metastasis in *Drosophila* has been to examine the in situ (within the organism) metastasis of tumor cells. In contrast to the transplantation model, in which a piece of tumor tissue is isolated from an organism in which a tumor suppressor gene is inactivated in all cells, the in situ approach involves generating marked clones of cells lacking specific tumor suppressor genes in otherwise normal tissues. The cells metastasize within the organism under certain conditions. The clonal aspect of the tumors models human cancers, in which tumor cells are adjacent to normal cells and receive signals from their environment. When mutant clones of the tumor suppressor gene, *scribble*, were generated in eye imaginal discs of larvae, the mutant clones did not overgrow (Brumby and Richardson 2003). This is in contrast to *scribble* mutant larvae, which exhibit neoplastic imaginal discs and brains (Bilder and Perrimon 2000). The *scrib* clones did grow, however, when combined with oncogenic Ras or Notch mutations (Brumby and Richardson 2003). This approach of studying clonal tumors in situ can be applied to identifying genes that promote progression from noninvasive masses to tumors with dissemination at distant sites. One study (Pagliarini and Xu 2003) undertook this approach and found that mutations in the *scrib* gene, as well as the tumor suppressors *lgl* and *dlg*, caused the progression

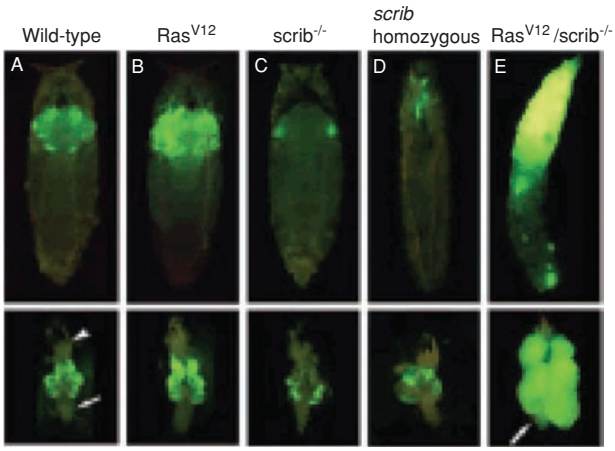

Figure 2.4. Tumor progression phenotypes in *Drosophila* with mosaic clones expressing GFP (top panel) and cephalic complexes dissected from third instar larvae (bottom panels). The anterior is the top in all panels. The mouthhook (arrowhead in A) is at the top and the ventral nerve cord (arrow in A) is pointing down in all bottom panels. Metastatic tumor cells caused an extended larval period, and flies died as bloated larvae (E) with cells invading the VNC (arrowhead in E) (Pagliarini and Xu 2003).

of Ras^{V12} tumors (Figure 2.4). These mutations cause large primary tumors and metastases to distant sites that could be visualized by marking the mutant clones with GFP. In this system, the tumor suppressor gene did not cause metastatic tumors alone, presumably owing to the presence of normal cells surrounding the *scrib*$^{-}$ clones.

An important question was whether mutations in other genes necessary for the maintenance of cell polarity and morphogenesis would also promote tumor progression. Indeed, mutations in *bazooka*, *stardust*, and *cdc42* promoted the progression of Ras^{V12} tumors. This method for detecting the metastasis of *Drosophila* tumor cells has since been adapted for other studies. Another group (Vidal et al. 2007) found that high Src levels in Ras^{V12} mutant cells led to metastatic growth. The authors propose that high Src levels in human tumors may also combine with oncogenic Ras in progression to promote metastasis.

Border Cell Migration: Modeling Cancer Cell Migration in *Drosophila*

A hallmark feature of metastatic tumor cells is increased motility. A well-studied *Drosophila* model for investigating the migratory behavior of cells is the egg chamber border cell model. A key morphogenic event in the developing egg chamber is the well-characterized migration of a group of epithelial cells called border cells. At a specific point in development, particular follicle cells called polar cells recruit a group of four to eight cells from the anterior epithelial follicle cells to form a border cell cluster that detaches from the epithelium, invades the nurse cell complex, and migrates to the anterior oocyte (Montell 2003; Nallamothu et al. 2008). Genetic screens have uncovered the molecular controls regulating this process. These screens have identified various pathways that are required for border cell migration, including the PDGF/VEGF, EGF, JAK/STAT, Notch, and JNK signaling pathways (reviewed in Montell 2003). One protein found to be a key regulator of border cell migration is Slbo, a basic region/leucine zipper transcription factor homologous to C/EBP (Montell et al. 1992). Whole-genome microarray analysis of border cells has complemented genetic approaches and demonstrated the complexity of the control of migratory behavior of cells (Wang et al. 2006; Borghese et al. 2006). Both microarray studies analyzing migration genes identified hundreds of genes (300 to 400) that were upregulated in migratory cells, compared with nonmigratory cells of the egg chamber. Border cells from *slbo* mutants had approximately 150 genes that were expressed at lower levels compared with wild-type border cells. Of these genes, about 100 overlapped with the genes that were enriched in the migratory cells. The genes important in migration were enriched for cytoskeleton regulators and components of the secretory and endocytic pathways.

The border cell migration model was used to investigate the function of Awd, the *Drosophila* homolog of the metastasis suppressor gene, Nm23, in migrating cells. Nm23 was identified more than twenty years ago as a cDNA downregulated in highly metastatic melanoma cell lines (Steeg et al. 1988) that was capable of inhibiting metastasis and motility when expressed in metastatic cell lines (Leone et al. 1993). Whereas cellular functions such as nucleoside diphosphate kinase (Biggs et al. 1990) and histidine-dependent protein kinase (Engel et al. 1995) activity have been assigned to Nm23, the cellular function of Nm23 in metastasis has remained a subject of investigation. Although Awd is expressed at high levels before border cell migration begins, its expression decreases rapidly prior to delamination from the epithelium and remains low as the border cell migrates (Nallamothu et al. 2008). Reexpression of Awd specifically in border cells blocked border cell migration (but not border cell cluster formation) and rescued the migration-inhibiting phenotype of constitutively active Pvr (*Drosophila* homolog of the PDGF/VEGF receptor). Awd activity brought Pvr down to control levels, rescuing the migration defects. Awd was also found to be a negative regulator of the surface receptor, Domeless, which is required for nuclear translocation of STAT. Experiments studying the relationship of Awd and Domeless or Pvr suggest that Awd controls the expression of surface receptors important in cell migration by interfering with dynamin-dependent endocytosis, which is normally required for precise temporal and spatial controls of migration.

Nm23 may play a similar role in the regulation of tumor cell motility through the control of endocytosis of multiple cell surface receptors. Supporting this hypothesis, the lysophosphatidic acid receptor EDG2 was found to be overexpressed in metastatic breast cancer cells with mutant Nm23-H1 (Horak et al. 2007).

Tumor Biology of *Drosophila* Neoplasias

Conclusions drawn from *Drosophila* neoplastic tumors models are in concert with many key concepts for signal transduction pathways and functions in mammalian tumors. Many of the molecules associated with metastasis in mammalian tumors also play a role in the *Drosophila* tumors, validating *Drosophila* tumors as models that can provide insights into human cancer biology, potentially including the therapeutic intervention of metastatic tumors in humans. Experiments on *Drosophila* tumors have shown that the metastasis of these cells is an active process, not simply a passive movement of cells floating from one region to lodge in another and grow. Furthermore, as in human metastatic cells, the metastatic behavior of the cells is associated with changes in expression of specific proteins that, if reversed, can block metastasis. For example, *Drosophila* $Ras^{V12}/scrib^{-/-}$ tumors are able to migrate from their site of origin in the developing eye to invade the ventral ganglion (Pagliarini and Xu 2003). Confocal microsopy showed that the leading edge of these invading cells had high levels of F-actin expression at the periphery, as is common to actively migrating cells. Furthermore, the cells were localized within the ventral ganglion, which indicated that invasion had occurred. Experiments that examined the integrity of the basement membranes of $Ras^{V12}/scrib^{-/-}$ tumors showed that the basement membranes surrounding mutant eye discs were disrupted at many points, indicating degradation by the mutant cells. Additional studies confirmed the requirement for basement membrane remodeling prior to the metastasis of *Drosophila* tumors. One study found that the *Drosophila* matrix metalloproteinase 1 (MMP-1) was upregulated in metastatic $Ras^{V12}/scrib^{-/-}$ tumors and contributed to the invasive behavior of these cells (Srivastava et al. 2006). Partial suppression of the invasive phenotype was achieved by $Ras^{V12}/scrib^{-/-}$ clones in MMP-1–mutants. Expression of tissue inhibitor of metalloprotease (TIMP) together with expression of another protease inhibitor, reversion-inducing-cysteine-rich protein with kazal motifs (RECK) completely blocked invasion (Srivastava et al. 2006). Another study (Beaucher et al. 2007b), used the *Drosophila* ovariole invasion model to show that MMP-1 facilitates metastasis in *lgl* but not in *brat* tumors. The expression of MMP-1 increased in *lgl* tumors, and removal of MMP-1 reduced the frequency of ovariole micrometastases in *lgl* tumor cells. In hosts that were transplanted with *brat* (but not *lgl*) mutant brain tissue, the expression of MMP-1 was increased in the host ovaries. MMP-1 expression is therefore a critical component of the metastasis machinery required for invasion of the ovariole, although the regulation of MMP-1 expression differs, depending on the mutation of the primary tumor. Expression of TIMP decreased metastasis in both *lgl* and *brat* tumors. The difference in control of MMP-1 expression observed in *lgl* compared with *brat* tumors presents an opportunity for probing the molecular events that occur downstream of specific tumor suppressor mutations.

ZEBRAFISH CANCER MODELS

The use of zebrafish (*Danio rerio*) to model cancer is an approach that has been growing in popularity and is anticipated to be of expanding utility in at least three areas. First, the ability to perform genetic screens using this model is expected to aid in the identification of genetic modifiers contributing to the complex cancer process. Second, the fluorescent imaging properties of zebrafish are particularly useful for visualizing cancer progression and tumor–microenvironmental interactions. Third, the amenability of zebrafish to pharmacological testing provides a platform for screening chemical modifiers of cancer.

The major methods that are used for genetic interrogation in zebrafish and examples of existing cancer models are briefly discussed in this section. Various scenarios are introduced regarding how the use of fluorescent imaging has been effectively exploited to monitor several aspects of the cancer process. These include cancer cell dissemination and interactions between tumors and the vasculature. Finally, the unique strengths as well as needed areas of development with respect to the use of zebrafish to investigate tumor metastasis are considered.

Zebrafish Genetics

Zebrafish genetics are easily manipulated (Lieschke and Currie 2007; Stern and Zon 2003). Other factors that make zebrafish an attractive model system are external fertilization, high fecunditiy, rapid development, and high stocking densities. Embryos and larvae are transparent, which has contributed greatly to straightforward assessment and selection of developmentally apparent phenotypes. Furthermore, considerable genomic resources exist for zebrafish, including a complete sequence of the zebrafish genome, a resource that aids greatly in the identification of mutations (see http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish).

Chemical mutagenesis, coupled with forward phenotype-driven screens or reverse, candidate gene

approaches, has led to the availability of mutant lines of fish, some of which have clear relevance to mammalian cancer biology. Random mutagenesis also has been successfully carried out in zebrafish using retroviral integration. Forward genetic screens have been performed for screenable phenotypes such as proliferation defects or embryonic lethality, and subsequently adult phenotypes with increased incidence of specific cancers have been observed (Amsterdam et al. 2004; Shepard et al. 2007; Shepard et al. 2005). Most of the genes identified in such forward phenotypic screens have not been associated with mammalian cancers (e.g., *myosin, separase*, and multiple ribosomal proteins). Their relevance to human cancers has yet to be determined.

Reverse screening approaches following *N*-ethyl-*N*-nitrosourea mutagenesis directly select for genes with known or anticipated cancer relevance. Because ancestral whole-genome duplication occurred in zebrafish, gene duplications exist that in some cases may have resulted in subfunctionalization and neofunctionalization, as has been observed for the duplicated *pten* homologs, *ptena* and *ptenb* (Faucherre et al. 2008). Reverse genetic approaches have produced lines mutant for genes of proven human cancer relevance, such as *tp53, apc, ptenb*, and mismatch repair genes that display increased incidence of specific spontaneous or carcinogen-induced cancers (Berghmans et al. 2005; Faucherre et al. 2008; Haramis et al. 2006). The incidence of spontaneously occurring cancers in these lines is about 30 percent, and the onset of tumors is between seven and eighteen months (Feitsma and Cuppen 2008). These spontaneous tumors are often of types, such as nerve sheath tumors, that are not common in humans. On the other hand, liver and intestinal tumors, as well as sarcomas, also have been described.

Several common human tumor types have been modeled in zebrafish using transgenesis (Feitsma and Cuppen 2008; Goessling et al. 2007; Lieschke and Currie 2007; Stoletov and Klemke 2008). Transposon-mediated transgenesis can be accomplished by the injection of constructs into fertilized oocytes, resulting in greater than 50 percent efficiency of germline transmission. Zebrafish transgenic cancer models have been developed with the familiar principle of tissue-specific promoters driving oncogene expression. Commonly, fluorescent markers are coexpressed with the oncogene, allowing direct visualization of oncogene-expressing cells. Although each cancer model will need to be individually assessed, there is evidence for histological and molecular similarities between certain human and zebrafish tumor types (Lam et al. 2006; Langenau et al. 2007). Models have become increasingly more sophisticated with time, incorporating conditional expression using CRE-lox technology (Le et al. 2007) that has been used extensively in mouse models for manipulation of the genome.

Several leukemia models have been produced using the *rag2* promoter that is highly expressed in lymphoid cells (Goessling et al. 2007; Langenau et al. 2003). Alternatively, tissue specificity can be determined by the oncogene as is the case for the leukemic fusion protein, TEL-AML1 expressed from the ubiquitous β-actin promoter, which leads to B-lineage leukemia (Sabaawy et al. 2006). In addition, unexpected ectopic expression from a promoter construct has been exploited for model production in the case of *rag2*-driven expression of *kRASG12D* in undifferentiated muscle, leading to rhabdomyosarcoma (Langenau et al. 2007). Solid tumor models have been produced for rhabdomyosarcoma, melanoma, and neuroendocrine tumors (Langenau et al. 2007; Patton et al. 2005; Yang et al. 2004). As more tissue-specific promoters are characterized, the range of tumor histologies is expected to expand. The penetrance of cancer formation in the models is variable, suggesting that often, additional genetic events cooperate in tumor formation (Feitsma and Cuppen 2008; Lieschke and Currie 2007). Consistent with this, crossing two transgenic models or a transgenic model and a mutant cancer susceptibility line can result in increased tumor incidence, increased tumor aggressiveness, or decreased tumor latency (Chen et al. 2007; Patton et al. 2005).

Fluorescent Imaging of Zebrafish Tumors

A powerful and exciting aspect of zebrafish is the ability to image fluorescent cells at the single cell level in an intact in vivo vertebrate system (Stoletov and Klemke 2008). As mentioned earlier, zebrafish embryos are transparent, but adult fish are not. However, adult zebrafish are small enough to allow visualization of fluorescent organs or tumors inside the living body, although spatial resolution is limited due to normal opacification of skin and subdermal structures. The relatively recent development of transparent adult zebrafish (roy$^{-/-}$; nacre$^{-/-}$), the so-called casper mutant, has increased the sensitivity to observe and the ability to resolve and quantify fluorescent cells (White et al. 2008).

Fluorescent imaging has been used in transgenic models and in transplantation studies. In transgenic models, the use of fluorescent markers linked to oncogene expression allows visualization of developing tumors, including the time of onset, location, and growth rate. Importantly, fluorescently marked tumor cells also provide a means for analyzing the response of tumors to therapy. Fluorescence can also be used for tracking regulated CRE-lox–mediated recombination events, which identifies genetically modified cells in a heterogenous mixture (Langenau et al. 2005). Fluorescent markers driven by distinct differentiation-dependent promoters have been used to distinguish

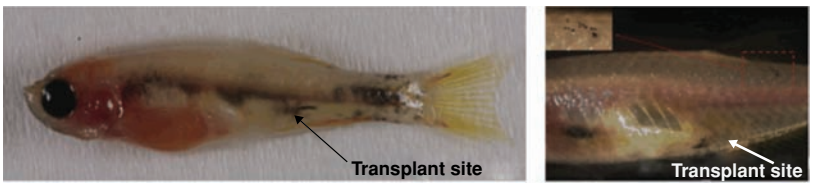

Figure 2.5. Transplanted melanoma cells migrate far from the transplantation site in *casper* mutant zebrafish. Single migratory (stellate appearing) melanoma cells (right, inset box) have migrated from the transplantation site and embedded in the dorsal skin (White et al. 2008).

cellular differentiation within tumors (Langenau et al. 2007). Finally, the expression of fluorescent markers in elements of the tumor microenvironment, such as blood vessels, provides a means to image host–tumor interactions (Lawson and Weinstein 2002).

Transplantation experiments of fluorescently labeled cells have been used for observing colonization and interaction with the host vasculature (Hakem et al. 2005; White et al. 2008). Adult or juvenile host fish are generally immunosuppressed prior to transplantation. Cells can be introduced systemically by intracardiac routes, or as a cell mass by intraperitoneal injection. Although cell lines derived from zebrafish tumors have not been established, it has been possible to transplant single-cell suspensions of primary tumors and to observe dissemination of small numbers of tumor cells at sites distant from the transplanted tumor (White et al. 2008). In addition, xenotransplants of human tumor cell lines have been performed. In one study, multicolor high-resolution confocal microscopy led to the observation of dynamic interactions between genetically manipulated human tumor cells and the zebrafish vasculature, resulting in vascular remodeling (Hakem et al. 2005). The combination of fluorescently labeled tumor cells, either endogenously developed or transplanted, and fluorescent host organs provides a stunning means to noninvasively observe tumors over time within the appropriate in vivo milieu.

Beyond Tumorigenesis: Zebrafish as a Genetic Model of Metastasis?

An exciting possibility for future exploration is the potential to extend zebrafish tumorigenesis models to the mechanistic study of metastasis (metastasis of tumor cells from the transplant site in zebrafish is shown in Figure 2.5). Two strengths of the zebrafish system for studying the molecular events underlying metastasis are its genetic tractability and the relative ease and efficiency of assaying pharmacologic agents in vivo. Zebrafish can absorb small-molecular-weight compounds directly from water, which makes this organism especially useful for carrying out chemical screens in a live vertebrate. The utility of zebrafish to carry out high- or medium-throughput screens to address mechanisms of metastasis has yet to be determined.

Either genetic or pharmacologic screens of phenotypes relevant to metastasis would depend on an efficiently generated and easily scored system, such as a transgenic fluorescent tumor model. The development of screen designs that can be automated and quantified with respect to defined tumor cell characteristics in vivo clearly will facilitate progress in this important area. One possible scenario of potential significance with respect to new gene or pathway discovery is screening for the progression to or suppression of tumor cell dissemination. However, a key issue concerns the natural history of tumor progression in the various transgenic models, and if tumor colonization at a distant site exists, whether it has resulted from vascular or lymphatic spread. Invasive motility of tumor cells within tissues in the absence of vascular intravasation is possible as well. Additional fundamental characterization of progression in zebrafish tumor models is needed before the range of usefulness of such models to investigate metastasis mechanisms with relevance to mammalian cancer progression will be known.

Another scenario is screening for genetic or chemical modifiers of genes such as *RAS* that are known to contribute to cancer progression and metastasis in mammals, without regard to the progression phenotype in zebrafish. In this case, the screen could analyze effects, for example, on tumor cell survival or metabolism. The development of tumor models in zebrafish has suggested the potential for using these systems to study cancer progression and possibly identify lead drug compounds with relevance to the treatment of metastasis. Although much has yet to be learned and developed, zebrafish models have begun, and will likely continue, to occupy a unique and important niche in cancer biology research.

CONCLUSIONS

When various organisms and animal models to study human cancer and metastasis are used, there are significant differences that must be taken into account between key physiological properties of model systems and human biology. However, vast commonality in fundamentally conserved signaling pathways and their functional consequences renders genetically tractable models that are valuable resources that complement clinical samples, mammalian models, and cell lines. Despite the differences inherent in *Drosophila* or zebrafish tumors compared with those in humans, these models provide unique opportunities for both forward and reverse genetic screens to identify genes that

influence tumorigenesis and/or metastasis, and allow manipulations that are difficult or impossible to apply in other models. The relationships of proteins occurring in the same signaling pathway or cross-regulating other signaling pathways have often been uncovered first in nonbiased genetic screens. Most recently, advances in imaging technology have allowed sophisticated genetic techniques to be combined with dynamically monitored in vivo physiological processes relevant to cancer and metastasis. The future undoubtedly holds additional creative and powerful approaches for these useful model systems.

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3 Computational Models

Wayne S. Kendal

Computational models are mathematical models executed by computer and used to simulate the behavior of complicated systems. When such models are used to analyze biological systems, they are given the added descriptor *in silico* to emphasize their ancillary role to in vitro and in vivo experiments. The computational approach allows us to analyze components of biological systems, understand complex data, predict system behavior, test hypotheses, and develop new hypotheses.[1] Good computational models should be consistent with both observation and biophysical principle. Ideally, it should be possible to take smaller computational models, each a component of a larger system, and then construct a larger model to represent the complete system.[1] One further criterion of a good computational model is falsifiability, since, by Karl Popper's rationalism, a model that cannot be disproved should be considered unscientific.[2] Models that employ many adjustable parameters or constructions can thus be problematic, as they might conform to a large range of potential observables and thus not be falsifiable.

Computational models used to study cancer metastasis are as varied as the many facets of metastasis. Models have been developed to study the ways in which tumor cells can dissociate from the primary tumor to invade into their local microenvironment. Zaman et al.,[3,4] for example, created a three-dimensional model of tumor cell migration. Their model showed how adhesive forces, propulsive forces, and viscous effects from ligands could influence such movements. In another approach, Shields et al.[5] examined how transcellular gradients of chemotactic factors could affect the interstitial migration of tumor cells toward lymphatics. Frieboes et al.[6] simulated the growth of glioma spheroids and showed how their surfaces could be affected by cellular proliferation and cohesion. On the basis of their work, Frieboes et al. postulated that heterogeneities in oxygen and nutrient concentrations could have a major effect on tumor growth and invasion.

Other computational models have examined the clinical manifestation of metastases. Koscielny et al.[7] studied metastasis in 2,648 breast cancer patients, and proposed a model in which metastasis was initiated only after the primary tumor had reached a threshold volume. By means of Monte Carlo simulation, they were able to optimize growth times for metastases as a function of the doubling time of the tumor. Their analyses led them to conclude that clinically apparent metastases could be reduced by about one third if the primary cancers were treated a year earlier. In another model, Retsky et al.[8] assumed that the growth of breast cancer metastases passed through three sequential phases: an initial dormancy of isolated metastatic cells, a second phase of avascular replication, and a third phase of vascularized growth. Their model was used to explain a bimodal relapse distribution from 1,173 cases of untreated early breast cancer. By means of these simulations, Retsky et al. were able to explain how chemoresistance to adjuvant therapy leads to delayed relapse.

Still other models have concentrated on the sizes, numbers, and anatomic locations of metastases. Farshid et al.[9] examined how the intranodal distribution of metastatic foci from breast cancer could affect the histological sampling of sentinel lymph nodes. They compared nodal sectioning protocols in common use and found that these protocols had a high false-negative rate, particularly for small metastatic foci. In another study, Bernhardt et al.[10] examined how the burden of metastatic disease could affect cure after radionuclide therapy. They analyzed serial computed tomography (CT) scans of a single individual with hepatic metastases from hepatoma, and estimated the growth rate of the primary tumor and the rate of formation of metastases. By assuming that metastases grew at the same rate as the primary tumor, they were able to simulate

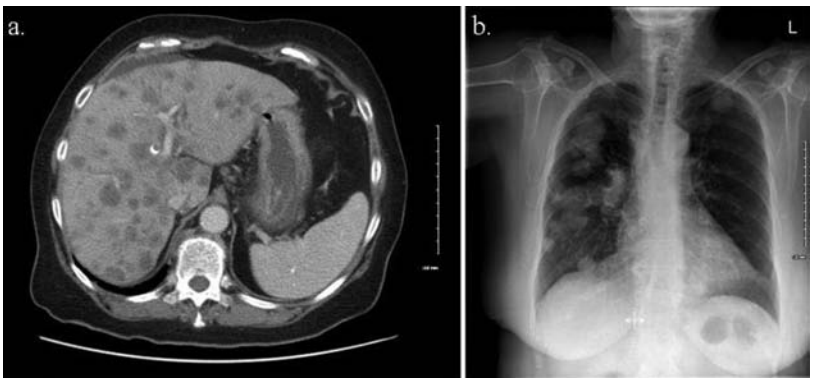

Figure 3.1. Clinical examples of metastases. (a) Hepatic metastases secondary to pancreatic adenocarcinoma. This computed tomogram demonstrates extensive multiple metastases that were present in this patient on initial staging. Note the variable sizes of the metastases and their spatially heterogeneous distribution within the liver. (b) Pulmonary metastases secondary to rectal adenocarcinoma. This chest X-ray documents advanced pulmonary metastases, which first appeared 5 years after definitive surgical resection. Note that these metastases also are variable in size and distributed unevenly within the lungs.

a size distribution for metastases, and, with this estimate, tumor control probabilities. Bernhardt et al. described further simulations, based on the assumption of a log-uniform distribution for the metastatic burden, and demonstrated how metastatic burden, cross-irradiation, and the type and concentration of radionuclide could affect cure.[11]

Bernhardt's studies[10,11] exemplify how the burden of metastatic disease can affect cancer treatment. In this chapter, factors related to this burden are examined in depth. The aim here is twofold: first, to present an alternative computational model for occult metastatic disease, and second, to provide an introductory tutorial for computational modeling. It is hoped that a reader who is unfamiliar with computational modeling might thus gain sufficient insight to begin to explore his or her own models. Because good computational models should be based on a clear understanding of the process of interest, this tutorial will begin with a review of the pathogenesis of metastasis.

BIOLOGICAL FOUNDATIONS OF METASTASIS

Certain individuals with cancer will develop extensive metastases (Figure 3.1). Why this happens to some individuals and not to others remains unclear, although much emphasis has been given to the role of the particular tumor's biology.[12–15] Most cancers are thought to start from a single stem cell from the tissue of origin.[16] Following this *transformation event*, a clone of cells may arise that has escaped normal tissue growth controls.[17] With further proliferation, an accumulation of somatically heritable changes and the selection of advantageous changes, subpopulations of cells might arise that are capable of invasion and metastasis, thus expressing a cancerous phenotype.[17] This process, leading to the development of a cancer, is called *carcinogenesis*. Clonal evolution can continue after carcinogenesis to allow further subpopulations of cells to develop and express growth and metastatic advantages.[18–20] Cancerous phenotypes are presumably determined by these accumulated genetic and epigenetic changes, subject to modulation by the local tissue microenvironment. In this context, when we speak of a particular tumor's biology, we are thus referring to these cell biologic properties and processes.

Cancer cells metastasize from the primary tumor to another part of the body by one of three means: direct extension, lymphatic spread, and hematogenous spread.[20] Hematogenous metastasis presumably reflects a series of discrete steps, starting with the transformation event, growth of the primary tumor, tumor angiogenesis, and vascularization, to be followed by tumor cell invasion through the vascular endothelium; embolization of tumor cells; their circulation, arrest, and adherence to the vascular endothelium of the target organ; and then the extravasation, proliferation, and angiogenesis within the nascent metastatic colony.[21] An analogous series of steps is also thought to occur with lymphogenous metastasis.[22]

There has been some debate as to whether metastasis to specific organs is a consequence of the growth advantage of selected tumor cells within certain tissues[23] or a consequence of the preferential organ blood flow to these tissues.[24] In fact, both mechanisms appear to be involved.[21,25] Videomicroscopy has further revealed that individual cancer cells can be efficiently entrapped within the hepatic and pulmonary vascular beds, where they subsequently can extravasate.[26] Only a small fraction of these extravasated cells eventually form micrometastases, although even fewer of these micrometastases become vascularized to form an expanding metastasis.[26]

It has been argued, by virtue of the propensity for metastases from certain tumors to affect particular organ targets, that metastasis is a nonrandom process.[21,23] Furthermore, the demonstration that some clones within tumors form metastases, whereas others do not, has also been used to argue for a selective element within metastasis.[21,27] Considering that the vascular transport and entrapment of cancer cells within organs is largely a passive and mechanical process[26,28] and that the rheological behavior of blood flow is variable,[29] there would seem to be an element of random

chance in the transfer of individual cells.[27] Moreover, whether a tumor cell that has extravasated within a distant organ will successfully form an expanding metastasis would also appear to influenced by chance, albeit modulated by intrinsic tumor and extrinsic organ factors.[26,27]

SIZE DISTRIBUTION OF METASTASES

The CT from Figure 3.1a shows that individual metastases within the liver can vary in size. As part of a quantitative estimate for the total burden of metastatic disease, it would be useful to derive a probability density function that could represent the sizes of metastases within an individual. In fact, Iwata et al. have provided us with such a function, based on the assumption that the rate of production of metastases is proportional to the number of tumor cells within the primary tumor in contact with the vascular tree, and that this tree should have a fractal structure.[30] They further argued that cellular proliferation should be described by a von Foerster growth equation, governed by Gompertzian kinetics, to yield a four-parameter model. This model agreed well with CT measurements from forty-eight hepatic metastases derived from a single case of hepatoma.

Another model was proposed by Hanin et al. in which metastasis is represented by a Poisson process with rate proportional to the size of the primary tumor.[31] Hanin et al. allowed for a variety of different growth kinetics. In the case of exponential growth, their model agreed well with CT/positron emission tomography (PET) measurements taken from thirty-one bone metastases in a single individual with breast cancer.

Both models described here were verified against measurements from CT or CT/PET images. However, clinical imaging by these means might not allow for particularly stringent testing of quantitative models. More precise measurements for the sizes of human metastases can be obtained by direct measurement from autopsy and surgical specimens. For example, Yamanami et al. used stereoscopic analysis of sequential formalin-fixed sections and light microscopic analysis of stained paraffin-embedded sections to measure hepatic metastases to within a fraction of a millimeter.[32] They provided data from thirty-one examples of primarily gastrointestinal tumors that gave a total of more than 968,000 metastases, and when analyzed, these data led Yamanami et al. to conclude that a lognormal distribution could represent the size distribution of metastases. Despite this extensive analysis, they did not offer any mechanistic explanation for their result.

Because of the size of their database, Yamanami et al. did not publish all their measurements. An earlier autopsy study by Douglas, however, provided a complete tabulation for the sizes of metastases from eighteen cases of human liver and lung metastases, secondary to cancers of the colon, rectum, breast, stomach, pancreas, lung, kidneys, and soft tissues.[33] These data furnished a further opportunity to test the lognormal distribution for the sizes of human hematogenous metastases.[34] Douglas's measurements were made to a tolerance of 1 mm for more than 3,900 macroscopic metastases. Because Douglas did not include metastases of less than 1 mm diameter, a truncated lognormal distribution had to be fitted to his data.[34] This truncated distribution had three parameters: one to define the truncation point and the other two being the shape and scale parameters of the lognormal distribution.

The lognormal size distribution can be explained mechanistically: Metastasis involves a series of discrete steps, taken from the transformation event, through each cell division, and to each step of invasion and metastasis. If the time periods required for each step are randomly distributed, mutually independent, and uniformly bounded, then the central limit theorem[35] would imply that the sum of these times should be normally distributed. Since the times of the transformation event and when the metastases are measured represent two defined points, and if the period of time from transformation to the initiation of each metastasis is normally distributed, then the time period remaining up to their measurement should also be normally distributed. If metastases are assumed to grow exponentially, it would then follow that their size distribution should be lognormal.[34] In this context, the growth of metastases within an individual can thus be described by modification of a pure birth process,[35] such that the growth times are normally distributed,[36]

$$N_i(t) = \int_0^\infty p_i(t)\frac{e^{-(t-\mu)^2/2\sigma^2}}{\sqrt{2\pi}\sigma}dt \Big/ \int_0^\infty \frac{e^{-(t-\mu)^2/2\sigma^2}}{\sqrt{2\pi}\sigma}dt \qquad (3.1)$$

$N_i(t)$ gives the probability that an expanding colony of tumor cells will contain i cells at time t, where μ is the mean growth time, and σ is its respective standard deviation. The next step toward a description of the burden of metastatic disease involves the description of the numbers of metastases sustained by individuals with cancer.

DISTRIBUTIONS FOR THE NUMBERS OF METASTASES

Lymphogenous Metastasis

An empirical probability density function for the number of involved lymph nodes within populations of breast cancer patients was obtained by analysis of fifteen published studies, which provided data from 24,757 axillary dissections.[37] These data agreed well with a negative binomial distribution. Two different

mechanisms could explain these findings: an *apparent contagion model*, in which the metastasis of metastases from involved nodes could seed uninvolved nodes along the lymphatic system, and a *spurious contagion model*, in which the number of involved nodes within any individual would be distributed in accordance with a Poisson distribution and the mean number of metastases would be influenced by population heterogeneity and be gamma distributed.[35]

A further examination of numbers of involved lymph nodes was derived from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) registry.[38] Data from 224,656 breast, 12,404 gastric, 18,015 rectal, 4,117 cervical, and 2,443 laryngeal cancers, as well as 9,118 melanoma cases, were reviewed. The numbers of both involved and sampled nodes obeyed negative binomial distributions. Since a negative binomial distribution for the number of sampled nodes could be more easily explained by population heterogeneity, this suggested that, to some degree, the numbers of involved nodes should be affected by a similar heterogeneity. Whether both mechanisms could contribute to the numbers of involved lymph nodes remains to be determined.

Hematogenous Metastasis

A model for the distribution of the numbers of organ metastases was proposed by Michor et al., based on the assumption that only a single mutation is required to render a tumor cell capable of metastasizing.[39] A further assumption, that the number of established metastases is proportional to the number of metastatically capable cells within the primary tumor, allowed them to derive a frequency distribution for the numbers of metastases. This model was presented in theory only, without comparison with observed data.

Observations are important, as they can provide an insight into the nature of this distribution and a means to test one's hypotheses. Experimental metastasis data have revealed considerable variability in the numbers of experimental metastases sustained by identically treated mice. Such data indicated clustering, whereby certain mice from the same treatment group sustained more metastases than can be expected from Poisson-distributed data.[40,41] There is another test for clustering, used commonly by population ecologists, that provides further insight into hematogenous metastases.[42] This test is based on the relationship between the variance of the number of metastases per animal σ^2, calculated from groups of identically treated animals, and the respective mean number of metastases μ. If this relationship takes the form of a power function $\sigma^2 = a\mu^b$ (with the constant of proportionality a and the exponent b), the exponent can be used to test for clustering. When $b > 1$, clustering exists; if b and a both equal 1, this would be consistent with a Poisson distribution. Plotting the logarithms of the variances and means can easily test for the power function. We then have $\log(\sigma^2) = \log(a) + b\log(\mu)$, which gives a linear relationship with slope b.

Preliminary tests showed that murine experimental metastasis data agreed with this power function, and that b ranged between 1.3 and 1.7.[41] Further collective analyses of twenty-two published experimental metastasis studies (with a total of 2,145 mice), and eight published spontaneous metastasis studies (with 1,020 mice) also revealed power functions, with the exponents both equal to $b = 1.5$.[43] The power function was also demonstrated with data from thirty-three published pathology studies of human metastasis, which involved 5,582 cases.[44]

According to the mechanical theory of metastasis, cancer metastases are deposited in proportion to regional blood flow.[26,28] Regional organ blood flow can be measured by the intravenous injection of radiolabeled microspheres, which are passively carried in the venous circulation to lodge in the vascular bed of organs. The target organ is then removed and cut into equal-sized sections, the radioactivity in each piece is quantitated, and the regional blood flow sustained over the injection period is thus determined. The relative dispersion of blood flow $RD(m) \equiv \sigma/\mu$ (i.e., the ratio of the standard deviation to its mean) for tissue samples of mass m has been empirically shown to obey the relationship,

$$RD(m) = RD(m_{ref})\left[\frac{m}{m_{ref}}\right]^{1-D}, \qquad (3.2)$$

where m_{ref} is a reference mass, and the exponent D is the spatial fractal dimension.[45] This power function relationship has been confirmed empirically for the heart,[45] lung,[46] brain,[47] skeletal muscle,[48] and other organs. Equation 3.2 can be related to the variance to mean power function $\sigma^2 = a\mu^b$, provided that $b = 4 - 2D$.[49] The observed range for the fractal dimension D in blood flow experiments is $1 < D < 1.5$, which corresponds to the range $1 < b < 2$ observed from metastasis studies.[49] One might then ask whether the variability in numbers of metastases could be at least partly attributed to blood flow heterogeneity.[50]

The equation for regional blood flow heterogeneity (Eq. 3.2) can be explained by a mechanistic model based on the theory of *exponential dispersion models*.[49] This theory was originally developed to describe error distributions for generalized linear models.[51] Particularly germane to the blood flow model is a family of exponential dispersion models characterized by the mathematical properties of scale invariance, additivity, and reproducibility that are known as the *Tweedie exponential dispersion models*, in honor of the mathematician who first described them.[52] The Tweedie models

exhibit a power function relationship between the variance and the mean. One of these models, the compound *Poisson-gamma* (PG) *model*, is further characterized by its power function exponent b being constrained between 1 and 2. In its additive form, the PG model has the cumulant generating function,

$$K^*(s) = \lambda\kappa(\theta)\left\{\left(1+\frac{s}{\theta}\right)^{\alpha}-1\right\}. \tag{3.3}$$

In this equation, s is an abstract parameter on which the generating function is constructed, λ is the index parameter, θ is the canonical parameter, the cumulant function is

$$\kappa(\theta) = \frac{\alpha-1}{\alpha}\left(\frac{\theta}{\alpha-1}\right)^{\alpha}, \tag{3.4}$$

and the constant α relates to the exponent from the variance to mean power function, $\alpha = (b-2)/(b-1)$. The probability density function corresponding to Eq. 3.3, $P^*(z;\,\theta,\,\lambda,\,\alpha)$, is expressed in terms of an infinite series,[51]

$$P^*(z;\theta,\lambda,\alpha) = c^*(z;\,\lambda)\cdot\exp\left[\theta\cdot z-\lambda\kappa\,(\theta)\right],\ \text{with}$$

$$c^*(z;\lambda) = \begin{cases} \dfrac{1}{z}\displaystyle\sum_{n=1}^{\infty}\lambda^n\cdot\left[\left(\frac{\alpha-1}{\alpha}\right)\cdot\left(\frac{-1}{(\alpha-1)\cdot z}\right)^{\alpha}\right]^n \\ \qquad /\left[\Gamma\,(-\alpha\cdot n)\cdot n!\right] & \text{for } z>0, \text{ and} \\ 1 & \text{for } z=0. \end{cases} \tag{3.5}$$

Equation 3.3 yields a variance to mean power function with the form $\sigma^2 = \lambda^{1/(\alpha-1)}\mu^b$.

From the algebra of generating functions, Eq. 3.3 implies a compound Poisson distribution generated by a gamma distribution. That is, this equation specifies the summation (convolution) of N number of identical and independently distributed random variables, which themselves obey a gamma distribution, such that N itself is a random variable governed by the Poisson distribution. One could postulate a blood flow model whereby, within equal-sized samples of tissue, there exists a random (Poisson-distributed) number of potential vascular entrapment sites through which the local blood flow (mL/min) obeys a gamma distribution. The probability that an injected microsphere can become entrapped would be directly related to the blood flow through the entrapment sites.[49] If this blood flow were required to be scale invariant and additive, then this model would obey Eq. 3.3.

One could thus propose that the frequency distribution for the number of metastases should be affected by Poisson statistics, modulated by stochastic variations in regional blood flow.[53] The distribution of numbers of metastases per individual would then be described by the discrete analog to the PG distribution, with the probability-generating function,

$$\begin{aligned} G(s) &\equiv \sum_{n=0}^{\infty} p_n\cdot s^n \\ &= \exp\left[\lambda\frac{\alpha-1}{\alpha}\left(\frac{\theta}{\alpha-1}\right)^{\alpha}\left\{\left(1-\frac{1}{\theta}-\frac{s}{\theta}\right)^{\alpha}-1\right\}\right]. \end{aligned} \tag{3.6}$$

This generating function describes the *Poisson negative binomial* (PNB) *distribution*,[53] with mean $\mu = \lambda\left[\theta/(\alpha-1)\right]^{\alpha-1}$. The variance-to-mean relationship for this PNB distribution is

$$\sigma^2 = \lambda^{1/(\alpha-1)}\mu^b + \mu. \tag{3.7}$$

When tested against real data, Eq. 3.7 is practically indistinguishable from the variance-to-mean power function $\sigma^2 = \lambda^{1/(\alpha-1)}\mu^b$. One may take the Taylor series expansion of Eq. 3.6 to obtain its probability density function P_n,

$$P_n = \frac{1}{n!}\frac{\partial^n G(s)}{\partial s^n}\bigg|_{s=0}. \tag{3.8}$$

Its cumulative distribution function Q_n can then be obtained by the summation of the sequential terms of P_n,

$$Q_n = \sum_{i=0}^{i=n} P_i. \tag{3.9}$$

Numerical evaluations of these probability density and cumulative distribution functions can be derived using a symbolic manipulating program such as MAPLE (Waterloo Maple Inc., Waterloo, Ontario, Canada).[53]

The variance-to-mean relationship in Eq. 3.7 can be fitted to the experimental metastasis data from B16 murine melanoma.[41] Indeed, the power function seemed to agree better with such data than other variance-to-mean relationships, such as those corresponding to the Poisson, geometric, or negative binomial distributions. But by itself, an acceptable fit of the variance-to-mean power function does not provide a compelling reason to accept the PNB model. Nevertheless, further evidence in favor of this model comes by comparison with the empirical cumulative distribution functions (derived from these data) with the theoretical PNB model.[53]

In this metastasis model, the total blood flow through restrictive sites in the target organ's microcirculation is assumed to be governed by the PG exponential dispersion model, Eq. 3.3;[49] the number of tumor cell emboli is then proportional to the blood flow within the target organ and the number of metastases per organ is Poisson-distributed, with intensity proportional to the number of injected tumor cells, the metastatic efficiency, and the blood flow through the organ. These assumptions seemed reasonable: We

know that plastic microspheres are passively carried and entrapped within the circulation in direct proportion to blood flow.[54] Human autopsy studies have shown that the anatomic distribution of organ metastases correlates with blood flow.[55] Videomicroscopic studies have shown that tumor cells of diameter 10 to 15 μm can be entrapped in the microcirculation to eventually form metastases,[56] much in the same way as plastic microspheres of the same diameter become entrapped.[54] Moreover, the PG model for regional organ blood flow[49] is supported by physiological studies[45] that have repeatedly demonstrated the scaling relationship that it implies (Eq. 3.2).

With mathematical models thus identified for both the number and size distributions for metastases, computational models to describe the burden of metastatic disease can now be described. Before we go on to these descriptions, it would be helpful to briefly review the simulation method to be employed.

COMPUTATIONAL MODELS FOR THE BURDEN OF METASTATIC DISEASE

Monte Carlo Method

Monte Carlo simulations employed here to execute these computational models were based on the *inverse transform method*: [57] Given the probability density function $f(x_k)$, with discrete random variable x_k, one may integrate this density over the domain $0 \leq x \leq a$ to give a step function $F(x_k)$. We then have a function that expresses a series of discontinuous jumps at the points x_k that have the magnitude $f(x_k)$, and that take the values $u = F(x)$. If we generate pseudorandom numbers from a uniform distribution in the range $0 \leq u \leq 1$, we can estimate the inverse function $x = F^{-1}(u)$ for each value of u. We thus specify x_k so that $F(x_{k-1}) < u < F(x_k) \equiv \text{Prob}(x_{k-1} < x < x_k)$.

The software package Mathcad 2001*i* Professional (MathSoft Engineering & Education, Inc., Cambridge, MA) was used to execute these simulations. Parenthetically, it should be mentioned that the inverse transform method does not allow for the description of scale invariant correlations that might arise between events determined by the PG model (Eq. 3.3);[58] nevertheless, such correlations were not critical to the metastasis model presented here, and thus this method was used as a first approximation.

Modeling Chance Events in Metastasis

Simulation provides a means to study the contribution of random chance to metastasis. Earlier in this chapter, models were identified to describe both the numbers and sizes of metastases. Clinical experience shows that the number of metastases that have been resected with curative intent can predict survival in human cancers[59–63] and that the total volume of resected disease is similarly predictive.[64] Both clinicians[32,65–68] and experimentalists[40,69–72] believe that the number of metastases provides some indication of the intrinsic biological behavior of the cancer at hand. However, the inferences derived from the number of metastases have at times differed between these two groups. Experimentalists such as Fidler and Kripke[40] have shown that the number of experimental metastases varies considerably among identically treated mice, and thus their assessments of malignant potential relied on observations from multiple animals as well as statistical inference. On the other hand, clinicians have sometimes attempted to infer the malignant potential of tumors for individual patients, under the implicit assumption that spurious variability can be ignored.

To determine the degree to which spurious influences might affect such assessments, simulations were performed to estimate the number of metastases within individuals under conditions in which the intrinsic metastatic potential of the tumor's cells, the influence of growth and angiogenic factors, the emergence of variant tumor cells, the hosts' immune systems, and other such factors could be assumed constant. The Monte Carlo method was first applied to the PNB model (Eq. 3.6) to estimate the number of metastases in sixty cases, and the results were compared to Fidler and Kripke's experimental metastasis data.[40] In these simulations the parameters were chosen to emulate B16 clones of lower malignant potential. Indeed, Kruskal-Wallis analysis revealed no significant differences between the simulated data and the less malignant clones *a* through *e* (Figure 3.2).

With the initial parameters thus chosen, Monte Carlo simulations were repeated to estimate the numbers of hematogenous metastases within 4,000 individuals (Figure 3.3a).[36] The resultant numbers of metastases ranged widely. Some cases yielded more than forty metastases; others had none. Because the simulation parameters were the same for each case, this implied that the biological conditions had been constant and the observed variation in numbers of metastases was attributable to chance events alone.

Further simulations were performed to estimate the volumes of the metastases (Figure 3.3b). The growth of each metastasis was modeled after a stochastic birth process with normally distributed growth times (Eq. 3.1). It was assumed that the metastases had growth times of 16 ± 1 volume doublings, up until the resection of the primary tumor, and that the time from surgical resection to assessment represented a further twelve doublings. The growth rates of all metastases were assumed to be the same as for the primary cancer. Under these conditions, the metastases ranged in size from 10^5 to 10^{10} cells.

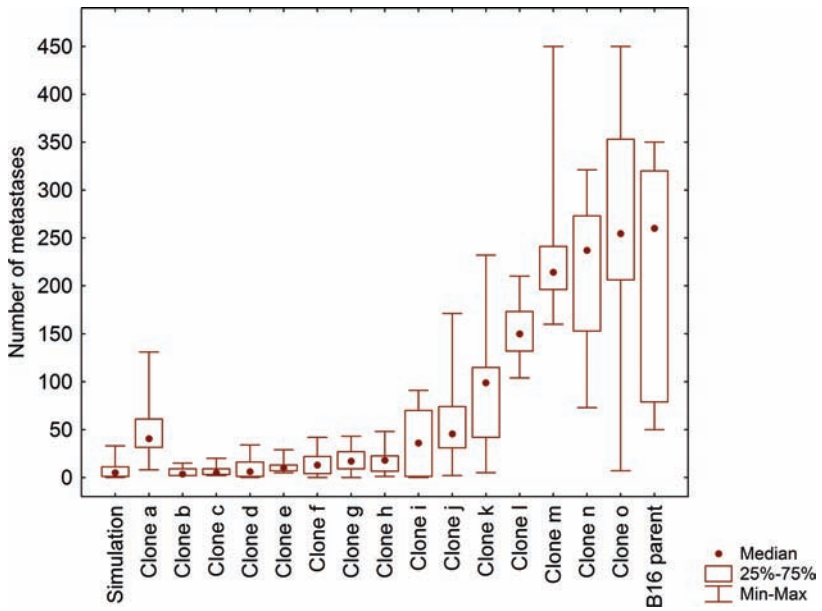

Figure 3.2. Experimental metastasis assays from B16 murine melanoma compared with simulation. Sixty simulations were performed using the PNB model for hematogenous metastases (Eq. 3.6) to predict the numbers of metastases within identically treated mice, with the parameters $\theta = -0.3307$, $\alpha = -1.030$, $\lambda = 0.200$, $\mu = 7.958$, $\sigma^2 = 56.81$, and $b = 1.493$. The predicted median and range for the numbers of metastases were compared with observations from parental and cloned B16 murine melanoma.[40] A Kruskal-Wallis analysis of variance, performed between the simulated and observed data, revealed significant differences ($H = 190$, $N = 263$, $d.f. = 16$, $p < 0.001$). However, when the Kruskal-Wallis analysis was restricted to the simulated data, and clones *a* through *e*, there was no statistically significant difference ($H = 10.05$, $N = 109$, $d.f. = 5$, $p = 0.07$). The simulated data thus were similar to those from B16 murine melanoma clones of low malignant potential.

The number and sizes of metastases for a series of fifty individuals were estimated.[36] Figure 3.4a gives the number of metastases sustained by each individual, ranked in increasing order. Below that, in Figure 3.4b, the sizes of the metastases corresponding to each individual are provided. Given a clinical detection threshold of 10^9 cells (i.e., 1 cm^3), most of these metastases would have been occult. Of course, if additional time had been allowed between surgical resection and assessment, a larger proportion of these metastases would have been detectable.

Figure 3.5 gives the percentage of clinically detectable metastases, plotted versus the time interval after resection of the primary tumor. Under the conditions assumed for these simulations, there was a latent period of about twelve volume doublings with essentially no detectable metastases, followed by a period of about three doublings during which most of the metastases would have become manifest. This simulation emulated the history of the individual recounted in Figure 3.1b, who had sustained a rapid appearance of multiple metastases some time after removal of the primary tumor. Such a rapid appearance of multiple metastases some time after the initial surgery of the primary tumor thus might, at least partly, be attributable to the growth time distribution of the metastases.

Burden of Metastatic Disease

The burden of metastatic disease is a major determinant for the success of cancer therapy.[73] Surgical extirpation of the primary cancer may be all that is required for cure, provided there are no residual metastases; if there are only limited metastases, it may be possible to excise them for cure.[59,74,75] In addition, for individuals with clinically occult metastases, cure may still be achieved with adjuvant therapy granted that the burden of disease is small, and that effective therapy exists.[76]

Provided acceptable models for both the numbers and sizes of metastases, it should be possible to estimate the form of the distribution for the numbers of metastatic cells per individual. Radiobiologists have conventionally hypothesized a log-uniform distribution for this purpose, in which the logarithm of the number of metastatic cells obeys a uniform distribution.[77] Justification for this hypothesis came nonmechanistically, from an assumed exponential growth of tumors and the belief that the number of metastatic cells can range from zero to some very large number.[77] This log-uniform distribution has been employed widely in radiobiological assessments.[10,11,78–80]

An alternative model for the burden of occult metastatic disease is proposed here. Let $N(s;\, t)$ represent the probability-generating function for the size distribution of metastases at time t, as measured in terms of the number of tumor cells. Using Eq. 3.1 we would then have[81]

$$N(s;\, t) = \sum_{i=0}^{\infty} N_i(t) s^i. \tag{3.10}$$

On the basis of the results presented here, there are at least three possible probability-generating functions for the numbers of metastases: the PNB-generating function $G(s)$, for hematogenous metastases; the Poisson distribution-generating function $M(s) = e^{\mu(s-1)}$, for lymphatic metastases within isolated individuals (assuming one metastasis per involved lymph node and no metastasis of metastases); and the negative binomial-generating function $H(s) = \{p/[1-(1-p)s]\}^k$, for lymphatic metastases[38] sustained by a heterogenous

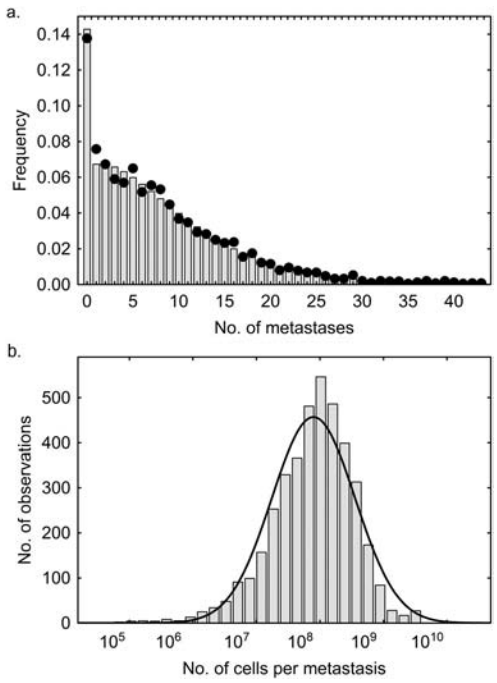

Figure 3.3. Metastasis simulations.* **(a)** Simulated numbers of metastases. Four thousand simulations were performed in accordance with the PNB model for hematogenous metastases parameterized in Figure 3.2. The hatched bars represent the theoretical PNB distribution, from which the simulations were based; the solid dots, the frequency histogram for the simulated metastases. The simulations were designed to provide a fraction (14%) of cases with no metastases, and other values consistent with B16 murine melanoma clones of low malignant potential, as well as other human tumors of similar behavior.[44] Even with this specification, a small number of the simulated cases sustained more than 40 metastases. **(b) Simulated sizes of metastases.** Four thousand simulations were performed, assuming a total growth time of 28 ± 1 volume doublings. With this parameterization the frequency histogram based on the logarithms of the numbers of cells per metastasis (hatched bars) approximated a normal distribution (solid line).
*Figures modified from Kendal 2005[36] and reproduced with permission of the author.

population of individuals (and/or the presence of metastasis of metastases).

Assume that all metastases are the result of seeding from the primary tumor, and that the growth of each metastasis is independent of other metastases. Let $N^{(j)}$ represent the number of tumor cells within the jth metastasis and let J represent the number of metastases within an individual. We have for the total number of metastatic tumor cells within the individual T,

$$T = N^{(1)} + N^{(2)} + \cdots + N^{(J)}. \qquad (3.11)$$

J is a random variable that is specified by one of the three generating functions: $G(s)$, $M(s)$, or $H(s)$. Because Eq. 3.10 represents the sum of a random number of identical and independently distributed random variables, we can use the algebra of generating functions[35] to construct the compound generating functions $G(N(s))$, $M(N(s))$, and $H(N(s))$ for the total number of metastatic cells. There is currently no known way to determine these corresponding probability density functions in closed form; we will thus rely here on Monte Carlo simulation to estimate these numbers.[81]

Figure 3.6 provides the results of 1,000 simulations used to estimate the distributions for the number of occult residual metastatic cells. Figures 3.6a and 3.6b represent simulations for lymphogenous metastases in individuals (with no metastasis of metastases); Figures 3.6c,d represent hematogenous metastases, and Figures 3.6e,f represent lymphogenous metastases within a population. Histograms were constructed for each case, in which the numbers of metastatic cells were

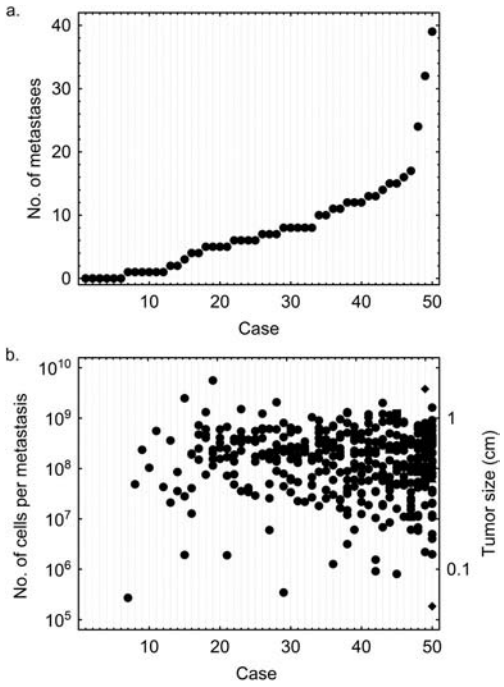

Figure 3.4. The residual burden of metastatic disease.* Fifty simulations were performed where the cellular and growth conditions of the tumors were assumed to be the same, and where the primary tumors were successfully resected. Parameterizations were kept the same as specified for Figure 3.2. The numbers and sizes of metastases were determined for each case. **(a) Numbers of metastases.** The metastases predicted for each case are plotted here, ranked in order of increasing number. Six cases sustained no metastases, whereas the remaining cases sustained anywhere from 1 to nearly 40 metastases. **(b) Number of tumor cells per metastasis.** The number of tumor cells per metastasis was simulated for each metastasis represented in Figure 3.4a. Each data point represents one metastasis, and the data corresponding to each case are provided as a scatter plot in line with corresponding ranked cases of Figure 3.4a. The number of tumor cells per metastasis is given on semilogarithmic coordinates (left vertical axis), along with the respective sizes of the metastases (right vertical axis). If the clinical detection threshold was 10^9 cells (1 cm^3), the majority of these metastases would be occult.
*Figures modified from Kendal 2005[36] and reproduced with permission of the author.

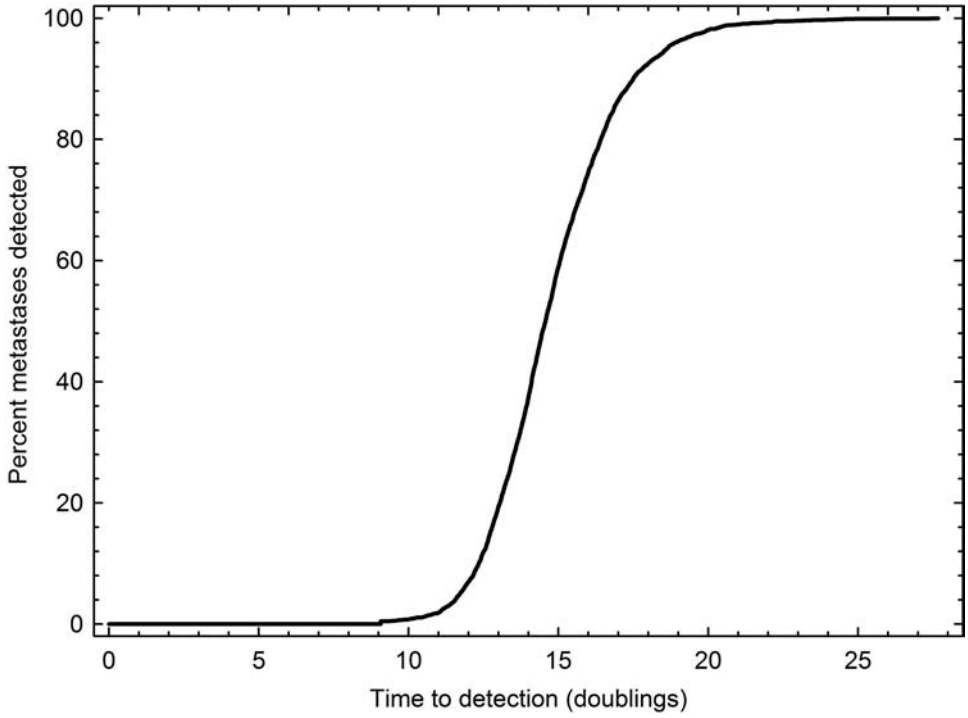

Figure 3.5. The time course of clinically detectable metastases.* Four thousand simulations were performed as described in Figure 3.2, but the measurement times were extended to allow further growth of the metastases. The percentage of clinically detectable metastases is plotted versus time to detection, assuming a detection threshold of 10^9 cells. There was a period between 10 and 20 volume doublings in which the percentage of detectable metastases increased relatively rapidly.
*Figures modified from Kendal 2005[36] and reproduced with permission of the author.

apportioned into equal-sized counting bins, specified in terms of the number of metastatic cells per individual, or the base 10 logarithm of that number. In accordance with the log-uniform hypothesis,[77] one would predict a uniform distribution on the semilogarithmic plots (Figures 3.6b,d,f). In fact, however, all three of these semilogarithmic plots were more consistent with normal distributions.[81] A lognormal distribution thus seemed more appropriate for the burden of occult metastatic disease in these cases.

This result should be considered preliminary. The simulations would have to be repeated over a wide range of parameters to determine the full range of applicability of the lognormal distribution to such situations. (At the time of writing this chapter, this work had not yet been done.) Simulations like these can potentially be applied to predict the results of cancer treatment.

How Well Do These Simulations Represent Reality?

These simulations were designed to emulate the spurious variability observed in both the numbers and sizes of metastases, under otherwise constant biological conditions. The lognormal size distribution used here was derived from observations of various human breast, gastrointestinal, and lung carcinomas, as well as soft tissue sarcomas from fifty pathological specimens with more than 970,000 metastases.[32,34] The models for the number of lymph node metastases were derived from studies of more than 295,000 human carcinomas of the larynx, stomach, rectum, breast, and cervix, as well as melanoma.[37,38] Furthermore, the model for the number of hematogenous metastases was derived from studies of more than 3,100 mice with more than 47,000 metastases from various adenocarcinomas, sarcomas, and melanomas,[41,43] and more than 1,400 humans with more than 992,000 metastases from head and neck, lung, breast, gynecological, and gastrointestinal carcinomas, as well as melanomas and sarcomas.[44]

One might be tempted to dismiss the mathematical models thus attained as the result of just curve fitting, citing that a variety of other models might acceptably fit these data. However, the mathematical models employed here were also based on plausible biophysical mechanisms: the size distribution for metastases assumed that the growth times for metastases within an individual should be normally distributed.[34] This was justified by the central limit theorem's presumed effect on the summed time increments for the sequential steps of metastasis. The Poisson distribution for the number of involved lymph nodes within individuals was justified by its applicability to counting statistics, in situations with minimal metastasis of metastases.[35]

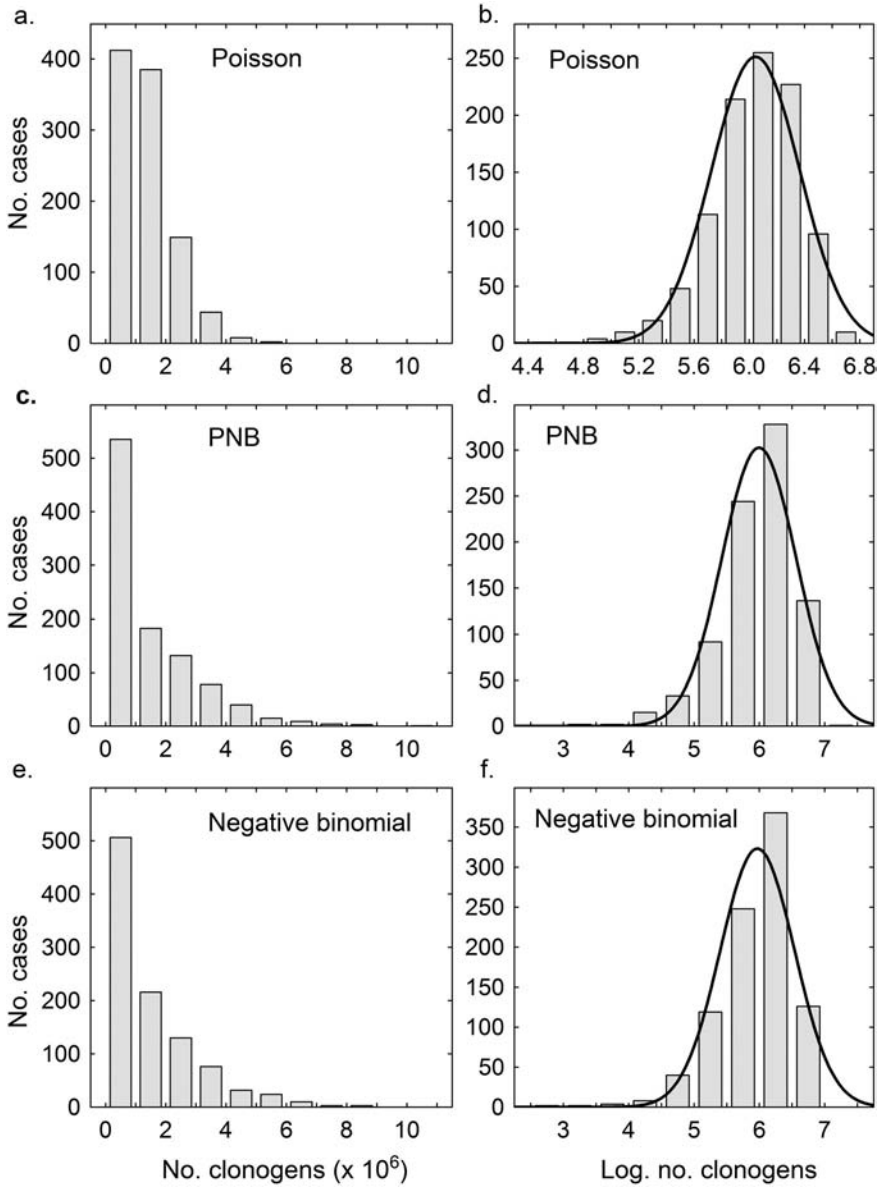

Figure 3.6. A lognormal approximation for the burden of residual metastatic disease.* One thousand simulations were performed to estimate the residual burden of metastatic disease after successful surgical removal of the primary tumor. The simulations were conducted for each of three situations: lymph node metastasis in identical individuals, hematogenous metastases within identical individuals, and lymph node metastases within a heterogeneous population of individuals. **(a) Frequency histogram for the burden of lymphatic metastases in individuals.** The numbers of nodal metastases were assumed to obey Poisson statistics; the sizes of the metastases, lognormal statistics. The frequencies of individuals with specified numbers of residual tumor cells were enumerated using equal-sized counting bins, demarcated for incremental increases of a thousand tumor cells (shaded bars). **(b) Lognormal approximation for lymphatic metastases in individuals.** The data from Figure 3.6a were redistributed among counting bins defined by the logarithm of the number of residual tumor cells. Each bin therefore represented an order of magnitude increase in the number of tumor cells. The bell-shaped solid line represents a normal distribution fitted to the logarithmically transformed histogram (hatched bars). **(c) Frequency histogram for the burden of hematogenous metastases in individuals.** The numbers of metastases were assumed to obey the Poisson negative binomial (PNB) distribution; the sizes of metastases, lognormal statistics. The frequency histogram (hatched bars) was based on a linear scale with equal-sized counting bins. **(d) Lognormal approximation for hematogenous metastases in individuals.** The data from Figure 3.6c were redistributed among counting bins based on increments of an order of magnitude. On this logarithmically transformed scale the redistributed histogram (hatched bars) revealed an approximate agreement with a fitted normal distribution (solid line). **(e) Frequency histogram for the burden of lymphatic metastases in a heterogeneous population.** The numbers of lymphatic metastases were assumed to obey the negative binomial distribution; the sizes of metastases, lognormal statistics. The frequency histogram (hatched bars) was based on a linear scale with equal-sized counting bins. **(f)** Lognormal approximation for lymphatic metastases in a population. The data from Figure 3.6e were redistributed among counting bins based on increments of a order of magnitude. The solid line represents a fitted normal distribution which agreed approximately with the frequency histogram (hatched bars).

*Figures modified from Kendal 2007[81] and reproduced with permission of Taylor & Francis Journals.

The negative binomial distribution for the number of involved lymph nodes was justified for heterogeneous populations and/or significant metastasis of metastases. The gamma distribution, used to represent the population heterogeneity associated with the negative binomial distribution,[82] could be justified by the convergence of exponential mixtures used to describe the frequency of nodal failure.[83] For hematogenous metastasis, the numbers of metastases were based on Poisson counting statistics modulated by the heterogeneity of regional blood flow.

The choices of parameters used in these models were consistent with observation. The means and variances for the numbers of metastases were within the range of observation, as was the power function exponent employed in Eq. 3.7. Detailed measurements regarding the growth kinetics of metastases within individuals, however, were not available; growth times were assigned arbitrarily, and thus further research with different parameterizations is warranted. Nonetheless, the models employed here were both plausible as first approximations, and potentially falsifiable. Within the limits of our current understanding of metastasis, these simulations could therefore be considered both appropriate and representative.

CONCLUSION

Much has been written about the nonrandom nature of metastasis,[84–89] particularly with respect to the seed and soil hypothesis[21,23] and anatomic routes of spread.[22,28] However, the role of chance in metastasis has not been as thoroughly examined. Laboratory studies, such as those of Fidler and Kripke[40] and others,[69–72] have revealed that there is considerable spurious variability associated with metastasis. One could conclude from such laboratory work that the total metastatic burden sustained by an individual reflects the combined influence of the tumor's phenotype, its interactions with the host, and its natural history, as well as random events. The simulations provided herein demonstrate that these random events can be a major determinant to the burden of disease. Indeed, as suggested later in this section, characterization of these random events provides a means to understand the underlying biophysical mechanisms and permits realistic computational modeling of the processes involved.

Earlier in this chapter it was mentioned how clinicians sometimes will attempt to characterize the biological behavior of an individual's tumor from the number and bulk of metastases.[65–68] Granted, these measures have prognostic importance;[59–64] however, they do not necessarily imply a more aggressive metastatic potential. Laboratory assessments of metastatic potential have therefore come to rely on multiple replicate experiments to compensate for random effects,[40,69–72] but in the clinics one deals with individuals. Any individual assessment of the biological properties of the tumor would consequently require more information than can be garnered from the burden of disease alone. Furthermore, any attempt to redefine such biological properties to include chance effects represents a confounding of basic biological and physical processes.

Erwin Schrödinger considered the distinction between biological and physical processes at some length, particularly in relation to the way random disorder enters into physiological systems.[90] Chemical reactions, which form the basis of life, are driven by the thermal motion of atoms, which leads to a perpetual introduction of randomness to biological systems. The precision of these reactions depends on the number of atoms and molecules involved. In most cellular processes, these numbers are sufficiently large to provide for predictability in the essential processes. Nevertheless, the fidelity of DNA replication and other molecular processes can be significantly influenced by chance events. At larger scales, biological systems may sustain an element of unpredictability consequent to nonlinear interactions, as might be seen with turbulent fluid flow,[91] diffusive growth,[92] and other nonequilibrium systems.[93] In this chapter use of the term "random" was implicitly intended to include any unpredictability that might arise from such chaotic processes.[91] The point to be emphasized here is that this randomness can be attributed to physical processes.

Metastasis was described earlier in this chapter as a complex sequence of biological and physical processes. The factors that influence the numbers and sizes of metastases presumably reflect the combined action of these multiple processes, each with its own potentially spurious contribution. To a certain extent, when the effects of such multiple processes are summed together, the end result can be predictable. When many small and independent random measurements are added together, the central limit theorem would predict that the sum should be normally distributed, even if the individual measurements are not. For this reason, many complex natural processes reveal measurable features that are normally distributed.

The central limit theorem can be generalized in a number of different ways.[94] In one approach, the Tweedie exponential dispersion models can be shown to serve as the foci of convergence for the error distributions of a wide range of statistical models.[51] These Tweedie models include the normal distribution, used here to justify the lognormal size distribution for metastases; the PG model, used to describe blood flow heterogeneity; the Poisson distribution, used to describe nodal metastases in individuals; and the gamma distribution, used to describe populational heterogeneity. One additional (and unrelated) convergence property was used in this chapter to justify the lognormal

distribution for the burden of residual metastatic disease, based on the approximate convergence of summed lognormal size distributions for metastases.[95] Such convergence behaviors, particularly those seen with the Tweedie models, reflect properties of complex systems, and, indeed, appear to underlie many other biological processes.[58,96–98]

Earlier, it was mentioned that the Tweedie models were characterized by their scale invariance, additivity, and reproducibility. Scale invariance would seem a desirable property for models such as one for blood flow, as such a model would remain valid with different measurement scales and units. Additivity would similarly seem desirable for the numbers of metastases, as one might expect the same statistical rules to apply to metastases for equal-sized portions of an organ as for the whole organ and, in the case of paired organs, for the right and left sides counted together. Likewise, reproducibility would seem to be important, as the mean number of metastases, obtained from a volume-weighted average, presumably should be governed by the similar statistical rules as for the individual samples. Moreover, these properties provide additional aspects of these random processes that are predictable, and potentially amenable to experimental scrutiny.

These convergence and transformational properties provided theoretical justification for the choice of models used here, and for the exclusion of alternative models. To be sure, the negative binomial distribution (used here to describe the numbers of axillary metastases within populations) is not scale invariant, additive, or reproductive. However, negative binomial distributions from populational subgroups can summed to approximately yield a negative binomial distribution for the whole populations.[99] This approximation likely reflects the transformational properties of the gamma distribution, the continuous analog to the negative binomial distribution, and which is itself scale invariant, additive, and reproductive.[51] Similarly, the PNB distribution (used to describe the numbers of hematogenous metastases) fails to meet these criteria, whereas its continuous analog, the PG distribution, does. Thus, to a certain extent, such approximate behavior can reflect a more fundamental and concordant underlying process.

It is relatively easy to propose a computational model to describe a biological process; it is much more difficult to propose a model that is both representative of that process and useful. One way to ensure that a model is appropriate to the situation at hand would be to derive it from a large body of empirical observation, and to firmly base it on biophysical and statistical principles. Biological processes, however, can be complicated and cryptic. When one is investigating processes such as these, mathematical artifice should not substitute for sound empiricism.

At the heart of any computational model lies the method of simulation.[1,100] Many computational models employ the Monte Carlo method, which may be based on various random number generators to provide a uniform distribution of statistically independent numbers ranging between 0 and 1.[57] The approach employed here was to empirically identify a candidate distribution function to characterize the randomness inherent to the process of interest, and then to use the inverse transform method on this uniform distribution to emulate the process. As mentioned earlier, this approach has its limitations, particularly with nonlinear processes. The variance to mean power function $\sigma^2 = a\mu^b$, evident to the blood flow model and to the clustering of hematogenous metastases, can be shown algebraically to indicate correlations between adjacent events.[58] Conventional random number generators, with their statistically independent output, cannot account for such correlations. Scale invariant correlations, implied by such clustered data, can be simulated by alternative random number generators.[58] However, much theoretical work still needs to be done in this area, particularly with the foundations of the Tweedie models.

Computational models have not been exploited in the study of cancer metastasis as much as their potential could allow. We have seen here one approach to the modeling of this complex process. To summarize this approach, cancer metastasis has major underlying random components that can be empirically characterized. Probabilistic models were chosen to represent metastasis by their agreement with these observations, as well as with their transformational and convergence properties. These models were, in turn, explained in terms of plausible biophysical mechanisms and explored by *in silico* analysis. The inherent randomness, thus understood, has furthered our insight into metastasis.

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4 Intravital Microscopy to Visualize Invasion and Metastasis

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INTRODUCTION

Metastasis is a complex process involving the acquisition of cell motility, intravasation into blood and lymphatic vessels, survival in the vasculature, extravasation, and proliferation in the new tissue (Chambers et al. 2002). The study of metastasis has been hampered by several factors, however. In most studies, metastasis is evaluated by the presence of large metastases in fixed material. Although this is a reliable measure of the overall process, however, it provides little information about the various steps of the metastatic process.

Furthermore, metastasis usually happens in anatomical locations that are difficult to access, which makes observation of the various steps in the process difficult. This problem is compounded by the fact that relatively few cells from a primary tumor will form metastases. A typical metastatic primary tumor will contain tens of millions of cells but give rise to a much smaller number of metastases. Therefore, finding the small numbers of cells actually involved in the process of metastasizing is not straightforward. One approach to overcoming these problems is to perform high-resolution optical imaging of live tissue containing metastatic cancer cells, a technique called *intravital imaging* (Condeelis and Segall 2003; Sahai 2007).

This chapter describes how intravital imaging has contributed to our understanding of metastasis. The intravital imaging techniques that can be used to visualize events within tumors are introduced, followed by a discussion of the ways in which these techniques have provided new insights into the process of metastasis.

INTRAVITAL IMAGING METHODS

Intravital imaging is critically dependent on the way the tumor is generated, the equipment used to capture the images, and methods for labeling components of the tumor. These are described in the following sections.

Generating Tumors to Image

Injection of tumor cells. The most common method used for generating tumors to image involves the injection of tumor cell lines into experimental animals, usually mice, to generate xenograft tumors. Frequently, cancer cells are injected under the skin of mice, and within a few weeks they will form macroscopically visible tumors. This method is very convenient, but the growth of tumors under the skin is often not the most appropriate anatomical site. The use of immunocompromised mice allows for human cancer cell lines to be employed but has the disadvantage that the role of the immune system in cancer metastasis can not be fully investigated. Other, more technically difficult, models involve injecting cancer cells into their original anatomical site; for example, breast cancer cells are injected into the mammary fatpad (Farina et al. 1998) or glioma cells are injected into the brain (Shah et al. 2008). Alternatively, cancer cells can be injected directly into the blood, and their spread and exit from the circulation can be followed.

All these methods allow for cancer cells to be labeled before injection to facilitate their subsequent detection. To aid in repeated high-resolution tumor imaging for long periods of time, a "window" can be surgically implanted in the skin overlying the tumor (Makale 2007; Kedrin et al. 2008). This prevents the need for additional surgical manipulation to expose the tumor. However, this is more labor-intensive and there can be concerns about the validity of growing a tumor against an artificial substrate such as glass.

Transgenic and chemical cancer models. A major drawback of injecting tumor cells is that the early stages of tumorigenesis and the progression from in situ to invasive disease are not recapitulated. The progression of cancer can be modeled by using chemical carcinogens to drive the formation of tumors (Mancuso et al. 2009). Alternatively, transgenic techniques can be used

to generate cells with a combination of oncogenes and loss of tumor suppressors that then give rise to tumors (Hutchinson and Muller 2000). The latter method can provide very powerful models of human cancer in mice. However, it is a laborious and expensive process, and additional genetic manipulations are needed to label the cancer cells.

Imaging Equipment

Whole-body fluorescence. This method involves noninvasive imaging of the whole animal (Hoffman 2005). The subject is illuminated with light of one wavelength, and fluorescent light emitted at longer wavelengths is detected. The resolution of this technique is currently limited to approximately 1 mm and there are problems with imaging deeper tissue reliably. However, equipment is constantly being improved, and imaging the same animal from different angles or with complex patterns of light sources enables tomographic reconstruction and much improved resolution in three dimensions (Montet et al. 2005). Hair can present a significant challenge to this type of imaging because it is both light-scattering and autofluorescent. To overcome this, shaved or hairless animals are often used.

Whole-body luminescence. This technique is similar to fluorescence except that it relies on a biochemical reaction, not fluorescence, to produce light. Cells expressing luciferase genes (see section on "Labeling Tumor Components") can be detected in animals that have been injected with an appropriate substrate for the reaction. The sensitivity and resolution of this method are broadly comparable to those of whole-body fluorescence.

Epifluorescence microscopy. This technique uses the same principles of fluorescence as whole-body imaging, but the resolution is much higher because microscope optics are used (Condeelis and Segall 2003; Hoffman 2005). The improved resolution comes at the cost of much reduced depth of imaging, however. As a result, it is usually necessary to perform some surgery to expose the area to be imaged. This limits the scope for repeated imaging of the same animal although the use of surgically implanted windows can overcome this problem.

Confocal microscopy. The major benefit of confocal microscopy (sometimes known as laser scanning microscopy) is the superior three-dimensional information obtained (Condeelis and Segall 2003). Epifluorescence microscopy captures much light from outside the focal plane, whereas confocal microscopy does not. This means that tissue can, in effect, be sectioned using purely optical means. Many variants of confocal microscopy have been used for intravital imaging; the two most popular are multiphoton confocal (Zipfel et al. 2003), which uses longer wavelength light to improve the depth of imaging, and spinning disc confocal, which can acquire images very rapidly (Egeblad et al. 2008).

Labeling Tumor Components

Visualizing cancer cells. To image cancer cells, the cancer cells need to be labeled in a way that distinguishes them from other cells in the animal. This can be done by labeling with colored or fluorescent dyes (Chambers et al. 1995); however, these have the disadvantage that they become diluted every time a cell divides, reducing their suitability for long-term experiments. In addition, dyes can be used only to label cancer cells that are subsequently injected, and are not suitable for more sophisticated transgenic models. Cancer cells can also be engineered to express genes encoding fluorescent proteins that are derived from jellyfish or coral (Hoffman 2005; Shaner, Steinbach et al. 2005). The most commonly used proteins are green fluorescent protein (GFP) and red fluorescent protein (RFP). An example of GFP-expressing cancer cells is shown in Figure 4.1. The genetic nature of this method means that cancer cells can be stably labeled and tracked for long periods. Different cell types can be labeled with different colored fluorescent proteins so they can be unambiguously identified, and their interactions studied or their behavior compared (Sahai et al. 2005). Furthermore, fluorescent proteins can be used in transgenic models (Ahmed et al. 2002). Together these reasons mean that fluorescent proteins are the method of choice for labeling cancer cells in most studies.

Genes encoding bioluminescent enzymes called *luciferases* can also be used to label cancer cells (Contag et al. 2000). The main disadvantage of using expression of either fluorescent or luminescent proteins is that there is very little scope for clinical development, as labeling tumors in cancer patients in this way is currently not possible. To address this problem, several groups have worked on fluorescently labeling antibodies to "cancer antigens" (Montet et al. 2005) or generating other probes to cancer-specific "activities" such as proteases (Weissleder et al. 1999).

Visualizing noncancer cells. In most cases, the noncancer cells come from the host animal into which cancer cells have been injected. This prevents them from being labeled in vitro in the way that the cancer cells are labeled. Transgenic manipulations can be used to drive the expression of fluorescent proteins in the host animal, either in all the cells or in a subset of cells (Yang et al. 2003; Wyckoff et al. 2004; Perentes et al. 2009). If different colored fluorescent proteins are used in the host and the cancer cells, the different cell populations can be unambiguously identified. Once again, the transgenic manipulations are laborious. Some noncancer cells can be labeled by injection of fluorescent dyes with which they can interact – for example,

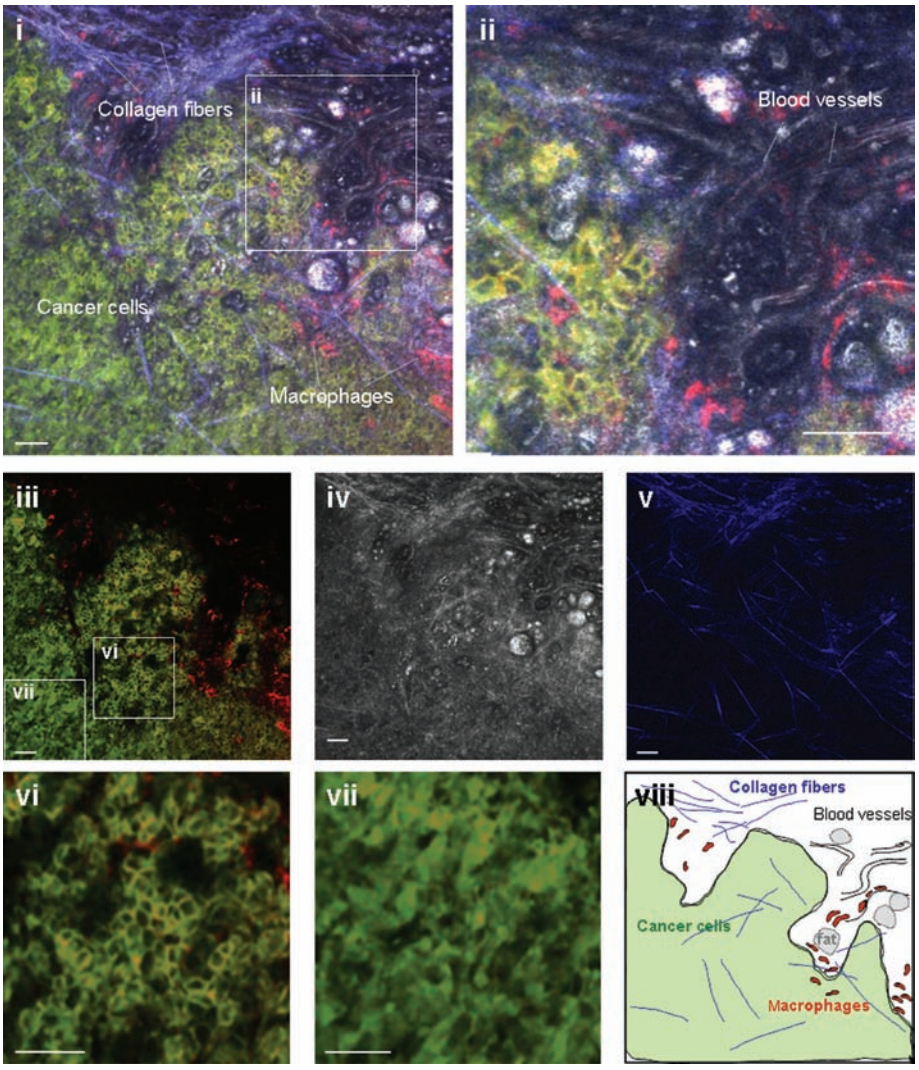

Figure 4.1. Intravital imaging of tumor microenvironment. Images show intravital imaging of a breast tumor in a mouse using multiphoton confocal microscopy. Breast cancer cells are expressing a green fluorescent protein (GFP) membrane marker (green in panels i, ii, iii, vi, and vii). Injection of TRITC-dextran (red in panels i, ii, iii, vi, and vii) enables phagocytic cells to be labeled – these are host macrophages. Reflectance imaging (white in panels i, ii, and iv) in white shows various structures, including collagen fibers, blood vessels, and fat droplets. Second harmonic imaging of collagen fibers is shown in blue (blue in panels i, ii, and v). Panels vi and vii contrast the morphology of cancer cells in two areas of the tumor (shown in panel iii); panel vi shows a well-organized area of cells with clear localization of the membrane marker to cell–cell contacts, whereas panel vii shows an area of tumor cells with a more disorganized and less epithelial organization. Panel viii shows a schematic illustration of panel i. Scale bar is 50 microns.

phagocytic cells such as macrophages can be visualized by their ability to take up fluorescently labeled dextrans (Figure 4.1). Reflectance imaging can reveal the presence of dense vesicles within cells and, in some cases, this can show the overall cell shape.

Visualizing blood vessels. Blood vessels can be identified as dark structures if the bulk of the tumor consists of fluorescent cells (Figure 4.1) or by the injection of fluorescent dyes into the venous circulation (Figure 4.2).

Visualizing the extracellular matrix. The extracellular matrix provides the physical support for most tissues and also provides a barrier to the movement of cancer cells. Reflectance imaging can provide information about fibrous matrix structures, and collagen fibers can be more specifically visualized if illuminated with near infrared light based on second harmonic generation (emission of light half the wavelength of the illuminating light) (Zipfel et al. 2003) (Figure 4.1). These techniques enable the interplay of cancer cells with the extracellular matrix to be studied in detail.

Visualizing protein dynamics. In addition to visualizing cells and the surrounding matrix, the location and dynamics of specific proteins can be visualized (Philippar et al. 2008; Wyckoff et al. 2006). This is normally

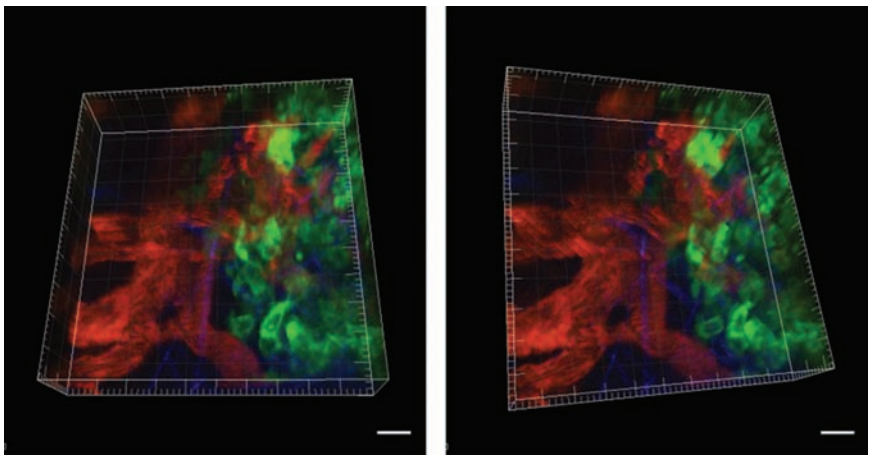

Figure 4.2. Three-dimensional reconstruction of tumor cells and blood vessels. Images show two different views of a three-dimensional reconstruction of a squamous cell carcinoma in a mouse using multiphoton confocal microscopy. Squamous cell carcinoma cells are expressing a GFP cytoplasmic marker (green). Injection of TRITC-dextran into the vasculature (red) enables blood vessels to be labeled. Second harmonic imaging of collagen fibers is shown in blue. Scale bar is 20 microns.

achieved by fusing the gene encoding the protein of interest to a gene encoding a fluorescent protein. This fluorescent fusion protein is then stably expressed in the cancer cells. Figure 4.3 shows breast cancer cells stably transfected with myosin light chain (MLC) fused to GFP. MLC can clearly be localized in the cleavage furrow of a dividing cell in vivo (marked with asterisks in Figure 4.3). Different colored fluorescent proteins can be simultaneously expressed in cells to enable the localization of multiple proteins to be studied at the same time. Furthermore, energy transfer can occur between different colored proteins when they are in very close proximity, a process called fluorescence resonance energy transfer (FRET), and this can provide information about the interaction of the proteins to which they are fused (Stockholm et al. 2005).

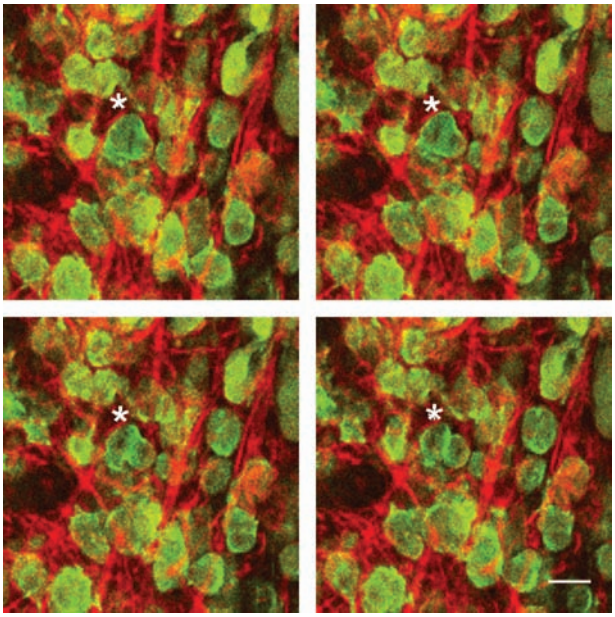

Figure 4.3. Mitotic cell dividing in vivo. A tumor generated from breast cancer cells expressing myosin light chain (MLC) fused to GFP is shown at time intervals three minutes apart. MLC is shown in green and second harmonic imaging of collagen is in red. The cell with the asterisk is dividing from metaphase (upper left) to late telophase (bottom right). The localization of MLC to the contractile body is clearly seen and the space occupied by the chromosomes appears as a dark area in the green fluorescence. Scale bar is 20 microns.

Visualizing gene expression. The activity of particular promoters can be visualized by placing the promoter of interest upstream of a gene encoding a fluorescent protein (Fukumura et al. 1998; Welsh and Kay 1997). The expression of the fluorescent protein then becomes dependent on the activity of the promoter. Imaging of the levels of the fluorescent protein provides a "readout" of promoter activity.

PARACRINE INTERACTIONS IN THE PRIMARY TUMOR REVEALED BY INTRAVITAL IMAGING

Tumors are composed of multiple cell types that interact with each other. In some cases, these interactions promote metastasis (Figure 4.4).

Interplay with Macrophages

Confocal imaging of various tumor models, including breast and melanoma, has shown that the behavior of cancer cells varies in different areas of the tumor. This heterogeneity is found even in xenograft tumors generated from genetically homogeneously cell lines, suggesting that it is extrinsically specified. Imaging of macrophages in tumors that were labeled either by their uptake of fluorescent dextran or by a transgene driving

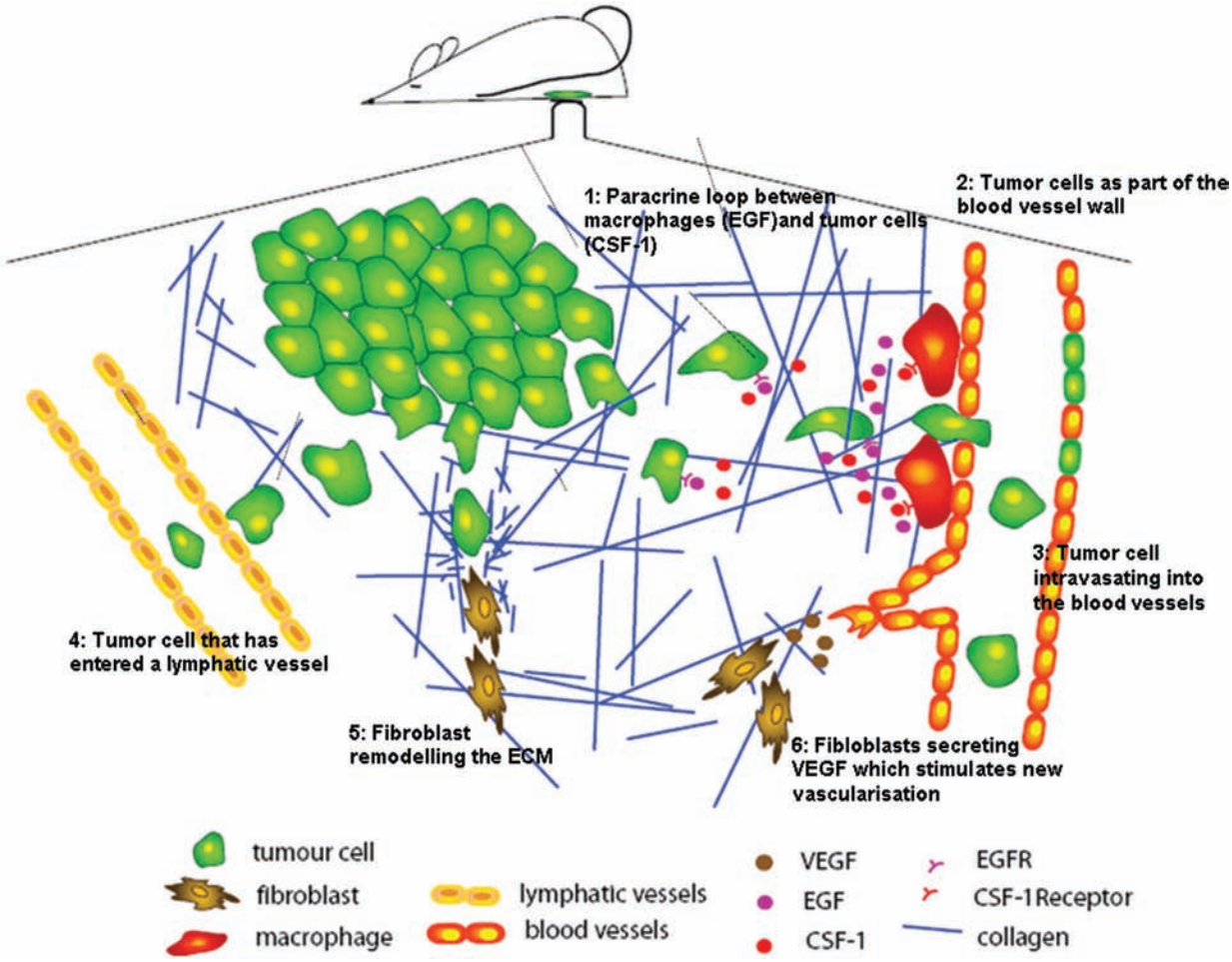

Figure 4.4. Schematic representation of the tumor microenvironment illustrating processes studied using intravital imaging. **1:** Macrophage-cancer cell interactions – EGF produced by CSF-1-stimulated macrophages promotes the invasion of carcinoma cells. **2:** Tumor cells can form part of blood vessel walls. **3:** Tumor cells can be observed entering blood vessels. **4:** The development of tumor lymphatics and subsequent entry of cancer cells can be imaged. **5:** Remodeling of the extracellular matrix by fibroblasts. **6:** VEGF expression in fibroblasts.

GFP expression from a macrophage-specific promoter revealed that high numbers of tumor-associated macrophages were present in the areas with motile cancer cells (Condeelis and Pollard 2006; Wyckoff et al. 2007). Complementary analysis using knockout mouse models (Lin et al. 2001) and in vitro co-culture systems (Goswami et al. 2005) demonstrated that macrophages promote the invasion of carcinoma cells by producing epidermal growth factor (EGF), which is a chemotactic ligand for carcinoma cells (Figure 4.4). In addition, production of colony-stimulating factor (CSF)-1 by the cancer cells increases EGF production by the macrophages (Goswami et al. 2005).

Interplay with the Immune System

In addition to macrophages, other immune cells can modulate the behavior of cancer cells, although the consequence of the interactions is not always clear. Splenocytes have been observed interacting with pancreatic tumors, and the extent of the interactions increases as the tumor develops (McElroy et al. 2008). The relevance of this observation in primary tumors is unclear, but similar interactions can promote metastatic colonization of the liver, suggesting that the splenocytes may aid tumorigenesis in the pancreas (Bouvet et al. 2006).

The immune system is also capable of attacking tumor cells expressing unusual cancer-specific antigens. Numerous studies have visualized T-cell-mediated killing of tumor cells (Mrass et al. 2006; Boissonnas et al. 2007). Although escape from immune attack is clearly important for cancer development, it is not clear whether this plays a specific role in metastasis.

Diverse Roles of Tumor-Associated Fibroblasts

Fibroblasts within the tumor can promote cancer progression (Bhowmick et al. 2004). Imaging of the collagen matrix in normal and cancerous breast tissue has

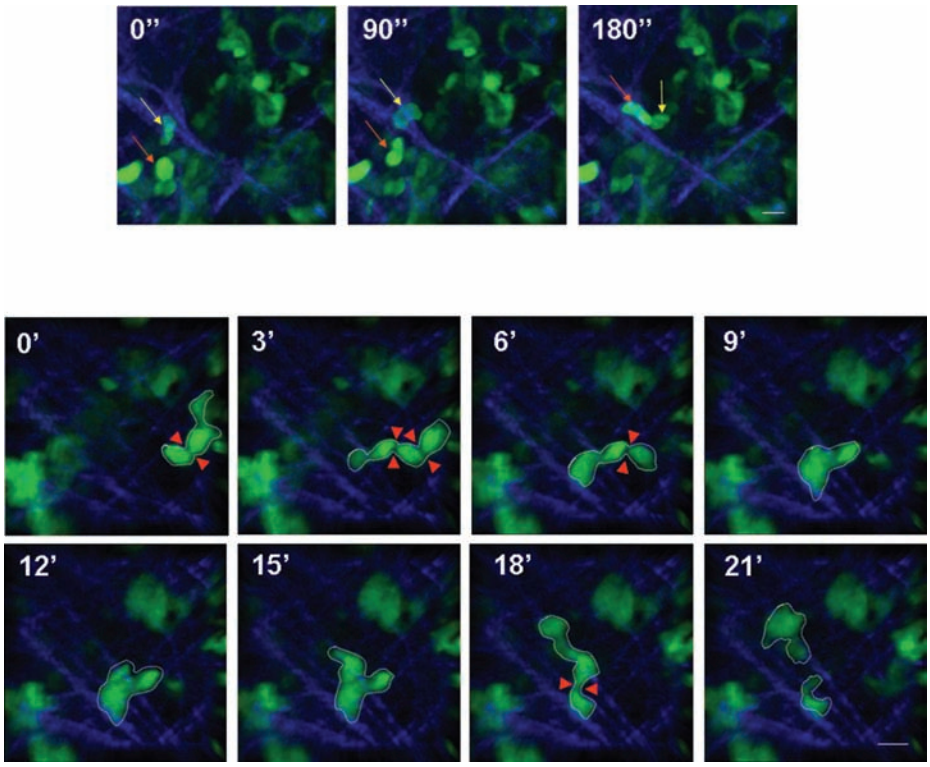

Figure 4.5. Cancer cells moving within the tumor. Images show squamous cell carcinoma cells expressing GFP cytoplasmic marker in green (with moving cells indicated with yellow and orange arrows). Second harmonic imaging of collagen fibers is shown in blue. Upper panels show two cells moving in the same path (moving cells indicated with yellow and orange arrows) and changing direction when contacting a collagen fiber. Lower panels illustrate the highly amorphous morphology of some motile cells (white dashed line indicates one cell). Constriction points that enable squeezing through gaps are shown with red arrows. Also a fragment of the cell being left behind can be seen in the final panel. Scale bar is 10 microns.

shown that there is a dramatic change in its organization (Provenzano et al. 2006). In normal breast tissue it is arranged in curved partially aligned fibers, whereas in invasive tumors, large linear collagen fibers are observed "radiating" from the tumor. It is likely that a significant amount of the alterations in collagen architecture are the result of the activity of fibroblasts. Repeated imaging of collagen fibers and fibroblasts has demonstrated that tumor fibroblasts remodel collagen (Perentes et al. 2009)(Figure 4.4). Other studies have shown that remodeling of the matrix by fibroblasts promotes the invasion of cancer cells (Gaggioli et al. 2007). Consistent with this observation, cancer cells can frequently be observed moving along collagen or changing direction when encountering a new fiber (see below) (Figure 4.5). An additional function of fibroblasts has been suggested by intravital imaging of a mouse containing the vascular endothelial growth factor A (VEGF-A) promoter driving the expression of GFP (Fukumura et al. 1998). This revealed that the VEGF promoter is very active in fibroblasts, arguing that fibroblasts are a significant source of VEGF in tumors and promote tumor angiogenesis (Figure 4.4).

CELL MOTILITY IN PRIMARY TUMOR AND INVASION OF SURROUNDING TISSUES

Cancer Cells Move Rapidly

Cancer cells need to become motile to "escape" from the primary tumor and colonize new tissues. The highly dynamic nature of cell motility means that repeated intravital imaging to generate "time-lapse" sequences has been very valuable in studying cancer cell motility. This field was initiated fifty years ago by Wood, who used dyes to label cancer cells before they were injected into the ear of a rabbit (Wood 1958). This study remains a seminal work, even though huge technological improvements have been made since then.

One key feature of intravital imaging studies of tumor cell motility is that relatively few cells are motile. The most likely reason for this is heterogeneous distribution of pro-motility cues in the tumor environment. EGF is an important pro-motility stimulus and, as discussed previously, its levels are likely to be highest in areas of tumors with high levels of macrophages (Condeelis and Pollard 2006). Experimental elevation of

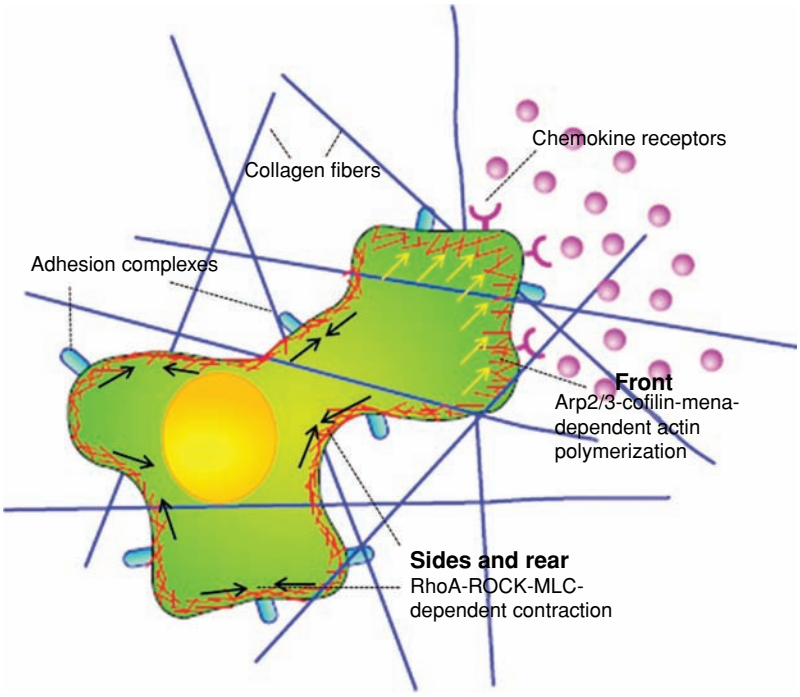

Figure 4.6. Schematic representation of a cancer cell moving in vivo. Cell migration is promoted by chemokines (represented in lilac) binding to receptors (purple). This subsequently triggers actin polymerization through the combined action of Arp2/3, cofilin, and Mena. Actin polymerization drives the front of the cell forward (yellow arrows). Movement of the rest of the cell is promoted by actomyosin interactions around the cortex of the cell (black arrows).

EGF signaling by overexpressing its receptor increases the proportion of motile cells in tumors and correspondingly promotes metastasis (Xue et al. 2006). EGF signaling is specifically required in the moving cells because in a mixed tumor in which only half the cells overexpress EGF receptor (EGFR), it is only the EGFR-overexpressing cells that show increased motility (Xue et al. 2006).

Strikingly, intravital imaging of cancer cell movement has shown that it can be fast (up to 10 μm/min) (Condeelis and Segall 2003). This is faster than the speed with which cancer cells move in vitro. The reasons for this discrepancy are not entirely clear. It is possible that gradients of chemoattractants may direct movement of cancer cells in vivo. Certainly, cancer cells are generally observed moving in common directions (Figure 4.5). The path of cell movement is also affected by the structure of the extracellular matrix. Figure 4.5 shows a cancer cell moving over a collagen fiber and changing direction on a second one. As mentioned previously, the organization of the collagen fibers may be largely determined by the activity of fibroblasts (Perentes et al. 2009). The fibroblasts can thereby have a significant effect on the invasion of cancer cells.

The shape and direction of motile cancer cells can change very rapidly, which has led to their movement being called "amoeboid." Changes in cell shape can be exploited to enable cancer cells to squeeze through small gaps (Figure 4.5, lower panels). This may circumvent the need for proteolysis to make a larger whole in the matrix. This type of amoeboid movement is not blocked by acute administration of matrix metalloproteinase (MMP) inhibitors, although proteolysis is likely to be important in establishing a matrix that permits amoeboid movement (Wyckoff et al. 2006). In extreme cases, the combination of cell movement and cell constriction may lead to fragments of the cell breaking off (last panel in Figure 4.5)

Cell motility requires the coordination of actin polymerization, cell adhesion, and actomyosin contraction (Figure 4.6) (Olson and Sahai 2009). The differences in cell speeds observed in vivo and in vitro have made it hard to directly extrapolate lessons learned from cell culture systems to the tumor environment. Recently, direct intravital imaging and experimental manipulation of actin regulators have been performed in tumors. This has revealed that the regulator of actin polymerization Mena is located at the front of moving cells, where it promotes actin polymerization together with cofilin in response to EGF (Philippar et al. 2008). Increased expression of Mena or activation of cofilin by interference with LIMK function both promote actin polymerization and metastasis (Wang et al. 2006; Philippar et al. 2008). Other studies have implicated the actin-related proteins Arp2 and Arp3 in actin polymerization in cancer cells (Olson and Sahai 2009).

To move, cells need some adhesion to or friction with their environment. In cell culture, cell–matrix adhesions are distinct foci, called *focal adhesions*. These have not yet been observed during intravital imaging, however, suggesting that the organization of cell–matrix adhesions may differ between cell culture and tumors (Philippar et al. 2008). This could be caused by differences between the three-dimensional, somewhat elastic, matrices found in tumors and the two-dimensional rigid substrates used in most cell culture studies.

Further evidence of discrepancies between cell culture and intravital imaging studies comes from the imaging of myosin organization. Interactions between F-actin and myosin generate contractile force and in cell culture studies of cancer cells, F-actin and myosin are organized in parallel arrays of thick linear cables that link to focal adhesions. However, intravital imaging of MLC demonstrated that this distributed around

the cell cortex and not in stress fibers (Wyckoff et al. 2006)(Figure 4.3). The organization of MLC in tumors is dependent on the function of the ROCK kinases. These kinases can also activate LIMK and thereby suppress cofilin activity. This mechanism probably helps to ensure that F-actin polymerization and actomyosin contractility occur sequentially and in different parts of the cell (Figure 4.6).

In addition to the rapid movement of a small proportion of cancer cells, it is likely that many cancer cells move much more slowly. In an elegant study, cancer cells were engineered to express a photoswitchable fluorescent protein. This enabled a patch of tumor cells to be labeled a different color in situ. The distribution of these cells was then examined after twenty-four hours, and it was found that numerous cells had only moved a few cell diameters (~5 μm/h) (Kedrin et al. 2008). This type of motility is currently not well understood.

INTRAVASATION

Cancer cells can spread to distant organs through the vasculature. The first stage in this process is the entry of cells into vessels, called *intravasation*. In most cases this involves cancer cells passing through the layer of endothelial cells that line blood and lymphatic vessels and then detaching completely. Cells are then carried by the flow of the blood or lymph.

In most tumors, a relatively low proportion of cancer cells intravasate; this has hampered the direct study of the process. Nonetheless, some researchers have successfully imaged cancer cells entering the vasculature. Breast cancer cells with different metastatic potential were observed to enter the vasculature differently (Wyckoff et al. 2000). Highly metastatic cells polarize toward blood vessels in the primary tumor. The polarization of cancer cells toward the blood vessels suggests a chemotactic stimulus that originates from the vessels. This could result from the EGF/CSF-1 paracrine loop between macrophages and cancer cells (described earlier) if macrophages are associated with blood vessels (Figure 4.4). In support of this theory, intravital imaging studies have shown that intravasation is most common in tumor areas with high levels of macrophages around blood vessels (Wyckoff et al. 2007).

The importance of EGF in intravasation is suggested by the observation that breast cancer cells with elevated EGF receptor levels show increased intravasation levels (Xue et al. 2006). Interestingly, nonmetastatic cells are not completely defective at intravasation. In fact, some of them can be seen to start entering blood vessels, but they often fragment as they enter the vasculature (Wyckoff et al. 2000). This suggests that the ability to withstand the shear stress caused by blood flow will be important for metastasis. Currently, we know very little of the molecular mechanisms that enable metastatic cells to cope with shear stress.

Tumor imaging of fluorescently tagged cancer cells and endothelial markers has suggested another mechanism by which cancer cells may enter the vasculature. Tumor cells can form part of the vessel walls (Changet al. 2000; di Tomaso et al. 2005) (Figure 4.4); in these circumstances, cancer cells could enter the blood by detaching from the vessel walls without the need to cross an endothelial cell layer. Although this has been suggested by imaging studies, it has not yet been observed directly.

To date the majority of studies have focused on the entry of cells into the bloodstream but many tumors also spread via the lymphatic system. Injection of fluorescent tracers inside tumors allows visualization of the lymphatic vessels (Dadiani et al. 2006). This has enabled the modification and growth of the lymphatic network in response to a growing tumor to be studied. Expression of VEGF-C in cancer cells promotes lymphangiogenesis and increases the rate at which lymph drains from tumors and, consequently, the number of cancer cells that arrive in lymph nodes (Hoshida et al. 2006). Cancer cells have been observed moving within lymphatic vessels, but the process of entry into lymphatic vessels has not been investigated in detail.

EXTRAVASATION AND INITIAL SURVIVAL AT SECONDARY SITES

Once metastatic cancer cells enter the vasculature, they flow in the blood or lymph before exiting the vessels (extravasating) into the secondary metastatic sites. Most of what is known about tumor cells circulating in the blood has been revealed from ex vivo experiments. However, direct visualization by intravital imaging has revealed that tumor cells attach to the vessels and extravasate using different mechanisms.

Arrest in Vasculature

The simplest way for cells to arrest in the vessels is size restriction. Relatively large tumor cells can become physically stuck in narrow capillaries (Figure 4.7). For example, the arrest of carcinoma cells (Ito et al. 2001) has been directly observed by intravital videomicroscopy in liver sinusoids. An alternative mechanism of arrest involves active adhesion to either endothelial cells or the basement membrane that underlies endothelial cells (Figure 4.7). Intravital imaging has shown that human fibrosarcoma cells can arrest in the lungs by attaching to the matrix underneath the endothelial cells. This process depends on the adhesion protein integrin $\alpha 3\beta 1$ expressed in the tumor cells that binds to laminin-5 in the basal membrane underlying the endothelium (Wang et al. 2004). Both active

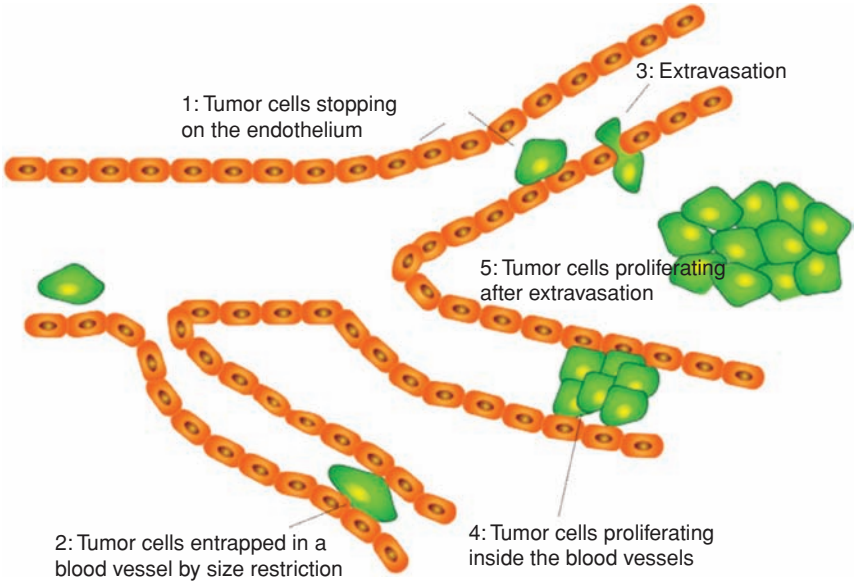

Figure 4.7. Cancer cells in the blood vessels and extravasating. The different ways cancer cells adhere to the blood vessels are represented. **1:** Arrest can be mediated by active adhesion of cancer cells to either endothelial cells or the basement membrane that supports them. **2:** Cancer cells can be trapped because they are larger than the capillary through which they are passing. **3:** Extravasation through the endothelial cell layer. **4:** Following arrest, cancer cells can begin to proliferate in vessels. **5:** Alternatively, they may extravasate before beginning to proliferate.

and passive methods of arrest can be enhanced by the association of cancer cells with platelets. The rapid formation of thrombi around tumor cells arrested in the vasculature was already noticed in the late 1950s (Wood 1958). More recent studies using fluorescently labeled antibodies show that tumor cells' interaction with coagulation components, such as platelets or fibrinogen, is required for the retention of cancer cells in the lungs (Im et al. 2004). Independent support for this work is provided by the reduced incidence of metastasis in fibrinogen-deficient animals or following depletion of platelet function (Camerer et al. 2004; Palumbo et al. 2000).

Exit from the Vasculature

Once cells have arrested, they typically cross the endothelium and enter the secondary tissue within a few hours (this process is called *extravasation*; see Figure 4.7). Most of our knowledge about this process comes from a combination of in vitro models and experimental metastasis that measure the number of cancer cells able to form large metastases. Therefore, our current understanding is largely based on inference; there is considerable scope for direct observation of extravasation by intravital imaging to shed more light on this process.

In some cases, extravasation is not needed for the early stages of cancer growth at secondary sites. Cancer cells have been observed proliferating within vessels once they have attached to the endothelium (Al-Mehdi et al. 2000). Colonies of cells inside the vessels grow larger with time, breaking the endothelium and entering the surrounding tissue (Figure 4.7).

Rapid Clearance of Cells at Secondary Sites

Following experimental introduction of cancer cells into the vasculature, the great majority of cells that initially arrest cannot be detected a few hours later (Figure 4.8). Several possibilities could explain this observation. First, the strength of adhesion to the endothelium may be critical for enabling cancer cells to resist pressures created by the blood flow. Alternatively, once cancer cells are removed from prosurvival signals in their tissue of origin, they may be more susceptible to apoptosis. The translocation of the apoptotic regulator BAD from the cytoplasm in healthy cells to the mitochondria in dying cells has been directly visualized by fusing BAD to GFP in a melanoma model of metastasis to the lungs (Kim et al. 2004). Changes in morphology of the nucleus typical for apoptosis have also been observed in cancer cells after arrest on the liver (Tsuji et al. 2006).

Cell death may also be actively triggered by the endothelial cells that surround the newly arrived cancer cell. Increased nitric oxide (NO) production by the endothelial cells, which is cytotoxic for the tumor cells, has been imaged in vivo using a fluorescent probe (DAF-2) sensitive to NO production (Qiu et al. 2003). Melanoma cells die as a result of NO production in

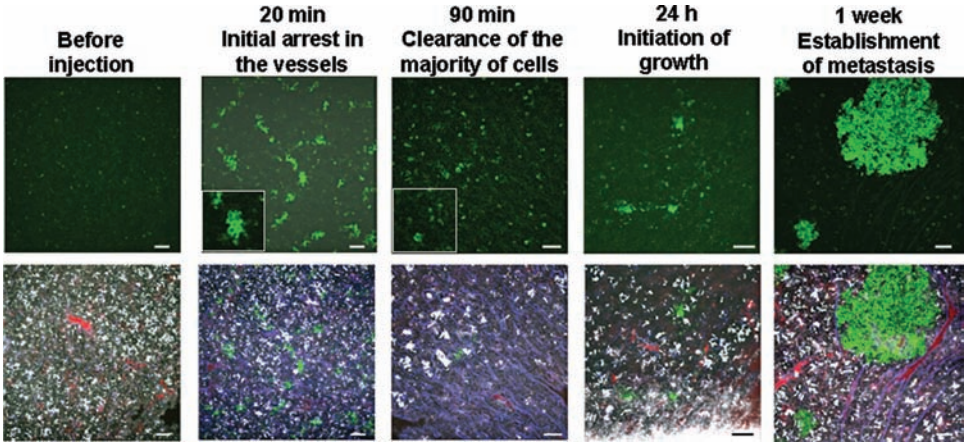

Figure 4.8. Temporal analysis of breast cancer cells arriving in the lungs. Images show cancer cells expressing a GFP membrane marker (green) in the lungs at different times after injection. The upper panels show the cells alone. In the bottom panels blood vessels can be seen in red labeled with a marker injected in the tail vein just before imaging, lung tissue imaged by reflectance imaging (white), and autofluorescence in blue. At 20 minutes, numerous cells have arrested in the lungs, sometimes as clumps (inset panel). At 90 minutes, less-intact cells are observed, although numerous small fragments are visible (inset panel). After 24 hours, only a few cells remain. After one week, some cells have formed large metastases (top right), whereas others have only formed small metastases (bottom left). Scale bar is 50 microns.

nearby endothelial cells; this does not occur in endothelial nitric oxide synthase (eNOS)-deficient mice. This correlates with a higher number of metastasis in these mice. However, the effect of NO in tumors is controversial and seems to depend on the cell type and tissue that produce it, as NO production by tumor cells has been found to promote tumor progression (Fukumura et al. 2006). Cancer cells may also be attacked by parts of the immune system at secondary locations. Several studies have shown that various immunosuppressive agents can promote metastasis, although immune-cell–mediated killing of cancer cells at metastatic sites has not yet been imaged (Tsuji et al. 2006).

GROWTH AT THE SECONDARY SITES

For macrometastases to form, cells arriving at secondary sites need to proliferate. Whole-body imaging is a simple but effective tool to measure this process. Cancer cells can be modified in order to express genes encoding both fluorescent or bioluminescent proteins to enable their tracking in animal models. This technique has enabled the tracking and quantification of metastasis produced by melanoma cells in different organs, such as brain, liver and bones (Yang et al. 2000) or in the spleen, bowel, lymph nodes, and liver in the case of pancreatic tumor cells (Bouvet et al. 2002). It is clear from both clinical and experimental studies that not all cells arriving at secondary sites have the same ability to form large metastases. Figure 4.8 shows that after one week, some experimentally generated lung metastases contain only a few cells, whereas others comprise more than 100 cells. This could potentially be explained by the cancer stem cell hypothesis (Bjerkvig et al. 2005). In this theory, only a subset of cancer cells have the ability to replicate indefinitely and thereby generate a large metastasis. Smaller metastases would be generated by cells with more limited replicative potential. However, additional studies will be needed to verify this.

Delayed Growth

Even more strikingly, single or small metastases have been found in cancer patients years after surgery to remove the primary tumor. These cells are frequently termed "dormant" (Naumov et al. 2002). Critically, in cancers such as breast cancer, growth of metastases can occur after a delay of several years. This is a major clinical problem, as it prevents assessment of whether surgery has been curative or whether patients should remain on a chemotherapeutic regime. The reasons for the regrowth of small metastases are controversial. One possibility is that an additional mutation in a dormant cell causes it to resume proliferation. Alternatively, a change in the local environment may lead to altered paracrine interactions and, hence, restored proliferation. As discussed earlier, this may even begin while cells are still within blood vessels (Aguirre-Ghiso 2007; Naumov et al. 2008).

PARACRINE INTERACTIONS AT METASTATIC SITES

Different tumors metastasize preferentially to different organs. For example, breast and pancreatic tumors

form metastases in the bones, whereas colon tumors metastasize to the liver. In part, this can be explained by patterns of blood flow (the liver is the first capillary bed through which cells exiting the colon will pass). It has also been argued for many years that there must be something in the tissues in which cells metastasize that promotes the survival and proliferation of specific cells and not others. Intravital imaging has helped to uncover paracrine interactions that are specific to particular metastatic locations in the body. Similar to primary tumors, cancer cells interact with other cells at secondary sites in a manner that supports their continued growth. By introducing cancer cells labeled with a fluorescent protein of one color into a mouse that is labeled with a second color, these paracrine interactions can be observed. Colon cancer cells arriving in the liver are frequently associated with splenocytes. By experimentally manipulating the levels of splenocytes that arrive in the liver together with the colon cancer cells, it has been shown that splenocytes promote metastatic colonization (Bouvet et al. 2006). However, the mechanism by which splenocytes help in the colonization of the liver by tumor cells is not fully understood.

Imaging of signaling by the cytokine TGFβ has demonstrated that it is particularly high in bone metastases (Kang et al. 2005). Further molecular analysis demonstrated that numerous target genes of TGFβ signaling can promote osteoclast function, including interleukin (IL)-11 (Kang et al. 2003). Therefore, elevated TGFβ signaling in cancer cells enables them to coopt osteoclasts to degrade bone tissue and generates space for the proliferation of the tumor cells (Kang et al. 2005). In another study, imaging of the bone marrow in the mouse thin skull has shown that leukemia cells expressing CXCR4 metastasize preferentially to bone marrow microdomains containing its ligand SDF1 (Sipkins et al. 2005). Thus, regions of the bone marrow that are able to activate chemokine signaling in cancer cells are favored for colonization. Other studies have shown that SDF-1 signaling can activate the integrins that mediate adhesion of hematopoietic cells to blood vessels (Peled et al. 2000). It is likely that SDF-1 signaling may help cancer cells to adhere to vessel walls and thereby promote metastasis.

Bone-marrow–derived cells can also promote the metastatic colonization of sites outside the bone. For example, VEGFR-1 positive bone-marrow–derived cells can promote lung metastasis. Detailed temporal analysis has shown that these cells arrive in the lung tissue before the cancer cells do. Exactly how these cells promote metastasis is not clear, although production of SDF-1 is once again implicated (Kaplan et al. 2005). The mobilization of these cells from the bone marrow is driven by the systemic production of factors from the primary tumor. This raises the possibility that paracrine interactions that promote metastasis are not just localized events, but can also occur at the systemic level.

INTRAVITAL IMAGING AS A TOOL FOR THERAPEUTIC TREATMENTS

Tracking Metastasis in Animal Models

Metastasis is a major clinical problem; therapies that reduce its prevalence will have a significant impact on cancer treatment. The multistep nature of metastasis provides many opportunities for intervention. However, demonstrating which targets are most critical for metastasis in preclinical models and subsequent development in the clinic remains a significant challenge.

Intravital imaging is rapidly becoming an important tool in the preclinical evaluation of antimetastatic agents. First, whole-body imaging can provide excellent longitudinal data about the spread and growth of tumors following different genetic or pharmacological manipulations. The power of this approach has been shown in studies of bone metastasis. As described earlier, bone remodeling in osteolytic metastases is performed by osteoclasts that are coopted by breast cancer cells (Kang et al. 2005). Bone remodeling can be prevented using bisphosphonates, and whole-body imaging has shown that these agents reduce the growth of bone metastases in breast cancer models (Abdelkarim et al. 2009). This work demonstrates how targeting non-tumor cells can be effective at reducing metastasis. This methodology is also useful to test combinations of chemotherapies in reducing metastasis (Gupta et al. 2007).

Evaluating Efficacy of Agents at the Cellular or Molecular Level

Higher-resolution intravital imaging can also be used to assess whether agents are specifically blocking the process they are designed to target. Numerous drugs have been developed that can reduce the invasion of cancer cells in vitro. Evaluating their efficacy in vivo is more challenging, however, because they would not be expected to affect simple parameters such as tumor size.

The oncogenic tyrosine kinase c-src can disrupt the adherens junctions that bind epithelial cells together. Breakdown of these junctions is an important early step in the metastatic process. Imaging of the dynamics of adherens junction proteins in control and src-inhibitor–treated tumors has demonstrated that src does indeed modulate adherens junction function in vivo. Imaging of this kind could provide a very useful means of evaluating the efficacy of anti-src drugs with cellular resolution in preclinical development (Serrels et al. 2009).

As described previously, high-resolution imaging has the power to actually observe moving cancer cells, and this can be combined with the administration of pharmacological agents. Inhibition of matrix metalloproteinases (MMPs) was hoped to stop the invasion of cancer cells and thereby reduce metastasis; however, intravital imaging has shown that, in fact, this has little effect on the number of rapidly moving "amoeboid" cancer cells observed in vivo. This could explain the modest effects of drugs against MMPs in clinical trials (Coussens et al. 2002). In contrast, pharmacological inhibition of the kinases ROCK1 and ROCK2, which regulate actomyosin contractility, reduces cancer cell motility in vivo (Wyckoff et al. 2006). Interestingly, cancer cell motility is not completely blocked by ROCK inhibition, and a population of cells with a distinctive morphology that were not affected can be observed (Sanz-Moreno et al. 2008). This example illustrates how very detailed analysis of drug action at the cellular level can be performed and how it can provide early indication of either heterogeneous responses or resistant cell populations.

Anticancer therapies can also target non-tumor cells that support the cancer, and the action of these can also be investigated by intravital imaging. Metastases above a certain size need a blood supply to provide nutrients and oxygen and therefore could be susceptible to antiangiogenic therapies in the same way that primary tumors are (Folkman 2007). Intravital imaging has shown how two different antiangiogenic agents modify vascular networks and blood flow in liver metastases. Furthermore, these agents reduce the growth of liver metastases (Varghese et al. 2004). Targeting the lymphatic vasculature may also have benefits in reducing the lymphatic spread of tumors. VEGFR-2 and VEGFR-3 antagonists reduce the extent of the draining lymphatic network from an experimental fibrosarcoma (Hoshida et al. 2006). This is also associated with a reduction in the number of fibrosarcoma cells arriving the lymph nodes. However, VEGFR antagonists were effective only if administered before the establishment of lymphatic vessels in the tumor. This suggests that they would not be able to reduce lymphatic spread from established tumors and therefore may not function well in a clinical setting.

Repeated imaging of stromal fibroblasts and collagen fibers in mice treated with the hormone relaxin has demonstrated how this hormone affects the remodeling of collagen by fibroblasts (Perentes et al. 2009). Given the importance of collagen fibers in modulating the migratory behavior of cancer cells in vivo, it is likely that relaxin treatment may modify the invasive behavior of cancer cells; however, this remains to be tested directly.

The detailed analysis of the action of anticancer agents on cellular behavior described here is still in its early stages, and the links to metastatic behavior have not always been established. Nonetheless, the detailed information these studies provide can help to predict and overcome problems in therapeutic strategies in preclinical models before the more laborious and costly business of clinical trials.

Assessing Delivery of Agents to the Target Tissue

At a more technical level, imaging can assess the efficiency with which agents reach tumors, and even specific areas within the tumor. This can enable modifications to be designed that improve the delivery of agents to their target tissues. An example of this is the tracking of the anti–breast-cancer antibody trastuzumab (Herceptin), which targets ErbB2, a member of the EGFR family overexpressed in breast cancer cells. The distribution of fluorescently tagged trastuzumab and various derivatives has been studied in mice bearing breast cancer or gliosarcoma. Intravital imaging showed that trastuzumab went to bind the breast cancer tumor specifically (Montet et al. 2005). Moreover, the distribution of different variants of this antibody could be compared by measuring the kinetics for tumor retention or in accumulation in the kidney (Dennis et al. 2007).

An alternative approach to improve the delivery of drugs is to modulate the barriers that reduce their access to the tumor. The surrounding extracellular matrix (ECM) can reduce the rate of diffusion of molecules from the vasculature into the tumor. As described earlier, the hormone relaxin can reduce the density of the ECM; intravital imaging has demonstrated that this can lead to an increase in the diffusion rates of molecules in tumors (Brown et al. 2003). These studies raise the possibility that strategies to improve drug delivery in the clinic could be designed and tested using intravital imaging approaches.

CONCLUSIONS

For many years, the study of metastasis was termed "black box" research because the only things that could be evaluated were the starting point and the endpoint of the process. This meant that many of our ideas about the process were based on inference or in vitro models. The application of imaging to metastasis research has been critical in enabling direct monitoring of metastasis in action and has greatly increased our understanding of the process. Nonetheless, there is still much to learn; in particular, we are only just beginning to unravel the ways in which paracrine interactions affect the likelihood and location of metastasis and what determines whether cancer cells grow or remain dormant once they arrive at secondary sites.

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5 Metastasis-Promoting Genes

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Accurately defining whether a tumor is benign or malignant is critical in determining the most appropriate course of therapy and, consequently, clinical outcome. Benign tumors, which are characterized by hyperproliferating cells, can, in most cases, be effectively treated by surgical removal if the tumor is located in an accessible and nonessential site in the body. However, when a tumor becomes malignant – acquiring dysplasia, dedifferentiation, and metastatic properties – treatment becomes extremely difficult because of profound genetic and epigenetic changes in the tumor that counteract host defense mechanisms, as well as exogenously delivered therapeutics [1]. Additionally, effective delivery to metastatic lesions can be difficult and inefficient. This chapter focuses on the genes and their products that have been identified and shown to be involved in regulating metastasis.

Metastasis is a dynamic process in which a transformed tumor cell migrates from its initial site of origin and colonizes at new locations in the body. In the biological cascade of metastasis, distinct steps have been delineated [2, 3]:

(1) Tumor cells become less adherent to the surrounding stroma and are shed from the primary tumor.
(2) Tumor cells acquire motility, degrade the surrounding extracellular matrix, and invade into it.
(3) The tumor cells enter into the circulation and survive (these processes are characteristic of the "intravasation" component of metastasis).
(4) Tumor cells emerge from the circulation and attach and enter new tissue (these processes are characteristic of the "extravasation" component of metastasis).
(5) Tumor cells proliferate and generate new secondary colonies of cells that are dependent on formation of a new blood supply – the process of angiogenesis. To perform each specific task, specific proteins (gene products) are necessary.

For a normal cell to be transformed into a tumor cell, an initial transforming event (or events) must take place. This event might be brought forth by activation of an oncogene that triggers aberrant cell growth and/or inactivation of a tumor suppressor that releases restrictions to cell cycle checkpoint controls. Genes that are not transforming or tumor-suppressing in their own right, but can contribute to expression or suppression of the transformed state – that is, progression-elevated and progression-suppressed genes, respectively – have also been identified [4, 5]. These sets of genes function as "master regulators" that control expression of the "effectors" and orchestrate the sequential events leading to and promoting metastasis.

THE MASTER REGULATORS

Receptor Tyrosine Kinases (RTKs)

Aberrant growth factor/growth factor receptor signaling is one of the principal events regulating initiation of tumor development and progression [6]. Epidermal growth factor (EGF) is an essential growth factor for almost all cells. It binds to its cell surface receptor EGFR, a receptor tyrosine kinase, and activates the mitogen-activated protein kinase (MAPK) signaling cascade that ultimately leads to phosphorylation and activation of specific transcription factors controlling genes compulsory for cell proliferation (Figure 5.1) [7]. One of the key transcription factors, regulated by MAPK, especially by extracellular signal-related kinase (ERK), is activator protein-1 (AP-1), which is a heterodimer of two proto-oncogenes, c-Fos (and its family of proteins) and c-Jun (and related family members) [8]. AP-1 regulates a plethora of genes, to be discussed later in this chapter, that are involved in regulation of metastatic events [9]. Many tumors are often characterized by mutations in the EGFR, so it becomes constitutively active in the absence of EGF [7].

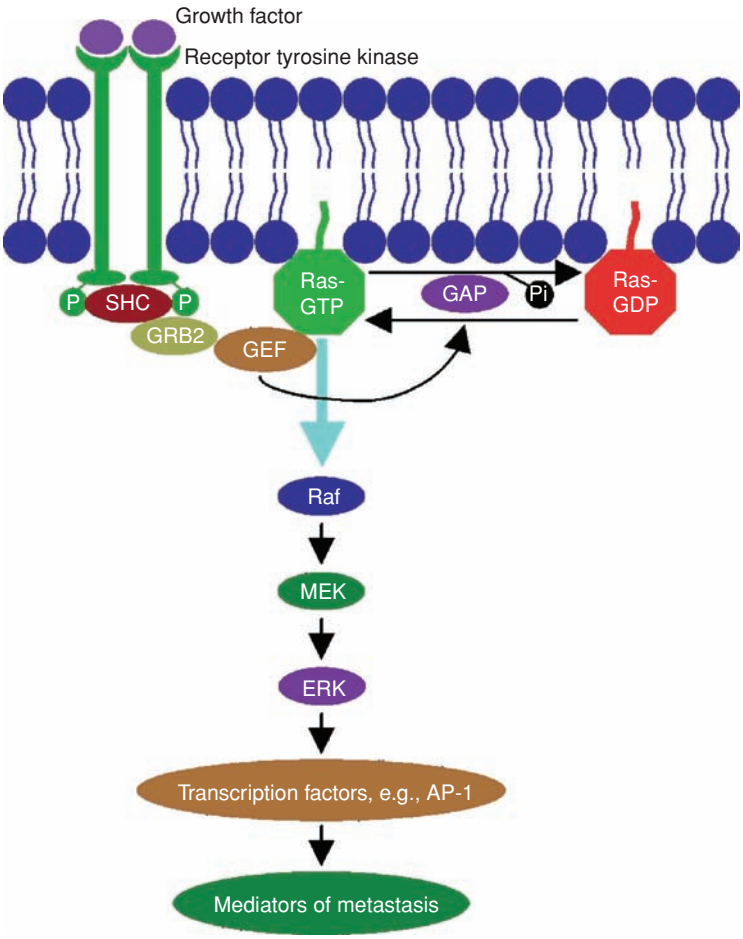

Figure 5.1. A simplified schematic of the pathway from identified master regulators to the effectors of metastasis. Binding of a growth factor (e.g., EGF) to its cognate receptor tyrosine kinases results in activation by autophosphorylation. This results in sequential activation of Ras (the mechanism described in the text), Raf, MEK, and ERK, which activates specific transcription factors regulating expression of genes mediating the different steps of metastasis. Activated Ras also activates several important pathways directly influencing the tumorigenesis process (not shown here).

The constant signaling induced by EGFR to proliferate leads to hyperproliferation of cells; eventually, EGFR signaling induces the expression of secondary genes crucial in mediating malignant transformation and metastasis.

Similar to EGFR, activated mutations and/or overexpression of other growth factor receptors, such as platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR/met), and other members of the EGFR family such as ERBB2 (Her2/neu) and ERBB3, have also been detected in many tumors [6]. There are fifty-eight known RTKs distributed among twenty subfamilies, aberrant expression of many of which has been shown to be involved in tumor initiation and progression [10]. Recent studies have also demonstrated that multiple RTKs can be activated in the same tumor [11].

Ras

One of the key molecules linking growth factor signaling to the MAPK pathway is Ras [12]. Ras is a small guanosine triphosphate (GTP)-binding protein that, in its inactive form, is bound to guanosine diphosphate (GDP) [13]. When EGF binds to EGFR, dimerization of EGFR occurs so the receptors phosphorylate each other. The phosphorylated receptors bind to an adapter protein, such as Grb2 or Shc, and the adapter protein then binds to guanine nucleotide exchange factors (GEFs) that interact with Ras, allowing it to exchange the bound GDP for GTP (Fig. 1) [14]. Ras-GTP is the active form of the molecule which interacts with several families of effector proteins stimulating their catalytic activity. One such effector is Raf kinase, which initiates MAPK signaling. Ras itself has intrinsic GTPase activity, so it can rapidly switch to a Ras-GDP "off" state. This reaction is also controlled by GTPase activating proteins (GAPs), keeping the constant activation of Ras in check. Activated mutations of Ras and Raf and inactivation mutations of GAPs, so Ras function is no longer kept in check, are observed in many tumors [12].

In addition to Raf, Ras also interacts with many other key molecules regulating cell growth and proliferation, such as phosphoinositide-3-kinases (PI3Ks), Ral-GDFs, and phospholipase C [12]. Activated mutations of all these genes have been observed in different tumors.

Nonreceptor Tyrosine Kinase, c-Src

c-Src is a nonreceptor tyrosine kinase belonging to the Src-family kinases, which include Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck, and Lyn [15]. Among these family members, c-Src is the most studied and has been shown to play an essential role in regulating the invasiveness of cancer cells. In certain cell types, such as fibroblasts, overexpression of c-Src augments cell proliferation, whereas in other cell types, such as colon cancer cells, c-Src overexpression does not affect cell growth but increases invasiveness [16, 17]. Increased c-Src activity has been observed in gastrointestinal cancers, as well as in breast, ovarian, and lung cancers [15]. c-Src protein is composed of a C-terminal tail containing a negative regulatory tyrosine residue (Tyr530 in humans), four Src homology (SH) domains, and a unique amino-terminal domain [18]. SH1 domains contain the autophosphorylation site (Tyr419 in

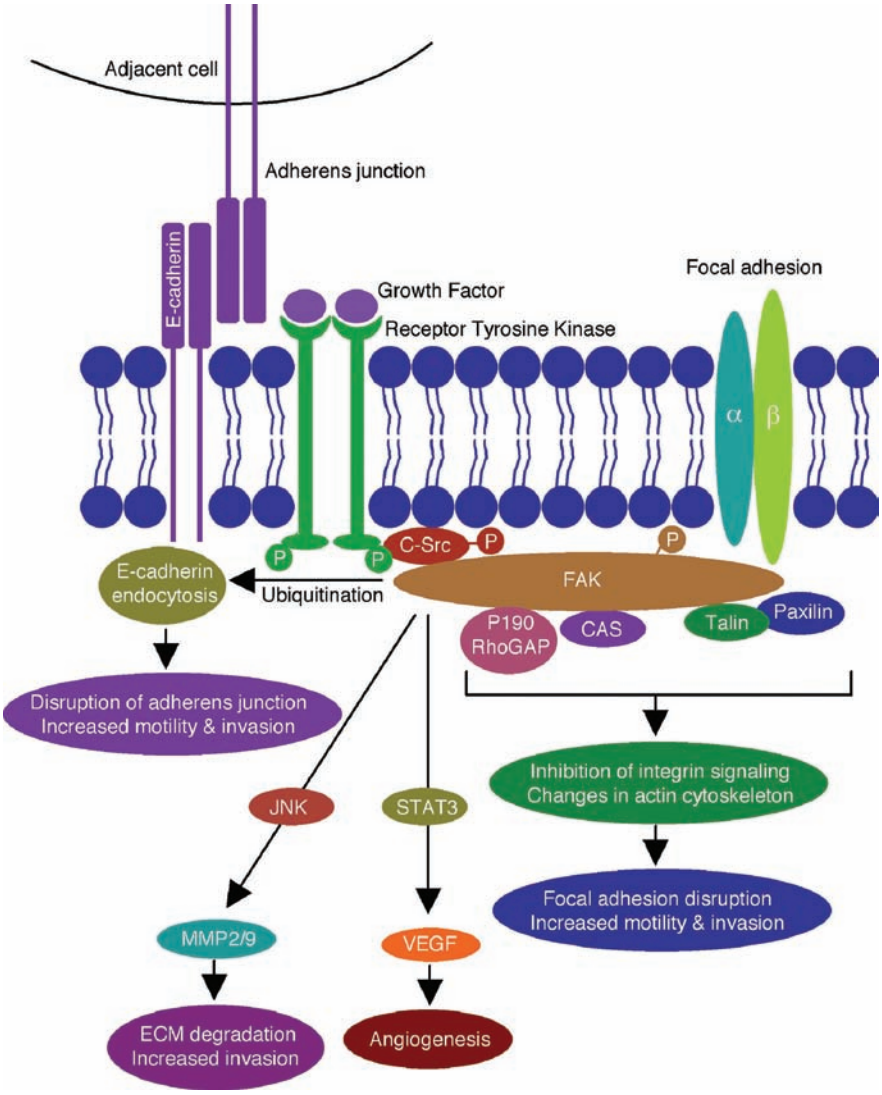

Figure 5.2. A schematic showing a proposed mechanism of increased motility and invasion during metastasis. Binding of growth factor receptors activates c-Src, which phosphorylates focal adhesion kinase (FAK), leading to focal adhesion disruption. Activated c-Src induces ubiquitination and endocytosis of E-cadherin, thus disrupting the adherens junction. Both these events lead to decreased adhesion, increased motility, and invasion. E-cadherin is also downregulated by other mechanisms (described in text but not shown here). Activation of c-Src results in increased MMP2/9 by activation of JNK and increased VEGF by activation of STAT3 that contribute to increased invasion and angiogenesis, respectively.

human); the SH2 domain interacts with PDGFR and the negative regulator Tyr530; the SH3 domain establishes intramolecular contact with the kinase domain in the active form of the protein; and the SH4 domain contains a myristoylation site that allows c-Src to dock into the cell membrane [15].

Extracellular signaling via RTKs can activate c-Src. It interacts with EGFR, PDGFR, ERBB2 (Her2/Neu), FGFR, colony-stimulating factor-1, and HGFR/met, which results in c-Src activation (Figure 5.2) [15]. Inactivation of c-Src by phosphorylation of the negative regulatory C-terminal tyrosine is mediated by c-Src tyrosine kinase (CSK) and its homolog CHK; a reduced level of CSK might be involved in c-Src activation in human cancers [19]. Alternatively, the C-terminal phosphate of c-Src might be removed by tyrosine phosphatases such as PTP1B, which is highly expressed in breast cancer cell lines, resulting in dephosphorylation and activation of c-Src [20]. In addition, interaction with focal adhesion kinase (FAK) and CRK-associated substrate (CAS) results in activation of c-Src (Figure 5.2) [21, 22]. The molecular mechanism by which c-Src increases cell

adhesion, motility, and invasion is discussed later in this chapter.

EFFECTORS

The effector molecules play specific roles in the cascade of events necessary for metastasis. Overexpression and/or activation of these effectors have been observed in metastatic cells, and experimental inhibition, by pharmacological or genetic approaches, abrogates the metastatic potential of the neoplastic cells. These molecules are described in their association with the different steps of metastasis.

Loss of Adhesion to Stroma

The first event in metastasis is the ability of tumor cells from a primary tumor to disengage from attachment to the stroma and acquire motility. Focal adhesions and adherens junctions are two important structures regulating adhesion, invasion, and motility [23, 24]. Focal adhesion is primarily a cell–matrix attachment structure, in which integrins connect the actin cytoskeleton to extracellular matrix (ECM) proteins (Figure 5.2) [23]. Accordingly, focal adhesions not only provide the structural and mechanical platform necessary for adhesion but also relay signaling information from the ECM, regulating cell proliferation and gene transcription. They are composed of more than fifty different proteins, such as talin, vinculin, α-actinin, c-Src, FAK, CAS, and paxillin, which form a complex supramolecular assembly. The cytoskeletal proteins, such as actin and myosin, which control the shape and motility of cells, are recruited to focal adhesions. Assembly of focal adhesion allows cells to adhere to ECM, and disassembly leads to cell suspension. This process is regulated by an intricate cross-talk between integrins and other cell-surface molecules, such as cadherins, selectins, syndecans, G-protein–coupled receptors, and RTKs, with the actin cytoskeleton regulating the Rho family of small GTP-binding proteins [25]. The mammalian Rho family consists of twenty intracellular signaling molecules, including RhoA, RhoB, RhoC, Rac1/2/3, and CDC42, which regulate cytoskeletal organization; Rho activation is mandatory for focal adhesion assembly [26]. A description of the role of the Rho family of proteins in regulating motility is provided later in this chapter.

c-Src modulates adhesion, motility, and invasion of cancer cells, particularly through phosphorylation of FAK (Figure 5.2). FAK is a substrate of c-Src and mediates growth factor and integrin-mediated motility, adhesion, and invasion, as well as cell proliferation and survival [27]. Concurrent activation of c-Src and FAK is observed in many tumors, resulting in increased invasion and metastasis [28]. c-Src cooperates with integrins to block RhoA-mediated downstream signaling through activation of p190 RhoGAP, leading to focal adhesion disruption (Figure 5.2) [29]. Overexpression of kinase-inactive c-Src results in the formation of large focal adhesions [30].

E-cadherin is a protein belonging to the adherens junction that maintains homotypic cell interactions (Figure 5.2) [31]. The highly conserved cytoplasmic tail of E-cadherin molecules binds a set of related proteins called *catenins*, which in turn link the E-cadherin/catenin complex to the actin cytoskeleton. The assembly and maintenance of the E-cadherin–catenin complex is tightly controlled. Loss of E-cadherin has been correlated with enhanced tumor cell invasiveness in vitro and in vivo and can be induced by inactivating mutations, epigenetic silencing, proteolytic cleavage, and proteosomal degradation [32]. Recent studies have shown that K-*ras* increases polysialylated neural cell adhesion molecule (NCAM) that interacts with E-cadherin, represses E-cadherin–mediated cell adhesion, and increases cell migration, thus establishing cross-talk between the "master regulators" and "effectors" [33]. E-cadherin repression might be produced as a part of epithelial–mesenchymal transition (EMT) in cancer cells upon activation of specific transcription factors such as Snail, Twist, and Slug [34].

EMT involves the transition of epithelial cells into a mesenchymal phenotype, thus allowing them to acquire a morphology that is appropriate for migration in an extracellular environment and colonization in new areas [35]. The Snail/Slug/Twist family of transcription factors is overexpressed in diverse cancers, including those of the breast, prostate, and pancreas, and they repress E-cadherin expression, thereby inducing EMT [34, 35]. EMT is also initiated by the activation of hepatocyte growth factor receptor (Met) that induces cell scattering [36]. Overexpression of Met is observed in tumors of the liver, kidney, thyroid, and other organs [37]. One of the consequences of E-cadherin downregulation is nuclear translocation of β-catenin, which is normally complexed with E-cadherin at the cell membrane [38]. β-catenin heterodimerizes with LEF/TCF transcription factor and regulates expression of genes promoting tumorigenesis and metastasis. Activation of β-catenin has been observed in cancers of the liver, colon, and other organs.

c-Src disrupts adherens junctions by membrane localization of E-cadherin and induces tyrosine phosphorylation and ubiquitination of the E-cadherin complex, leading to endocytosis of E-cadherin (Figure 5.2) [39]. As a result, c-Src releases cells both from attachment to the ECM and from each other. These effects also allow the cells to acquire motility and invasiveness. In addition, c-Src activation of FAK results in initiation of a signaling cascade that increases the expression of MMP-2 and MMP-9 (discussed later) that are necessary for invasion [40].

Tumor Cells Acquire Motility, Degrade Surrounding Extracellular Matrix, and Invade into It

The process of motility involves formation of cellular protrusions, namely lamellipodia and filopodia, at the leading edge of the cell, which allow the cell to move directionally; formation of stable attachments near the leading edge of the protrusions; and propulsion forward [41]. This is followed by release of adhesions, especially attachment of integrins to the ECM, and retraction of the rear end. As the cells become motile, at their leading edge, actin-rich ruffles called *lamellipodia* are generated by the formation of new actin filaments from the sides of existing filaments. *Filopodia* are thin protrusions that extend from the lamellipodia and contain parallel bundles of actin filaments and sense signals and establish directionality of movement. These events controlling motility are primarily governed by Rho family proteins, namely CDC42, Rac, and RhoA [42]. Similar to Ras, Rho-family proteins undergo lipid modification that targets them to the cell membrane; these proteins can shuttle between a GTP-bound "on" state to a GDP-bound "off" state [26]. Rho-guanine nucleotide exchange factors (Rho-GEFs) stimulate binding of GTP to Rho and Rho-GTPase activating proteins (Rho-GAPs) stimulate GTP hydrolysis. In addition, Rho-GDP dissociation inhibitors (Rho-GDIs) sequester GTP-bound Rho proteins in the cytoplasm away from the GTP–GDP cycle. Signaling from growth factor receptors and integrins stimulates exchange of GDP for GTP on Rho proteins, and GTP-bound Rho interacts with a number of effector proteins, many of which are kinases, to regulate cellular physiology, especially motility.

The two well-characterized effector kinases are p21-activated kinases (PAKs), which bind activated CDC42 and RAC1, and the Rho-associated coiled-coil-forming kinases (ROCKs), which bind active RhoA [42]. These kinases phosphorylate downstream molecules to promote motility. CDC42 is important for the extension of filopodia by facilitating actin polymerization. Both CDC42 and Rac regulate generation of lamellipodia. RhoA is involved in the generation of contractile force and moving the body and tail of the cell behind the leading edge.

Overexpression of Rho, Rac, and the CDC42 family of proteins has been observed in diverse tumors originating from different organs or tissues [42]. RhoC has been linked to lung metastasis of melanoma cell lines [43]. Growth factor signaling, such as that mediated by hepatocyte growth factor (HGF) through its receptor Met, modulates many of the activities controlled by the Rho family of proteins [44]. Recent studies have identified Nedd9, an adapter protein to FAK, in regulating motility and invasion by melanoma cells, as well as metastasis of breast cancer cells to the lungs [45]. Another adapter protein. melanoma differentiation associated gene-9 (*mda*-9)/syntenin, interacts with c-Src and activates FAK, thus facilitating invasion and metastasis by melanoma cells [46].

Invasion, in which tumor cells with acquired motility need to break down the extracellular matrix to create a passage through which they can move, is an important step in metastasis. Two major pathways of controlled proteolysis are involved in this process, the matrix metalloproteinase (MMP) pathway and the urokinase-type plasminogen activator (uPA) pathway [47, 48]. MMPs are zinc-dependent endopeptidases [47]. Two members of the MMP family, MMP-2 and MMP-9, have the highest enzymatic activity against type IV collagen, the main constituent of the basement membrane. MMPs are secreted as inactive zymogens (Pro-MMPs) that require proteolytic activation by extracellular proteases. MMP-2 is activated on the cell surface by a complex containing MMP-2, membrane type 1 MMP (MT1-MMP), tissue inhibitor of metalloproteinase 2 (TIMP2), and integrin$\alpha v\beta 3$ [49]. Initially, the carboxy-terminal of MMP2 binds to TIMP2, which in turn associates with MT1-MMP; this cleaves the amino-terminal of MMP-2, resulting in an intermediate form that binds to integrin$\alpha v\beta 3$ [50]. This interaction activates MMP-2, thereby localizing its proteolytic activity to the invasive front of cells. Inhibition of interaction between MMP-2 and integrin$\alpha v\beta 3$ suppresses growth of melanomas and gliomas and inhibits angiogenesis in animal models [51].

The serine protease uPA, bound to its cell surface receptor (uPAR), efficiently cleaves cell surface-bound plasminogen, activating the broad-spectrum serine protease plasmin [52]. Plasmin promotes tissue degradation and remodeling of the local extracellular environment directly by degrading ECM molecules and activating or releasing latent growth factors. Plasmin also potently activates pro-MMP-2 and pro-MMP-9. Expression of uPA is being used as an independent marker of poor prognosis in many cancers [53]. Interestingly, one of the inhibitors of uPA, plasminogen activator inhibitor type 1 (PAI1. also known as SERPINE1), is also overexpressed in many tumors and, together with uPA, is considered a poor prognostic marker for many cancers [53]. This apparent paradox indicates complex regulation of this pathway along with the non-uPA–dependent function of PAI1. On the other hand, tumor-associated expression of PAI2 (SERPINB2) is associated with increased survival in breast cancer patients, indicating that PAI2 function might be more focused on uPA [54].

Both MMP and uPA pathways might be activated by the transcription factor nuclear factor-kappaB (NF-κB). NF-κB is primarily activated by inflammatory signals, and chronic inflammation plays a major role in tumorigenesis [55]. NF-κB directly regulates genes necessary

for cell survival, as well as for migration, invasion, and metastasis. Activation of NF-κB is observed in tumors of diverse lineages, and pharmacological inhibition of NF-κB is being evaluated as a potential therapeutic strategy for multiple cancers.

Tumor Cells Enter into Circulation and Survive (Intravasation)

For successful metastasis, tumor cells need to develop strategies to survive against continuous death stimuli arising from nutrient deprivation, hypoxia, changes in extracellular adhesions, changes in cell shape during invasion, and exposure to new stromal microenvironments. Overexpression of antiapoptotic proteins, such as Bcl-2, Bcl-x_L, XIAP and survivin, protects cancer cells from these death stimuli and increases the efficiency of metastasis [56]. In a similar manner, loss of caspase 8, an apoptosis-initiating caspase, also facilitates invasion and metastasis by making tumor cells more resistant to death signals generated from loss of adhesion [57]. For example, genomic loss of caspase 8 in pediatric neuroblastomas has poor overall prognosis [58].

Tumor Cells Emerge from Circulation and Enter New Tissues (Extravasation)

Certain genes are important in mediating extravasation by increasing permeability of blood vessels in target organs. Vascular endothelial growth factor (VEGF) is a potent vascular permeability factor [59]. VEGF activates c-Src family kinases in endothelial cells, leading to disruption of endothelial cell junctions, which can facilitate metastatic extravasation. The time of extravasation also varies, depending on the tumor. Some tumors grow in the intravascular space attached to the endothelium until the tumor physically bursts through the restraining surrounding vasculature. In metastatic osteosarcoma cells, the cytoskeletal anchoring protein ezrin facilitates this process; inhibition of ezrin results in higher cancer cell death prior to metastatic extravasation into the lung parenchyma [60].

Seeded Tumor Cells Proliferate and Develop into New Colony of Cells

A significant criterion for metastasis is that tumor cells must proliferate and form colonies at new organ sites. This aspect of metastasis requires new blood vessel formation (angiogenesis) that sustains the growth of the tumor cells in the new environment. The tumor cells also need to interact with the stroma of the colonized organ. Breast cancer cells generate the chemokine CXCL12, which helps recruit circulating endothelial progenitor cells containing CXCR4, the receptor for CXCL12, thus facilitating angiogenesis [61].

Interestingly, an innate immune response to tumor development and progression, which includes infiltration of neutrophils, lymphocytes, and macrophages, can also contribute to growth of the colonized tumor cells [62]. Cells and cytokines that mediate chronic inflammation facilitate both tumor initiation and metastatic progression. Some of the metastatic events are promulgated by cytokine-mediated activation of NF-κB that regulates a number of genes mediating invasion, metastasis, and cell survival [55]. Cyclooxygenase-2 [COX-2) expressed by the inflammatory cells generates prostaglandins (PGs) that facilitate metastatic progression [63]. Tumor-associated macrophages, attracted to regions of hypoxia and necrosis, induce angiogenesis by secreting vasoactive factors, such as VEGF, interleukin-8 (IL-8), and PGE_2, as well as MMP-9 and uPA, which enhance their bioactivity [64]. These macrophages also produce growth factors, such as EGF, PDGF, and HGF, that may facilitate tumor cell proliferation and survival [64]. Mice with defects in macrophage lineage caused by mutations in CSF-1 rarely develop lung metastasis from breast cancer.

Tumor cells from different primary sites have the propensity to home in and metastasize to specific organs – for example, breast cancers metastasize mainly to the lung and liver, prostate cancers metastasize to the bone, uveal melanomas to the liver, and sarcomas to the lung [65]. One hypothesis is that the tumor cells express specific proteins and the target organ contains a corresponding receptor (such as CXCL12 or CXCR4) that facilitates "homing" of the tumor cells to a specific organ. Astrocyte elevated gene-1 (AEG-1/Metadherin) is expressed on the surface of breast cancer cells [66, 67]. It contains an extracellular lung homing domain that facilitates metastasis of breast cancer cells to the lungs. Interestingly, AEG-1 is overexpressed in advanced cancers of diverse lineages, and it is also localized in the cytoplasm and nucleus; thus, it might regulate tumor progression and metastasis by multiple mechanisms [68]. Downregulation of KISS-1, a secreted protein, is required for the tumor cells to divide and form colonies in the new organ. KISS-1–overexpressing melanoma cells complete every step of the metastatic cascade in vivo, except growth and new colony formation at ectopic sites [69].

OSTEOPONTIN: EFFECTOR THAT CONTROLS EVERY STEP IN METASTASIS

A secreted phosphoprotein, osteopontin (OPN), belonging to the small integrin-binding ligand N-linked glycoproteins (SIBLINGs) family, is overexpressed in many tumors and plays a seminal role in regulating every step in metastasis [70]. Elevated OPN levels can be used as a sensitive and specific marker in predicting disease progression in head and neck, renal, gastric,

hepatocellular, lung, and pancreatic cancers and uveal melanomas. OPN signals through $\alpha v\beta 3$ integrins and CD44 glycoproteins, especially CD44v6 [71]. Binding of OPN to CD44 leads to the activation of the prosurvival PI3K/Akt pathway, thus protecting tumor cells from apoptosis and facilitating the growth of the primary tumor. Expression of $\alpha v\beta 3$ integrin is consistently detected in breast cancer bone metastasis. OPN–integrin binding stimulates EGFR transactivation and ERK phosphorylation, leading to the activation of AP-1 [70]. OPN also activates the NF-κB pathway, leading to increased invasion by activation of MMP and uPA pathways.

OPN protects tumor cells by downregulating nitric oxide (NO) production in tumor-infiltrating macrophages. In addition, OPN and other SIBLINGs can activate complement factor H, which disables the formation of the membrane attack complex and subsequent lysis of cancer cells, thus favoring their escape from host immune defense [70]. OPN functions as an angiogenic factor by promoting neovascularization through integrin-mediated endothelial cell migration, prevention of endothelial cell apoptosis, and vascular lumen formation. On the other hand, OPN expression itself is regulated by numerous protumorigenic events and transcription factors, such as AP-1 and c-Myc, thus establishing a positive cascade that helps in the process of metastasis [71].

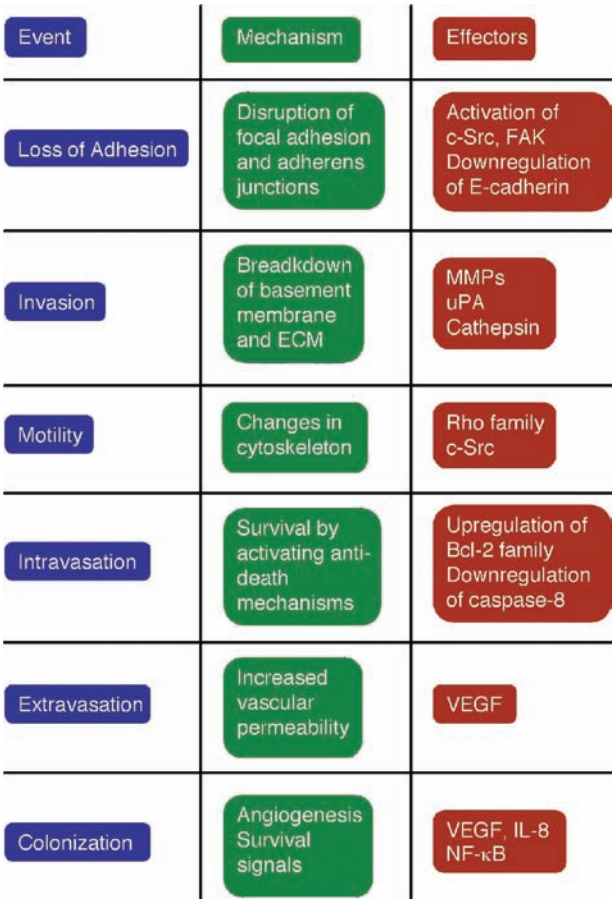

Figure 5.3. A simplified schematic of effectors potentially mediating different steps of metastasis. The effectors listed here are only illustrative examples; a large number of additional genes are involved in regulating every step in this complex process. FAK: focal adhesion kinase; ECM: extracellular matrix; MMP: matrix metalloproteinase: uPA: urokinase plasminogen activator; VEGF: vascular endothelial growth factor; IL-8: interleukin-8.

SUMMARY AND PERSPECTIVE

Metastasis is an extremely complex process involving the orchestration of multiple genetic and epigenetic changes in an evolving cancer cell (Figure 5.3). It is clear that there may potentially be additional metastasis-promoting genes that remain to be discovered. Failure to identify these genetic elements may occur because they are hidden by false-negative data based on experimental systems that are defective for a specific step (or steps) in metastasis.

By precisely defining the nature of the gene changes mediating and associated with metastasis, it will be possible to identify potentially new targets that can be used to develop pharmacological and genetic approaches to prevent this invariably fatal component of the cancerous process. Specifically, inhibiting seminal steps in this process – including extravasation, survival in the bloodstream, intravasation, and/or growth at a new organ site – could involve hindering some of the gene and gene family changes highlighted in this chapter. Comparative oncogenomic studies continue to identify specific gene expression signatures in metastatic cells compared with primary tumors. These gene signatures are uncovering previously unexplored genes and pathways involved in metastasis, thus facilitating newer insights into this complicated, multistep process. Future approaches employing pharmacological (including small-molecule drugs) and genetic approaches, including shRNA, siRNA, and microRNA, to alter the expression of specific metastasis-promoting genes or their downstream pathways may provide tangible benefits in inhibiting cancer metastasis.

ACKNOWLEDGMENTS

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6 The Role of Metastasis Suppressor Genes in Metastasis

Brunilde Gril, Russell Szmulewitz, Joshua Collins, Jennifer Taylor, Carrie Rinker-Schaeffer, Patricia Steeg, and Jean-Claude Marshall

In the 1970s and 1980s, clever scientific insight and innovation rapidly advanced our understanding of the molecular mechanisms of cancer biology. The discoveries of oncogenes and tumor suppressors, and the elucidation of their functions, greatly aided in studies aimed at a molecular understanding of the etiology of primary tumors. Despite this, cancer biologists had little understanding of the molecular aspects of metastasis. Considering the devastating consequences, scientists were anxious for a breakthrough. The first clue would come from the study of tumor suppressors.

Tumor suppressor genes were identified when it was discovered that their loss of function was critical to tumorigenesis. Prior to their discovery, researchers were of the mindset that the oncogenic phenotype was always dominant. In other words, a mutation need happen on only a single allele for a normal cell to be transformed into a tumor cell. However, not all disease incidence data seemed to fit neatly into this hypothesis. By studying retinoblastoma case histories, a "two-hit" hypothesis emerged, predicting that for at least some cancers, two mutations must occur (one on each allele) to successfully transform a cell [1]. Indeed, the retinoblastoma gene, or *Rb*, would become known as the first described tumor suppressor. We now know that the "two hits" need not come in the form of distinct somatic mutations but may be the result of any combination of germinal and/or somatic mutations, mitotic recombinations, gene conversions, and functional inactivation of genes owing to promoter hypermethylation. The list is extensive, but tumor suppressors currently include *Rb, p53, APC, PTEN, TSC1*, and *NF1*, among many others.

Making such headway in our understanding of metastasis was more problematic. The prevailing view of the day was that the process of metastasis was so complex as to be refractory to mechanistic studies. Further, once cancer cells escaped the primary tumor, it was only a matter of time before the patient succumbed to metastatic disease. It was on this scientific backdrop in 1988 that Patricia Steeg and colleagues proposed a hypothesis that analogous to tumor suppressor genes, there exist metastasis suppressor genes, the functional loss of which contributes to acquisition of metastatic ability [2]. To test this, she screened a cDNA library with ^{32}P-labeled mRNA probes generated from related melanoma cell lines possessing low and high metastatic potential [3]. Following differential hybridization, only a single cDNA clone, *Non*metastatic clone *#23*, or *NM23*, exhibited the expression pattern predicted by the hypothesis. *NM23* mRNA levels were highest in cells of low metastatic potential and decreased to undetectable levels in cells with high metastatic potential.

In vivo functional studies showed that both highly metastatic mouse and human cells transfected with *NM23* at physiologic expression levels and injected into mice significantly reduced metastatic potential independent of primary tumor growth. In particular, pulmonary metastases were reduced by as much as 96 percent. These data confirmed the place of *NM23* as that of the first metastasis suppressor gene [4, 5]. Moreover, this work provided direct molecular and biological evidence that tumor formation and metastasis formation are separable events, suggesting that metastasis formation could be specifically studied and targeted.

The discovery of *Nm23* ushered in a new functional class of genes, the metastasis suppressors, which currently number more than twenty. Metastasis suppressor genes (MSGs) are defined by their ability to suppress spontaneous metastases without affecting primary tumor growth in vivo. Although the field is young, research to date has shown that MSGs are involved in a variety of pathways regulating multiple steps in the metastatic cascade (Figure 6.1). For instance, MKK4, MKK6, and MKK7 are involved in the stress-activated MAPK pathway that regulates cell cycle

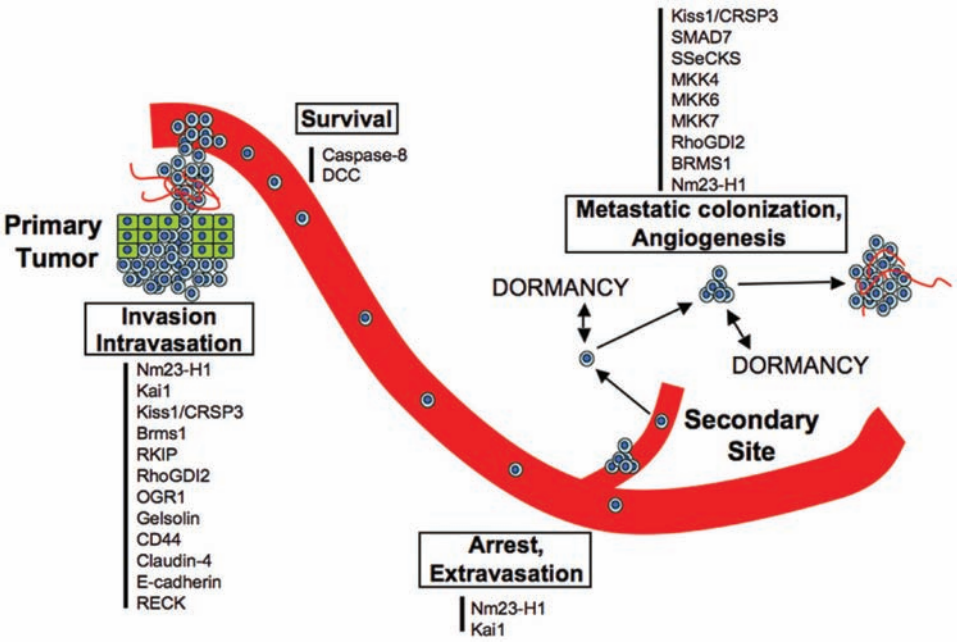


Figure 6.1. Schematic representation of the metastatic cascade and the possible steps in which various metastasis suppressor genes may interfere in the cascade.

progression and/or apoptosis, whereas *RhoGDI2* regulates cytoskeletal reorganization and motility by inhibiting *Rho*. The latter, then, inhibits metastatic invasion and the former inhibits metastatic colonization. In vivo mechanism-based studies have demonstrated that the metastasis suppressors can regulate rate-limiting steps of metastasis formation, making them attractive targets for molecular therapeutics.

MSG IDENTIFICATION AND VALIDATION STRATEGIES

The search for candidate MSGs has involved many techniques, including subtractive hybridization, differential display, microcell-mediated chromosome transfer (MMCT), and microarray analysis. The last two have been particularly profitable.

Early in the search for MSGs, many candidate genes were discovered using MMCT. Briefly, MMCT begins by blocking growing cells in mitosis and then chemically disrupting the mitotic spindle, allowing the condensed chromosomes to drift freely [6]. The cells are then allowed to reenter the cell cycle whereby the free chromosomes become membrane-bound, forming micronuclei that contain single and multiple chromosomes. Further chemical treatment, followed by differential centrifugation and filtration, results in single chromosome-containing microcells. Microcells containing the chromosome of interest are then fused to recipient cells – in this case, metastatic cancer cells. If the newly formed hybrid cells are found to have suppressed metastatic capabilities, then a variety of positional cloning techniques allow for pinpointing the genes of interest on the transferred chromosome.

The more recent development of microarray technology has greatly facilitated the search for MSGs. The microarray concept is simple – albeit in theory only. Oligonucleotide probes are chemically adhered to a substrate, usually glass or silicon, in an ordered array. Purified RNA that has been fluorescently labeled is then hybridized with the arrayed probes. The fluorescent intensities of the hybridized samples are measured relative to standardized samples, allowing for the determination of gene products that are either upregulated or downregulated. Thousands of genes can be analyzed at once using only picomoles of nucleic acid products. Candidate MSGs are identified on the arrays either by their reduced expression in metastatic versus nonmetastatic cell lines or in clinically resected tumors associated with metastatic versus nonmetastatic disease incidence.

After a candidate MSG has been proposed, usually a battery of validation tests ensues. A candidate MSG may display in vitro characteristics of metastasis suppression, including decreases in motility, invasion, anchorage-independent growth, and angiogenesis. However, a metastasis suppressor gene must be shown to function in vivo. This is in part because the metastatic cascade is far too complex to be adequately modeled by any number or combination of in vitro experiments, but also because of the need to monitor the effects on primary tumor growth.

Generally, in vivo validation of a potential MSG begins by transfecting the candidate gene into a highly

metastatic cell line at physiologic levels of expression. The transfected cells are then injected into animals in one of two basic methods [7]. The first approach, considered to be the gold standard, involves the orthotopic inoculation of the transfected, metastatic cells into the tissue site of origin. This allows for the formation of a primary tumor and subsequent generation of spontaneous metastases that have completed every step of the metastatic cascade. Metastasis incidence and number are then quantified.

The second approach involves the generation of "experimental" metastases by directly introducing the cells into the circulatory system of the animals, usually by intravenous (IV) or intracardiac injection. The metastases that develop are considered experimental because they have effectively skipped the beginning steps of the metastatic cascade. To conform to the definition of an MSG, transfected cells are also injected into the subcutis or, preferably, an orthotopic site for the determination of primary tumor size.

Experience with the model system can provide the required data to biostatistical collaborators who can identify the appropriate statistical approach or model for the study. This enables power calculations to be developed, thereby ensuring that the data will yield meaningful results. Because of the inherent heterogeneity of most metastasis models and the critical importance that data be incontrovertible, it is imperative that biostatistical approaches be part of every aspect of the design, implementation, and evaluation of metastasis studies.

Although there is a great deal of excitement regarding the use of optical and molecular imaging techniques for the assessment of in vivo metastasis, it is imperative that investigators recognize the strengths and limitations of each imaging technique. Imaging offers the potential benefits of increased sensitivity of detection, improved quality control of the experiment, and longitudinal studies in individual animals. However, it is not a shortcut or means to skirt the laborious and costly aspects of in vivo studies. It is also imperative that appropriate (additional) controls be included in the biostatistical design of the study and that imaging data be confirmed by histological examination of tissues (the gold standard).

Regardless of the approach, a gene loses the status of "candidate" and becomes accepted as an MSG if it fulfills the requirements of causing a significant decrease in the number of quantifiable metastases without affecting the growth of the primary tumor.

MSG AND METASTATIC COLONIZATION

Metastatic colonization begins after circulating tumor cells survive transport and lodge in the vasculature at secondary sites. The fate of such disseminated cells is complex. They may either extravasate into the surrounding tissue or remain within the vasculature. In either location they may undergo proliferation or apoptosis, or remain essentially quiescent for extended periods of time. Work from metastasis suppressor studies supports previous findings that growth in the primary tumor and metastatic site are not identical processes. A number of preclinical drug studies show disparate effects on primary tumor and metastatic growth [8], leading to the conclusion that applying the well-worn principles of primary tumor growth is unlikely to produce a complete understanding of metastatic colonization. Studies are only beginning to tease out the cancer cell–microenvironmental interactions that contribute to cell fate and, ultimately, metastasis formation. Metastasis suppressors are proving to be an important tool in these in vivo mechanistic studies.

Clinically, metastatic colonization may represent an optimal, and heretofore untapped, therapeutic target. Using breast cancer as an example, once a patient is diagnosed with a lymph-node-positive tumor, invasion has already occurred and it is probable that cells have already disseminated into the vasculature and lodged at secondary sites, implying that cells need only to survive and ultimately grow to complete the colonization process. The finding that some metastasis suppressors seem to specifically affect metastatic colonization provides a unanticipated application for their use in developing molecular therapeutics that target their functions and/or pathways. This may change disease management by extending the dormancy of the disseminated tumor cells, making the disease a clinically treatable chronic condition, or perhaps even killing disseminated cells in the adjuvant setting, thereby preventing metastasis formation altogether.

KNOWN METASTATIC SUPPRESSOR GENES

Since the identification of NM23 in 1988 [9], the number of candidate metastasis suppressors has increased to more than twenty, listed in Table 6.1 [10, 11]. In this section, we will focus on MSGs that have been the most thoroughly characterized with multiple cancer lines or in vivo spontaneous metastasis assays (reviewed in [11]).

Nm23-H1

Originally described as nonmetastatic clone 23, Nm23-H1 was identified by comparing the cDNA libraries of low- and high-metastatic melanoma cell lines. Its expression was quantitatively reduced in five highly metastatic cell lines, as compared with two related, less metastatic cell lines. The idea that one gene could affect the ability of cells to form distant metastasis was, at the time, controversial. In retrospect, it is logical, as

TABLE 6.1. Compiled list of all described metastasis suppressor genes and the evidence to support their role as MSGs.

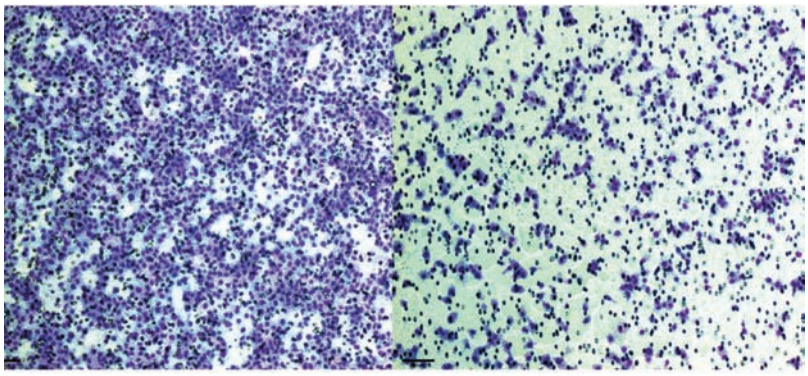
Metastasis suppressor gene	Evidence for metastasis suppressor gene	Potential steps in the metastatic cascade that are inhibited	References
KISS1	GFP-labeled tumor cells ectopically expressing KISS1 disseminate, but do not proliferate in secondary organs in experimental metastasis assays	Colonization of distant sites (lung)	[31]
Kai1	Binds to DARC on the surface of vascular endothelial cells to induce tumor cell growth arrest	Intravasation and survival in the bloodstream	[34, 74]
MKK4	(1) Exogenous expression prevents the development of overt metastases, but not micrometastatic foci in a spontaneous metastasis model (2) Activates MAPKs p38 and JNK (3) Kinase activity is detectable only in metastatic lesions, not in primary tumor	Migration and colonization at the secondary site	[22, 37, 38]
MKK7	(1) Exogenous expression prevents the development of overt metastases, but not micrometastatic foci in a spontaneous metastasis model (2) Activates MAPK JNK (3) Kinase activity is detectable only in metastatic lesions, not in primary tumor	Migraiton and colonization at the secondary site	[22]
Nm23 (-H1 and -H2)	(1) MPA stimulates Nm23-H1 expression and inhibits the growth of preestablished micrometastatic colonies in a breast cancer model (2) Inhibits MAPK ERK1/2 (3) Expression correlates with suppression of anchorage independent growth in vitro	(1) Ectopic expression of Nm23-H1 reduces survival of fluorescently labeled MDA-MB-435 cells in the lung (2) EDG2, a lysophosphatidic acid receptor important for motility, can restore metastatic potential to tumor cells ectopically expressing Nm23-H1	[9, 44, 51, 52, 56, 57]
BRMS1	(1) Reduces size, as well as incidence, of tumor metastasis in experimental metastasis assay (2) Metastatic foci from mice bearing BRMS1 overexpressing MDA-MB-435 tumors do not form grossly visible lesions	Survival in the bloodstream as well as colonization at the secondary site	[58, 62, 63]
SMAD7	(1) Reduces size, as well as incidence, of tumor metastases (2) Metastatic bone lesions derived from melanoma cells ectopically expressing SMAD7 continue to develop 14 weeks after the completion of an experimental metastasis assay	Potential regulation of cadherin expression, adhesive properties of the tumor cells	[26, 59]
SSeCKS	(1) Reduces size, as well as incidence, of tumor metastases (2) Mechanism is linked to VEGF downregulation (3) Inhibits anchorage independent growth with no effect on motility in vitro	Angiogenesis at the secondary site and migration	[24, 60]
RHOGDI2	(1) Reduces size, as well as incidence, of tumor metastases (2) Mechanism is linked to ET-1 downregulation, which is an important target for the antiangiogenic compound astrasentan	Migration and colonization at a secondary site	[25, 61]
CTGF	Reduced size of lung metastases by 15–25 % in an experimental metastasis model of lung adenocarcinoma	Expression correlates with inhibition of tumor cell motility and invasion in vitro	[18]

(Continued)

TABLE 6.1 *(Continued)*

Metastasis suppressor gene	Evidence for metastasis suppressor gene	Potential steps in the metastatic cascade that are inhibited	References
RKIP	(1) Inhibits MAPK ERK1/2 (2) Regulates expression of angiogenic genes (3) Associates with kinetochores and centrosomes and regulates the cell cycle spindle checkpoint	Expression correlates with reduced motility and invasion, but not anchorage-independent growth in vitro	[75–77]
DLC-1	Identified by its reduced expression in a non-metastatic subline of MDA-MB-435, which has a dormant phenotype, as compared with a metastatic subline	Migration and invasion of the tumor cells	[66, 67]
DCC	Cell-cell or cell-matrix adhesion	(1) Luciferase-labeled tumor cells exogenously expressing DCC reduced the number of tumor cells disseminated to lymph nodes and lungs (2) Induce apoptosis via caspase-8	[78]
Caspase-8	Induces apoptosis/anoikis	(1) Proapoptotic enzyme (2) Reduces tumor cell dissemination by triggering apoptosis via an integrin-linked mechanism	[79]
OGR-1	G-protein-coupled receptor signaling	(1) GFP-labeled cell tumor cells ectopically expressing OGR-1 do not appear to disseminate to secondary organs (2) Expression correlates with reduced motility and invasion, but not anchorage independent growth in vitro	[17]
Gelsolin	Actin regulatory molecule, cytoskeletal architecture and rearrangement	(1) Actin severing/capping protein that is involved in cell motility (2) Mutational analysis revealed that its metastasis suppressive effects correlate with its ability to suppress motility and cell spreading in vitro	[80]
E-cadherin	Cell-cell or cell-matrix adhesion	Regulates heterotypic cell adhesion via adherens junctions	[81]
Claudin-4	Expression correlates with reduced anchorage independent growth in vitro	(1) Mediates cell–cell adhesion via tight junctions (2) Expression correlates with reduced invasion in vitro	[82]
CD44	Cell–cell or cell–matrix adhesion, can bind to hyaluronic acid and osteopontin	(1) Transmembrane glycoprotein involved in cell migration and lymphocyte activation and homing (2) Expression correlates with reduced invasion in vitro	[83–86]

empirical data have shown that acquisition of metastatic competence requires multiple cellular functions, the impairment of which renders a cell non-metastatic [2]. Since its discovery, Nm23-H1's metastasis suppressor function has been confirmed in several cancer models, including melanoma [12], prostate [13], colon [14], breast [15], and oral squamous cell carcinoma [16]. The metastasis-suppressive effects of Nm23-H1 were also confirmed by a genetic approach by Lacombe and colleagues [17], who showed that in an Nm23 knockout mouse, the rate of hepatocellular tumor formation was unchanged between the knockout and wild-type mice; however, the knockout mice developed twofold more pulmonary metastases ($p < 0.001$). In vitro overexpression of Nm23-H1 has been shown to decrease the migratory ability of breast cancer cells (Figure 6.2). Increased expression of Nm23-H1 has not always been shown to correlate with suppression of metastasis. Indeed, in leukemia and neuronal cancers, Nm23 has been linked to increased aggressiveness. These findings have indicated that the metastasis suppressor function of Nm23 is context- and possibly tissue-dependent, although the exact reasons behind this difference remain to be elucidated.



A B

Figure 6.2. Migratory ability of Nm23-H1 overexpressing cells in response to fetal bovine serum as a chemoattractant. (A) shows vector control cells, and (B) shows cells overexpressing Nm23-H1.

The biochemical mechanism of action of Nm23 suppression of metastasis is likely complex. A total of eight different human Nm23 homologs have been identified, although only Nm23-H1 and -H2 have been studied extensively [18, 19]. Nm23-H1 has been shown to interact with an ERK1/2 MAP kinase scaffold protein, known as the kinase suppressor of Ras (KSR), to reduce ERK1/2 activation [20]. Tumor dormancy in other model systems has been characterized as a balance between p38 and ERK1/2 activation, where higher ERK1/2 activation promotes cell proliferation; high levels of p38 activation stimulate cell death; and a balance prompts dormancy, although this has not been tested formally [21–23]. Nm23-H1 inhibition of ERK1/2 may help regulate this balance. Nm23 specifically binds other proteins, which may also contribute to its mechanism of action, including casein kinase 2, Rad, latent antigen of the Eptsein-Barr virus, and heterotrimeric G proteins. Other biochemical activities have been attributed to Nm23 that may contribute to its metastasis suppressive effect, including histidine protein kinase, NDP kinase, and DNA exonuclease [24].

Gene expression profiling of control and Nm23-transfected cell lines have identified candidates downstream of Nm23 that may also contribute to its metastasis suppressive effect. Microarray analysis of downregulated gene products between a control and high-expressing Nm23 breast cancer cell line identified several proteins of interest, including EDG2. EDG2 and its homologs, EDG4 and EDG7, are receptors for lysophosphatidic acid (LPA), an abundant phospholipid that is a major constituent of blood serum. Previously these molecules have been linked to tumorigenesis and metastasis [25]. Specifically, LPA has been shown to act through these receptors to enhance metastasis in ovarian cancer. Nm23-H1 demonstrably inhibits expression of EDG2; this reduced expression is crucial for Nm23-H1-mediated suppression of motility, invasion, and metastasis model systems [25, 26]. In addition, overexpression of EDG2 was capable of overcoming Nm23-H1 suppression of tumor cell arrest, adhesion, and survival in the secondary site. Taken together, this work suggests the possibility that EDG2 may constitute another molecular therapeutic target for aggressive, low-Nm23-H1-expressing metastatic breast cancer. Levels of LPA in the blood remain relatively constant; however, several tumor cells produce and secrete autotoxin, which may lead to localized gradients of LPA. Targeting EDG2 in tumor cells could potentially inhibit the cells from continuing on to form metastasis.

Owing to their ability to inhibit metastasis, MSGs are an attractive therapeutic target. There has been some indication already of compounds that are capable of increasing Nm23 expression in mammalian tumor cells, with a corresponding decrease in metastasis. In "triple negative" (estrogen receptor [ER] negative, progesterone receptor [PR] negative, and Her-2 wild type) breast cancer cell lines, Nm23-H1 expression was increased twofold by treatment with medroxyprogesterone actetate (MPA). It is thought that this activation is the result of binding by MPA to the glucocorticoid receptor [27]. To test the hypothesis that MPA increased Nm23 expression, mice were injected with metastatic MDA-MB-231 cells IV; micrometastatic pulmonary lesions developed within four weeks, after which mice were randomized to MPA or vehicle. The mice on an eight- to ten-week MPA treatment regimen had a 27 to 40 percent reduction in incidence of metastatic colonization and a 44 to 48 percent decrease in the mean number of metastases. Side effects of MPA included weight gain, but no significant effects were noted on bone density, lean–fat ratio, or mammary fatpad histology. A Phase II trial examining high-dose MPA, alone and in combination with metronomic chemotherapy, has opened at Indiana University. Thus, over the past twenty years, NM23 has gone from concept to mechanism but has not been translated into the clinical setting. This has demonstrated the feasibility and utility of metastasis suppressors in studying the metastatic process. Findings from other suppressor and model systems are contributing to the rich, vibrant, and increasing knowledge of metastatic colonization.

KISS1

KISS1 was first characterized in 1996 when expression of the KISS1 cDNA led to inhibition of metastases in both breast and melanoma experimental models, with no effect on the primary tumor growth [8]. KISS1 is unique among the MGSs in that it encodes secreted polypeptides, known as kisspeptins. Multiple

kisspeptins have been described, including metastin, a 54-amino-acid polypeptide. Metastin has been described as binding to the G protein coupled receptor GPR54 and is believed to be a peptide responsible for metastasis suppression [28, 29]. The GPR54 receptor and its ligand, metastin, have previously been described has having a function in puberty [30].

Evidence suggests that KISS1/metastin promotes dormancy of solitary tumor cells at secondary sites [31]. Specifically, experiments using fluorescently labeled cutaneous melanoma cells were injected into athymic nude mice via the tail vein and shown to disseminate throughout the body. Stable production of the fluorescent protein enabled imaging of the cells throughout the course of the experiment. Cells that had been transfected with empty vector or a secretion signal deletion variant of kisspeptin formed detectable macrometastases in lung, bone, kidney, and eye at thirty-five days postinjection. In comparison, the melanoma cells ectopically expressing wild-type kisspeptins remained dormant in the multiple organs without any detectable growth for up to 120 days postinjection. These data indicate that secreted kisspeptins play an important role in maintaining the dormancy of these melanoma cells once they have arrived at a distant site. Paradoxically, the mechanism of KISS1-induced dormancy in melanoma cells did not appear to require the presence of GPR54, suggesting there may be another receptor at work, although further study is required to delineate its molecular mechanism. The fact that this is a secreted peptide makes it an attractive pharmacological target for patients with disseminated disease.

Kai1

Another metastasis suppressor that appears to confer dormancy of solitary tumor cells is Kai1, or CD82. It was first described in 1991 as a 267-amino-acid tetraspanin protein, thought to be involved in inhibiting cancer cell migration and invasion [32]. Tetraspanins are a large group of cell surface proteins with four transmembrane structures, which can form complexes with integrins [33]. A recent study showed that Kai1 also inhibited a later stage in the metastatic cascade, in addition to cell migration and invasion. Kai1 interacted with Duffy antigen receptor for chemokines (DARC), a seven-transmembrane protein, which is expressed on endothelial cells [34]. Using breast, melanoma, and prostate cancer cell lines, Kai1 high- and low-expressing cells were assayed for their ability to bind DARC-positive endothelial cells, with the high-Kai1-expressing prostate cells exhibiting a much higher binding affinity. Cancer cells binding to endothelial cells via Kai1/DARC induced growth arrest in the tumor cells and decreased in vitro colony formation without detectable initiation of apoptosis. Knockout DARC –/– mice were used to assay the effects in vivo, and showed that Kai1 + clones were able to develop significant lung metastases when introduced in the DARC –/– mice as compared with wild-type and heterozygous controls with breast and melanoma cancer cell lines. The data indicate that Kai1 requires DARC to suppress metastasis. The observed growth arrest in the tumor cells was associated with a decrease in TBX2 and an upregulation of p21, an inhibitor of the cyclin-CDK2, cyclin-CDK4 complexes. Previous studies have shown that TBX2 inhibited senescence by repressing p21 expression in melanoma cells, indicating that the p21 pathway may be important in dormancy and tumor progression [35]. Downregulation of Kai1 at a protein level has been demonstrated, implicating a ubiquitin ligase, Gp78, which targets Kai1 for degradation [36]. This degradation of Kai1 from the cell membrane was inhibited by suppression of Gp78, leading to a decrease in metastatic potential of the sarcoma cell line in vivo. The data may lead to a potential therapeutic target to maintain Kai1 levels in these cells by inhibiting Gp78.

An additional mechanism of action by Kai1 has been linked to its ability to inhibit Met and Src activation [37]. Human prostate cancer cells were used to explore the interaction between Kai1 and integrin-mediated signaling. The addition of Kai1 to these cells downregulated the ability of c-Met to be activated by its ligand, HGF. In addition, reexpression of Kai1 led to decreased activation of Src, along with reduced migration and invasion, both mediated by integrins, thereby indicating that the loss of Kai1 in tumor cells may lead to increased c-Met and Src signaling, which in turn increases motility and invasive ability of the cells, rendering them more aggressive. This revealed downstream molecules that could be targeted to mimic the effects of Kai1 in reducing the metastatic ability of tumor cells.

JNKK1/MKK4, MKK7, and MKK6

Using a combination of microcell-mediated chromosomal transfer, positional cloning strategies, and in vivo metastasis assays, JNKK1/MKK4 was identified, a MAP2K involved in the stress-activated signaling kinase casade, as a metastasis suppressor encoded by chromosome 17 [38]. Initial work showed that ectopic expression of JNKK1/MKK4 reduced the number of overt prostate cancer lung metastases by 90 percent [39]. A broader role for the protein in metastatic cancer was established using a xenograft model of ovarian cancer [40]. Human ovarian carcinoma cells expressing JNKK1/MKK4 formed significantly fewer overt metastatic implants and increased the life span of injected animals by 70 percent. In both model systems, JNKK1/MKK4's kinase activity was required for metastatic suppression [39, 41].

Mitogen-activated protein kinases (MAPKs) are proteins that translate an external signal into activation

of a series of downstream targets that direct cellular processes (reviewed in [42]). MAPKs involved in mammalian cells include ERK1/2, ERK5, JNK, and p38. The phosphorylation cascade is initiated by activation of MAP kinase kinase kinases (MAP3Ks), which in turn phosphorylate MAP kinase kinases, or MAP2Ks, which in turn phosphorylate the target MAPK. MAP2Ks phosphorylate a single MAPK target (except JNKK1/MKK4); however, MAP3Ks are thought to be able to phosphorylate multiple MAP2Ks, allowing for signaling specificity in response to diverse stimuli. Extracellular signal-related kinase (ERK) signaling has been a focus of the cancer literature because of its role in cellular proliferation, transformation, and malignant progression downstream of the Ras oncogene. The JNK and p38 MAPKs are part of the stress-activated protein kinase (SAPK) pathway, which is classically associated with growth arrest and apoptosis; they are therefore most commonly thought of as inhibitors of cancer formation and progression.

The involvement of JNKK1/MKK4 in clinical cancers is supported by data showing that approximately 5 percent of tumors have loss-of-function mutations in the *JNKK1/MKK4* gene. Moreover, multiple tumor types (e.g., lung, pancreas, breast, and testes) have loss of heterozygosity at the *JNKK1/MKK4* locus [43–46]. Xin et al. found that life expectancy is reduced by approximately 50 percent in gastric cancer patients with MKK4-negative tumors as compared with those with MKK4-positive tumors, and that JNKK1/MKK4 was more commonly expressed in primary tumors than in metastases [47]. Similarly, in a retrospective study of gastric adenocarcinomas, lack of JNKK1/MKK4 expression in the primary tumor was associated with a five-times greater risk of death [48]. Stark et al. found decreased JNKK1/MKK4 mRNA levels in breast cancer brain metastasis, suggesting a link between lack of JNKK1/MKK4 and metastasis formation [49]. In prostate cancer samples, a statistically significant inverse relationship between the level of JNKK1/MKK4 expression by immunohistochemistry and Gleason grade was reported [50].

Clinical findings have not always been consistent, however, highlighting the complex role of the SAPKs in cancer regulation. In certain contexts, JNKK1/MKK4, JNK, and less so p38, have also been linked to cancer progression (reviewed in [43, 51]). For example, in a study of pancreatic and breast cancer cell lines lacking JNKK1/MKK4, ectopic expression of JNKK1/MKK4 resulted in increased cellular proliferation and invasion [52]. In a preclinical study of pancreatic cancer, cells with a homozygous mutation in *MKK4* formed fewer experimental (intravenous) metastases and slower tumor doubling times as compared with cancer cells that were heterozygous for *MKK4* [53]. Furthermore, in contrast with the study by Kim et al., a recent study by Lotan et al. showed increased expression of JNKK1/MKK4 and MKK7 were associated with higher prostate cancer stage at diagnosis [54].

Elaborating further on the role of JNKK1/MKK4 in the regulation of metastatic cancer, in vivo functional studies show that its kinase activity, not just expression, is required for suppression of ovarian and prostate cancer metastasis formation [39, 41]. This suggests that the protein regulates cellular proliferation by acting in a kinase cascade to activate downstream substrates of JNK and/or p38. Interestingly, in vivo studies showed that JNKK1/MKK4 activity does not cause increased apoptosis in SKOV3ip.1 human ovarian cancer cells in an experimental model of metastasis [55]. Instead, a proliferative arrest was observed, as measured by decreased BrdU incorporation and phospho-histone-3 expression in JNKK1/MKK4-expressing microscopic metastases. This correlated with a statistically significant increase in expression of the cell cycle inhibitor p21Waf1/Cip1, suggesting that JNKK1/MKK4 is impairing metastatic colonization by inducing cell cycle arrest.

Additional mechanistic studies indicate that JNKK1/MKK4 acts through the p38 arm of the SAPK pathway in this model system [41]. On the other hand, data from the AT6.1 prostate cancer xenograft model indicates that JNKK1/MKK4 signals through JNK to suppress the growth of disseminated cells within lung metastases [39]. However, it remains to be determined whether this suppression is mediated through apoptosis or cell cycle arrest in this model system.

These in vivo preclinical findings have many implications for understanding the role of the SAPK signaling pathway in cancer progression. Understanding how cell type and microenvironment determine how a stimulus is transmitted via the same kinase to two different target molecules is essential to designing specific therapeutic agents. Additionally, although it is imperative to understand how JNKK1/MKK4 can lead to signaling divergence on a molecular level, it is equally important to acknowledge that it also leads to convergence in cellular outcome. Activation of both the JNK pathway in prostate cancer and the p38 pathway in ovarian cancer leads to suppression of metastatic outgrowth. Tools are now available to dissect the mechanism responsible for these findings.

BRMS1

Like JNKK1/MKK4, the BRMS1 metastasis suppressor was as a result of microcell transfer of chromosome 11 into a human breast carcinoma cell line, which inhibited metastasis formation [56]. Positional mapping of the antimetastatic activity of chromosome 11 led to the identification of the novel MSG breast cancer metastasis suppressor 1 (BRMS1) gene. The metastasis suppressor activity of BRMS1 has been verified in melanoma, bladder, and non–small-cell lung

carcinoma cell lines [57, 58]. The putative mechanism of BRMS1-mediated suppression of metastasis appears to be complicated, as the protein and has been implicated in the regulation of multiple cellular functions (e.g., gap junction formation) and expression of signaling modules (e.g., PI3K and EGF). A brief overview of each of these BRMS1-associated events is given in the following sections.

BRMS1 is unique among MSGs in that it has the demonstrated ability to regulate gap-junctional communication between tumor cells, which may modulate communication between tumor cells or between the tumor cells and the surrounding microenvironment, and thereby regulate metastatic growth [59]. In vitro expression of BRMS1 was shown to decrease the survival of breast cancer cells in response to hypoxia and to increase anoikis, the ability of cells to survive after detachment from a particular surface (usually modeled in two dimensions on tissue culture plastic), while also decreasing the adhesive ability of these cells [60].

The phosphoinositide-3-kinase (PI3K) signaling pathway [61, 62] is a major pathway in many cancers and plays a key role in regulating cell survival and growth. PI3K phosphorylates a downstream phospholipid known as phosphatidylinositol-4,5,bisphosphate (PIP_2) to form PIP_3, which in turn binds to a variety of downstream targets such as AKT. BRMS1-expressing cells have been shown to have significantly decreased levels of PIP_2, potentially impairing the activation of cellular pathways that influence cell survival and growth. Overexpression of BRMS1 in breast cancer lines can also decrease the levels of epidermal growth factor receptor (EGFR) [63, 64]. EGFR is a transmembrane receptor tyrosine kinase that is expressed in many normal cell types. Binding of the ligand causes phosphorylation of the tyrosine residues in the cytoplasmic tail and can initiate signaling through the Ras/MAPK cascade, or PI3K pathway, among others. Deregulation of EGFR is an important factor in a variety of cancer types, including breast, head and neck, and colorectal carcinomas.

Finally, BRMS1 has also been shown to interact with a key molecular regulator of cellular survival and apoptosis, NF-κB, and can also interact with histone deacetylase (HDAC) complexes. Because HDACs are responsible for the deacetylation of histones and their subsequent release from DNA to promote DNA transcription, this may be a viable target for future drugs. At this time it is unclear if BRMS1 functions represent independent or interacting activities, which ultimately modulate the metastatic ability of cells.

RHOGDI2

RHOGDI2 is located on chromosome 12p12.3 and was identified as a result of a DNA microarray analysis of human bladder cancer cell lines [65]. Theodorescu and colleagues analyzed more than 30,000 genes to identify those that have reduced expression in the more aggressive cell lines. This differential expression approach identified 2,368 gene candidates, of which ten were investigated further based on relative fold change; of those ten, RHOGDI2 was validated in human samples. Introduction of RHOGDI2 into bladder carcinoma cell lines had no effect on primary tumor growth in immunodeficient mice but did significantly reduce metastasis, meeting the criteria for an MSG. RHOGDI2 has been shown to inhibit Rho and Rac both of which are involved in invasion [8]. Rac and Rho, along with Cdc42 have a central role in cytoskeletal organization, adhesion, and motility. The mechanism of RHOGDI2 function is likely the result of its ability to inactivate Rho and thereby inhibit cytoskeletal reorganization and motility.

A microarray analysis approach was used to identify differences in gene expression between highly metastatic bladder cancer cells that expressed ectopic RHOGDI2 as compared with control cells [66]. Two gene products were found to be downregulated by the reintroduction of RHOGDI2 into these cells, endothelin-1 (ET-1) and neuromedin U (NMu), both of which are secreted peptide agonists of G-protein-coupled receptors. Examining the role of ET-1 signaling in metastasis was greatly enhanced by the fact that there already exists a compound, atrasentan, which blocks the ET-1 receptor. Atrasentan is currently in clinical trials for the treatment of other types of cancer [67] and other ET-1 antagonists are under development. Mice were injected with bladder cancer cells and treated with atrasentan for eight weeks prior to necropsy. Only 5 percent of the mice treated with atrasentan developed detectable lung metastasis, compared to 53 percent of the mice in the control group. This, as well as a previous study showing that atrasentan is well tolerated in patients, forms the basis of an ongoing clinical trial for the treatment of bladder cancer patients with low-RHOGDI2-expressing tumors.

RKIP

Raf kinase inhibitor protein (RKIP) was first demonstrated as an MSG in prostate cancer cells, and has since been shown to play a role in colorectal and breast cancer [68, 69]. RKIP has been mapped to chromosome 12q24.23 and appears to have a variety of functions, which are context-dependent [70]. RKIP has been shown to bind directly to Raf, thereby decreasing MEK-ERK1/2 activation, a pathway critical for several cellular functions, such as survival and motility. Reduced expression of RKIP can lead to increased activation of MAPK and subsequent reduction of apoptosis in cancer cells [71]. Validation of RKIP as an MSG has come from

clinical samples, where primary tumors with matched patient lymph node metastasis showed loss of RKIP expression in the metastases [68]. RKIP expression has also been characterized as a predictive marker of metastasis in patients with colon cancer [72]. Recent work has begun to elucidate the biological function and signaling pathways in which RKIP is involved [73]. RKIP was shown to have multiple mechanisms of action, inhibiting MAPK as well as G-protein-coupled receptor kinase and the NF-κB signaling cascades. It appears that RKIP functions to regulate these important cellular pathways, maintaining them in their correct states. It has been hypothesized that loss of RKIP can therefore lead to deregulation of key cellular functions such as replication, leading to cancer.

Newer Metastasis Suppressor Genes

In recent years, the number of described MSGs has increased considerably. Although a full description of each MSG is beyond the scope of this chapter, those that have been validated are listed in Table 6.1. The recent use of microarray-based techniques has accelerated the rate at which MSGs have been found, although many of the newest putative metastasis suppressors have yet to be functionally validated using in vivo metastasis assays. Of these, some may prove to be of considerable interest, such as CD44, Claudin-1 and 4, gelsolin, RECK, and Drg-1 (reviewed in [11]). Doubtless, there are more MSGs to be discovered, with the hope of finding more targets that can be translated to helping patients.

CLINICAL IMPLICATIONS

The ability to control primary tumor growth with surgery and radiotherapy, chemotherapy, or some combination of them provides adequate control at the initial site for most patients. For most solid cancers, mortality usually arises as a result of metastatic disease or a complication of treatment. Although the metastatic process is complex, the inhibition of even one step of the metastatic cascade will halt the entire process. Therefore, the ability to restore the function of an MSG into cancer cells prior to the completion of the entire metastatic process would be of clinical benefit. Metastatic colonization may represent the portion of the metastatic process most amenable to therapeutic intervention.

It is an unfortunate reality that most cancer therapeutic trials to date focus on the ability of a therapeutic agent to shrink a large metastatic lesion in a patient. Therapeutic agents that target MSGs would be expected to halt, rather than reverse, metastatic progression in patients. These compounds would be unlikely to meet current clinical response standards for early clinical testing. This would necessitate the use of tailored clinical trials to investigate MSG therapeutic compounds in an adjuvant setting. The future of study in this field is promising, as new techniques allow us to dissect areas of the metastatic cascade that were previously hidden. The push to introduce these into the clinic and targeting them as potential therapeutic targets, as currently in the case of NM23 and RHOGDI1, has opened the entire field of study for potential breakthrough into clinical use.

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7 Stromal-Derived Factors That Dictate Organ-Specific Metastasis

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The acquisition of a metastatic phenotype is the most deadly trait a tumor can develop. Secondary tumors compromise organ function, are refractory to standard chemotherapeutics, and ultimately lead to the demise of the patient. Although tumor progression contains stochastic elements, there is an emerging pattern of organotropism, as various cancers display a predilection to metastasize to distinct secondary sites. A common trait of highly metastatic tumors is the ability to adapt the topology of local and distant microenvironments to better aid their progression. Indeed, many metastasis-regulating genes are components of, or require interactions with, stromal cells or the extracellular matrix (ECM) to exert proper function [1–4]. As such, the propensity to metastasize to specific sites is controlled in part by endemic homing mechanisms that involve coordinated ligand–receptor interactions between the cancer cell and the host microenvironment. Despite large advances in our understanding of metastasis biology, the molecular mechanisms guiding these processes remain largely uncharacterized.

Combinatorial phage-display libraries are a powerful screening tool that can readily identify functional protein interactions *in vivo*. Their utility has revealed that the stromal microenvironment – specifically the vasculature – of an organ contains a unique "molecular address" that can be modulated during inflammation, tumor growth, and metastasis [3, 5–7]. This chapter explores the role of the stroma during metastatic progression and highlights how phage display technology has been used to discover novel endothelial markers that disrupt tumor progression and metastasis.

SITE-SPECIFIC METASTASIS

The process of metastasis is not random. Although it has been suggested that the patterns of blood flow from the primary site to the first capillary bed can dictate metastatic seeding [8], this paradigm does not hold true for all cancers. For example, the high incidence of liver metastasis in patients with advanced colorectal cancer can be attributed to the direct blood flow via the hepatic portal vein, whereas breast cancer metastasizes preferentially to the liver rather than to spleen or gastrointestinal tissues [9, 10]. Moreover, despite the lung's being one of the first distant capillary beds for breast and prostate cancer, these tumors have a higher predilection to metastasize to the bone [9].

An alternative hypothesis was proposed in 1889 by Paget [10], who reviewed a large breast cancer autopsy series and noted that the pattern of metastasis cannot be described by mechanical dissemination alone. Instead, he mused that a tumor cell is similar to a seed and that it will thrive (metastasize) only in a foreign soil (the secondary site) if it is congenial for growth. Although it dates back a century, this hypothesis remains steadfast, with modern genomic platforms supporting Paget's dogma. Indeed, the combination of animal models of metastasis and genome-wide screening platforms, such as gene expression arrays [11], shRNA libraries [12], miR libraries [13] and combinatorial phage libraries [14], have all proved fruitful in the identification of key genetic elements that control metastatic propensity and dictate site-specific colonization of cancer cells.

Lessons learned from genome-wide screens have also suggested that elements within both the cancer cell [15] and stromal compartments [16, 17] can contribute to whether a tumor will achieve full metastatic potential. Cell compartments that can regulate metastatic progression include the endothelium [4], mobilized immunomodulatory cells [3, 18, 19], adipocytes [20], fibroblasts [21], and coopted mesenchymal stem cells [22]. Likewise, non-cellular constituents within the ECM provide an alternative stromal compartment that contains regulatory elements that can influence metastasis [1, 23]. As such, the development of novel

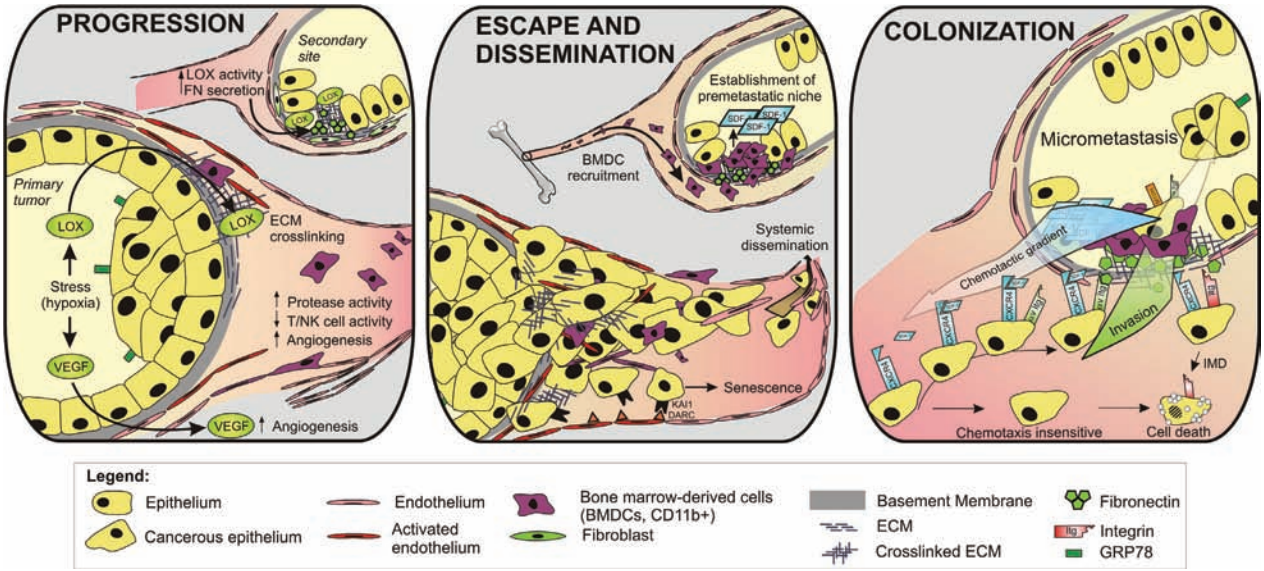

Figure 7.1. Stromal regulation of spontaneous metastasis. As the tumor develops, increasing oxidative stress elevates stress-inducible genes including lysyl oxidase (LOX), vascular endothelial growth factor (VEGF), and glucose-regulated protein 78 (GRP78). These, in turn, stimulate angiogenesis through the recruitment of quiescent vascular cells and the mobilization of bone-marrow–derived cells (BMDCs). Crosslinking of extracellular matrix (ECM) proteins in the basement membrane and interstitial ECM pave the way for cellular migration and invasion into and out of the tumor. Intravasating tumor cells must escape metastatic inhibitory cues from endothelial cells by downregulating metastasis suppressor genes such as caspase-8 and KAI1/CD82. LOX may also be involved in establishing potential future sites of metastasis, through its ability to crosslink ECM proteins in premetastatic niches that promote adhesion of both tumor cells and circulating BMDCs. Recruited BMDCs appear at secondary sites prior to the arrival of the tumor cell and can aid tumor cell establishment through the production of stromal-derived factor-1 (SDF1) chemoattractive gradients. Circulating tumor cells respond to SDF1 by altering its cell-surface receptor profile in a way that it is complementary to the extracellular topology of the secondary site. Tumor cells that create unfavorable adhesions are cleared from the system through necrotic and apoptotic mechanisms such as integrin-mediated death (IMD).

therapeutics would greatly benefit from a better characterization of functional cell–cell, cell–ECM, and ECM–ECM interactions that occur during tumor progression.

STROMAL–CANCER CELL INTERACTIONS DURING METASTASIS

As a prelude to metastasis, the primary tumor ECM becomes a volatile microecosystem, whereby "out-of-context" cellular and non-cellular communications between the cancer cell and the stroma lead to pathological progression [24, 25] (Figure 7.1). Although oncogenic conversion leads to the initial derailment of cellular growth, it does so in an environment that is dynamic and responsive to the demands of the growing mass. Inefficient energy use within starved cancer cells leads to acidosis, hypoxia, and the release of reactive oxygen species within the microenvironment. These stresses in turn, trigger the release of pro-angiogenic peptides such as angiopoietin [26], ephrin A1 [27], fibroblast growth factor-3 (FGF-3) [28] and vascular endothelial growth factor (VEGF) [28, 29], which induce sprouting, growth, and migration of endothelial cells toward the tumor in an attempt to "normalize" the tissue. As a result, microvascular homeostasis becomes disrupted through persistent pro-angiogenic signals from the tumor that tip the balance in favor of a heightened level of activity of the normally quiescent vasculature [30].

Despite elevated pro-angiogenic factors, inefficient blood flow remains a common feature in many tumors [31]. This has been attributed to a lag in the rate of neovascularization and a diminishing vascular space as the tumor expands, causing heterogenic pockets of hypoxia to be formed throughout the tumor [31]. Additionally, the haywire of signals derived from the angiogenic milieu leads to a chaotically arranged vasculature that consists of an immature and incomplete endothelium [32]. Newly developed endothelium is often hyperpermeable and has a slow rate of blood flow, which creates an interactive environment that is conducive to transvascular escape of the cancer cell.

In a parallel mechanism, sustained hypoxia recruits bone-marrow–derived cells (BMDCs) to the tumor in an attempt to restore vascular function. Instead it seems that BMDCs (of the $CD11b^{+}$ lineage) augment tumor progression through several mechanisms: they can integrate into the developing endothelium to further enhance tumor vasculogenesis [18, 33], activate latent proteases within the ECM [3], and suppress the anti-tumor actions of T- and NK-cells [34]. Recently, it has been demonstrated that under hypoxic conditions cancer cells secrete lysyl oxidase (LOX), an enzyme

that modifies the interstitial ECM by crosslinking the basement membrane proteins collagen IV and elastin [35]. These posttranslational modifications enhance the tensile strength of the matrix scaffold, producing a molecular track that is conducive for cellular attachment and motility of BMDCs and cancer cells [36]. Moreover, LOX activity is observed in distant tissues prior to the arrival of metastatic cells, suggesting that hypoxia-driven, posttranslational ECM modifications are a driving factor in the development of the pre-metastatic niche [3]. Indeed, systemic quenching of LOX activity *in vivo* prevented the mobilization and recruitment of BMDCs to the pre-metastatic niche and inhibited spontaneous lung metastasis [3]. Collectively, these studies implicate ECM remodeling as the driving force in site-specific metastasis. It follows that a better comprehension of the molecular topology of the pre-metastatic niche could pave the way for preventive therapies.

The interstitial ECM, proteolytically scarred and modified, provides a tactile scaffold on which cancer cells can migrate and eventually disseminate from the primary site. Cellular movement is orchestrated through the repetitive formation and dissociation of cell surface receptors with ECM ligands and is guided by chemotactic and haptotactic forces (reviewed in [37, 38]). A major contributor to ECM-mediated cellular movement is the integrin family of cell adhesion proteins [39]. The functional integrin receptor consists of the heterodimeric pairing of one of eighteen α-subunits and one of eight β-subunits, the combination of which mediates substrate-specific cellular attachment, survival, and migration [39].

Integrins have been implicated in cancer invasion and metastasis to distant sites where complementary ligands are abundant [40]. For instance, integrin $\alpha_v\beta_3$- and $\alpha_8\beta_1$-positive cancer cells have a predilection to metastasize to the lung, bone, and kidney, where their cognate ECM components laminin-511, osteopontin (OPN), and nephronectin are located [1, 41, 42]. Conversely, a lack of favorable ECM components prevents integrin–ECM ligation leading to an induction of caspase-8 dependent apoptosis, a phenomenon known as integrin-mediated death (IMD) [43]. IMD also occurs in normal cellular biology as demonstrated by the survival and guidance of $\alpha_v\beta_3$- and $\alpha_5\beta_1$-integrin-positive endothelial cells [43, 44]. With only a small fraction of cancer cells surviving past extravasation [45], overcoming IMD-specific death presents a major barrier to the prospective metastatic cell. Recently, Stupack et al. (2006) demonstrated that the loss of caspase-8 (but not other caspases) expression in human neuroblastoma cells allows the cells to become refractory to IMD signals provided within the primary stroma and endows them with spontaneous metastatic potential [2].

Endothelial-specific cell surface proteins can also regulate metastatic propensity at the primary site. Bandyopadhyay et al. (2006) discovered that surface expression of Duffy antigen receptor for chemokines (DARC) on endothelial cells can functionally interact with KAI1, a metastasis suppressor protein found on the surface of prostate cancers with low malignant potential. Intravascular interaction between KAI1 and DARC rendered the cancer cells senescent, whereas cells lacking KAI1 expression were not growth-arrested [4]. Moreover, when KAI1-positive tumors are engrafted into DARC$^{-/-}$ mice, the cancer cells were able to escape endothelial cell-induced senescence, intravasate, and metastasize to the lung [4].

Once in circulation, the cancer cell is shunted throughout the body, guided initially by vascular flow but arresting and colonizing distant tissues based on complementary molecular contacts and growth cues. Further, the architecture of distant capillary networks, including the retraction of endothelial cells, exposure of the sub-endothelial layer and fragmented basal lamina, provide a lucrative niche for tumor cell lodgment and metastatic colonization. While in transit, the cancer cell can form conglomerates with platelets and other circulating cells to resist the stress of shear flow, increase their chance to lodge in the capillaries, and enhance their response to chemotactic gradients [46].

Muller et al. (2001) have suggested that the dissemination of cancer cells is akin to the trafficking of leukocytes, with chemotactic gradients originating from distant organs dictating site-specific metastasis. Indeed, it has been demonstrated that breast and prostate cancer cells expressing the chemokine-responsive receptor CXCR4 can specifically home to vascular beds of the lung and bone based on chemoattractive gradients of stromal-derived factor-1 (SDF-1/CXCL12) originating from these organs [47, 48]. Vascular-derived SDF-1 activates VCAM-1, or α_5-, α_v-, and β_3 integrin receptors on the arriving cancer cell, which increases their compliance to attach to the foreign endothelium and to invade into interstitial ECM [49, 50]. Once in the secondary site, tumor cells may remain dormant for extensive periods, adapting to new microenvironmental growth cues and avoiding harmful cytotoxins until finally reestablishing proliferative mechanisms to form overt metastases.

Collectively, there is not one step in the metastatic cascade in which the cancer cells are functionally independent of the stroma. As such, studies aiming to assess metastasis must be performed using in vivo models that can take into account the changes that can occur during disease development. We have illustrated here how the stroma can react to a growing tumor to modulate its local and distant microenvironments, favoring tumor progression. Although largely unknown, the characterization of these reactive microenvironments will

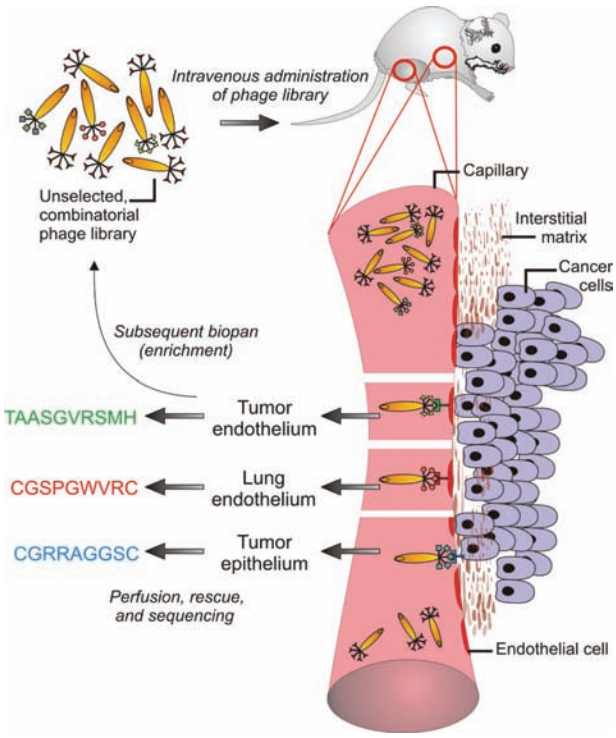

Figure 7.2. Phage biopanning for extracellular targets. Combinatorial phage libraries are injected into the circulation via the lateral tail vein. The library passively distributes with individual phage lodging wherever complementary protein–protein interactions occur. Organs, or cell populations of choice, are then removed, the phage isolated, and the DNA sequenced. Serial rounds of biopanning can selectively enrich for phage with specific homing properties.

provide benefit for the development of site-directed therapeutics.

PHAGE DISPLAY: A FUNCTIONAL MEANS TO IDENTIFY LIGAND–RECEPTOR INTERACTIONS

Genomic screens have identified hundreds of candidate metastasis-regulating genes; however, there remains a paucity of information in regard to their functional role or their potential to be translated to the clinic. Moreover, high-throughput sequencing and gene array approaches cannot describe the molecular heterogeneity and posttranslational modifications of the interstitial ECM – a critical, non-cellular element that can control metastasis. As such, profiling techniques that can identify biologically relevant, functional interactors within the extracellular space would be highly advantageous for the design of targeted diagnostic and therapeutic strategies.

Phage display provides a rapid and unsupervised means to select, isolate and characterize protein–protein interactions (Figure 7.2). This method has been used to fingerprint antibodies [51] and identify cell surface receptor–ligand complexes *in vitro* [52, 53] and *in vivo* [54, 55]. Combinatorial phage display libraries consist of ~10^9 unique cyclic polypeptide sequences, with each peptide expressed on the PIII coat protein of an individual filamentous bacteriophage [53, 56]. Through complementary protein–protein binding, the phage becomes captured by its target and can be recovered and the polypeptide identified by DNA sequencing. Target enrichment can be accomplished through successive rounds of biopanning the same sample with each refined library.

A major strength of the phage display method is its utility to identify receptor–ligand interactions in a physiologically relevant *in vivo* setting. When injected intravenously, combinatorial phage libraries circulate and can be recovered from various organs. Using this approach, we and others have identified a vascular "address system" that is unique between tissues [6, 54] and becomes altered during tumor progression [57–59]. Furthermore, peptides identified in this manner were able to deliver targeted angiogenic compounds to the tumor endothelium [60] and associated lymphatics [61], verifying the functional and clinical utility of *in vivo* phage display methods.

The combination of phage display and animal models of metastasis provides an ideal strategy for the systematic identification of peptides that are involved with disease progression. Indeed, several targets have already been established (Table 7.1). In this next section, we will examine several functionally validated targets that have been identified using various phage display methodologies.

Metadherin (MTDH). MTDH encodes a transmembrane protein that is involved with cell–cell adhesion and was recently established as a poor-prognostic marker in breast cancer. Using the highly metastatic mouse mammary tumor line 4T1, Brown et al. (2004) created a phage expression library that consists of secreted and transmembrane proteins. This library was subsequently screened *in vivo* to identify phage interactors that specifically home to the lung, of which MTDH was the prime candidate [14]. Phage displaying the MTDH peptide exhibited a twentyfold enhancement in binding to lung endothelium compared with controls, whereas the MTDH protein can be detected on the cell surface of 4T1 cells, suggesting that this interaction can occur *in vivo*. To prove this, siRNA-mediated MTDH gene ablation, or treatment with neutralizing MTDH antibodies largely inhibited the colonization of 4T1 cells within the lung [14].

Recently, studies from the Kang laboratory have identified elevated MTDH expression in breast tumors owing to a genomic amplification at 8q22 [62]. Similar to the study of Brown et al. (2004), reduced MTDH expression in human breast MDA-MB-231 cells inhibited metastasis to the lung, but not brain or bone, suggesting a site-specific role for MTDH during

TABLE 7.1. Functional interacting peptides identified using combinatorial phage display libraries.

Target Protein	Peptide Sequence(s)	Peptide Localization	Cancer	Reference
APN/CD13	CNGRC	Endothelium, tumor cells	Melanoma, non-small-cell lung cancer, prostate	[66, 78]
GRP78	WIFPWIQL, WDLAWMFRLPVG	Tumor cells	Most solid cancers	[68, 73]
HSP90	CVPELGHEC	Tumor cells	Ovarian	[51]
IL-11Rα	CGRRAGGSC	Endothelium, tumor cells	Prostate, osteosarcoma, breast	[74, 75]
αv integrin	CDCRGDCFC	Endothelium, tumor cells	Melanoma, breast	[63, 64]
NG2/HMP	TAASGVRSMH, LTLRWVGLMS	Endothelium, tumor cells	Melanoma	[60]
VEGFR-1, VEGFR-2, NRP-1	CPQPRPLC, HTMYYHHYQHHL, WHSDMEWWYLLG, ATWLPPR	Endothelium	All cancers	[79–82]
Metadherin	See reference	Tumor cells	Breast	[14]
Membrane dipeptidase	GFE	Endothelium	Melanoma	[59]
Galectin-3	ANTPCGPYTHDCPVKR, PQNSKIPGPTFLDPH	Endothelium, tumor cells	Breast, melanoma	[83]
E-selectin	IELLQAR	Endothelium, tumor cells	Melanoma	[84]

progression [62]. Furthermore, it was demonstrated that MTDH mediates chemoresistance in breast cancer cells [62], a result that was further enhanced if the cancer cells were co-cultured with endothelial cells. Taken together, MTDH is an ideal candidate for the therapeutic targeting of advanced cancer and further characterization of this largely novel protein is eagerly awaited.

α_v integrin. A phage displaying an Arg-Gly-Asp (RGD)-containing peptide (RGD-4C phage) with high specificity for both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was characterized in a mouse model of melanoma [63, 64]. The RGD-4C phage homed specifically to tumor-associated endothelium compared to the vasculature of non-tumor–bearing tissues, or phage displaying other RGD-containing motifs [64]. This finding is in agreement with previous work that suggests that α_v integrins are present and become activated in tumor angiogenic vasculature [65]. Because of the superior targeting of the RGD-4C phage, doxorubicin (dox) was conjugated to the peptide mimetic to assess the therapeutic validity of targeting α_v integrins in animal models of cancer. Compared to an equivalent dose of non-targeted or non-conjugated dox, targeted treatment with dox-RGD-4C resulted in lower toxicity, induced vascular death, inhibited tumor growth, and prevented metastasis to the lymph nodes and lung [66]. Moreover, α_v-specific neutralizing antibodies retarded the growth and metastasis of integrin α_v-positive, but not α_v-negative, melanoma xenografts [67]. These studies validate the strategy of α_v integrin-targeted therapeutics, with several compounds, including humanized monoclonal antibodies and small amino acid peptides in Phase II clinical trials for advanced metastatic disease.

Glucose-regulated protein-78 (GRP78). In an alternative strategy, phage display was employed to map the diversity of sera-derived antibodies from prostate cancer patients. In this technique, the humoral response characterizes the antigens presented by the tumor, and as a result the phage-displayed peptides that bind to these antibodies reflect these tumor-based antigens [68]. Identified in this screen were multiple peptides that mimic GRP78, a member of the heat shock protein-70 family of stress-induced factors [69]. GRP78 expression becomes elevated in response to cellular stresses such as acidosis, glucose starvation, and hypoxia that threaten to disrupt normal function of the endoplasmic reticulum [70]. As such, it is not surprising that GRP78 levels are low in resting cells, but become grossly overexpressed and localized to the cell surface during the progression of most solid cancers [70–72], which makes it an ideal candidate for targeted antitumor and antimetastatic therapy. To this end, we have fused the GRP78-targeting moiety (WIFPWIQL) to the pro-apoptotic 12-mer $_D$(KLAKKLAK)$_2$ and demonstrated that it can home to and inhibit DU145 prostate tumor xenografts [73] and spontaneous lung and bone metastasis derived from 4T1.2 tumors (unpublished data). Although GRP78 may not be a bona fide regulator

of site-specific metastasis, its elevated expression at the cell surface in both primary and secondary tumors makes it a practical therapeutic target.

Interleukin-11 receptor alpha (IL11Rα). IL11Rα was validated as the receptor that binds the IL11- peptide mimetic CGRRAGGSC, which was initially discovered in the organ-specific vascular mapping project of a patient [55]. The IL11Rα-targeting phage bound specifically to normal prostate tissue and subsequent verification found that enhanced IL11Rα expression correlates with prostate malignancy and bone metastasis [55, 74]. Studies by other groups have now demonstrated a functional role for IL11/IL11Rα in the growth of bone tumors [75] and cancers that metastasize to bone [15, 76, 77]. These data provide strong support for the utility of IL11Rα-directed therapeutics in advanced cancers with bone involvement.

SUMMARY

Mounting evidence points to a critical role for the stroma in disease progression. The development of cancerous disease inflames local and distant microenvironments, which alter the display of cell surface receptors and ECM topology to benefit metastatic progression. Presented here are several examples of how the molecular terrain of tumors can be functionally evaluated using phage display. Candidates such as IL11Rα and MTDH have been similarly identified using alternative genomics-based methods, thus cross-validating both platforms and streamlining target selection for therapeutic design. A more thorough understanding of the molecular interactions at the tumor–host interface will be important in the development of new therapeutic strategies.

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8 Metastasis Genes: Epigenetics

Amaia Lujambio and Manel Esteller

Metastasis is the sequence of interrelated steps by which primary tumor cells acquire the capability to invade adjacent tissue, enter the systemic circulation (intravasate), translocate through the vasculature, arrest in distant capillaries, extravasate into the surrounding tissue parenchyma, and, finally, proliferate from micrometastases into macroscopic secondary tumors [1, 2]. This metastatic process is the cause of 90 percent of deaths in patients with solid tumors [1, 2]. Therefore, unraveling the inner mechanisms of the pathogenesis of metastasis at systemic, cellular, and molecular levels has become a major goal of cancer research [1, 2].

In recent years, the contribution of epigenetics to the field of cancer has been of paramount importance, because cancer is both a genetic and an epigenetic disease [3] and because epigenetic alterations are also involved in the metastatic process [4]. Thus, cancer cells have to gain an epigenotype to disseminate from the primary tumor mass or to survive and proliferate at a secondary tissue site [4].

We are still in the early stages of deciphering the timing and hierarchy of these epigenetic lesions; we need to know how epigenetic mechanisms operate in normal and cancer cells to understand the epigenetic changes that occur in metastasis. This information will allow us to identify new metastasis-related genes, to discover new epigenetic biomarkers that may help identify the diagnostic signatures of metastasis, and to develop new cancer therapies based on epigenetic drugs [5]. In this chapter, we discuss the contribution of epigenetics to cancer progression and metastasis through the regulation of metastasis-related genes and miRNAs.

EPIGENETIC CONTRIBUTION TO NORMAL CELLS

Epigenetics is defined as the inheritance of changes in gene expression patterns that involve no changes in DNA sequence [6]. The term *epigenetics* was first introduced by C. D. H. Waddington in 1930 to name "the causal interaction between genes and their products, which bring the phenotype into the being" [3]. There are two main epigenetic events of key relevance to gene regulation, development, and carcinogenesis: DNA methylation and histone modification [7].

DNA methylation occurs almost exclusively on a cytosine in a CpG dinucleotide context, through the addition of a methyl group to the 5′ position of the cytosine ring [8]. This process is mediated by three DNA methyltransferases (DNMTs) [9]. DNMT1 is the maintenance methyltransferase that preserves methylation patterns at each cell division, whereas DNMT3A and DNMT3B are de novo methyltransferases [10].

Only 3 percent to 6 percent of all cytosines are methylated in normal human DNA, as methylation is restricted to CpGs [8]. Moreover, CpG sites are roughly depleted in the genome and are not evenly distributed, leading to CpG-poor regions and CpG-dense regions called CpG islands [11, 12]. The latter are often located at the 5′ end region of almost half of the protein-coding genes and are usually unmethylated in normal cells [13]. In contrast, sporadic CpG sites in the rest of the genome are usually methylated. The unmethylated status of CpG-island-containing genes ensures their transcription when the necessary transcriptional activators are available [13].

DNA methylation is an important phenomenon by which mammalian cells maintain their correct patterns of expression; it is involved in the establishment of imprinting and X-chromosome inactivation [9, 14]. Repetitive genomic sequences are densely methylated, maintaining chromosomal integrity by preventing the translocations, chromosomal instability, and gene disruption that could arise from the reactivation of endoparasitic sequences [15]. Finally, DNA methylation is required for germline-specific expression of some genes, such as those of the MAGE family [16],

and for tissue-specific gene silencing in cell types, such as maspin, in which they should not be expressed [17].

However, DNA methylation does not play alone; other epigenetic modifications join in the game. Histones are now recognized as being dynamic regulators of gene activity; they undergo many posttranslational chemical modifications, including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation [6, 18]. In general, some histone modifications, such as histone acetylation, are associated with active gene transcription, whereas others, such as the methylation of lysine 9 of histone H3, indicate condensed and inactive chromatin [19]. However, the "histone code" hypothesis postulates that the expression status of a particular region of chromatin depends on the given combination of histone modifications [20]. For this reason, deciphering the "rules of the game" is not an easy task.

Finally, the interplay between DNA methylation and histone modifications needs to be elucidated. The two mechanisms cooperate in controlling gene expression by maintaining a precise crosstalk in different complexes [21].

Taking all this into account, the disruption of the epigenetic patterns of a healthy cell will lead to different dysfunctions, including cancer and metastasis. Thus, the study of epigenetic alterations associated with cancer and metastasis has become one of the most important challenges in cancer research.

EPIGENETIC CONTRIBUTION TO CANCER PROGRESSION

The three main epigenetic alterations in human cancer, which affect both DNA methylation and histone modifications, are the hypermethylation of tumor suppressor genes, global DNA hypomethylation, and histone changes [22]. These alterations can also affect metastasis, but their contribution to this has not been thoroughly studied to date.

The discovery of "classical" oncogenes and tumor suppressor genes has attracted all the attention in the past thirty years. However, as it became generally accepted that major disruption of DNA methylation, histone modification, and chromatin compartments are common hallmarks of human cancer, the study of epigenetic alterations has come under cancer research's spotlight [22].

The contribution of epigenetics to cancer can also embrace metastasis. On one hand, metastasis has been characterized by complex gene signatures, which may be able to define the efficiency of the metastatic process – for instance, ensuring the ability of individual metastatic cells to survive at specific secondary tissue sites [23]. On the other hand, a proportion of these gene expression alterations could be the consequence of a failure in the epigenetic patterns of DNA methylation and histone modification [13, 24]. Changes in gene expression could result from epigenetic modifications that alter transcription either directly or through chromatin effects [23].

Recently, the epigenetic contribution to tumorigenesis has been widely reviewed, from the perspectives of mammalian epigenomics [25], cancer epigenomics [13, 26], and chromatin modifications [27]. These publications highlight the potential diagnostic, prognostic, and therapeutic applications emerging from this rapidly progressing field [13]. However, the contribution of epigenetics to metastasis is not very well understood. In this chapter, we try to summarize our current knowledge of the contribution of these three epigenetic alterations to tumor progression and metastasis.

Global DNA Methylation Alterations during Tumor Progression and Metastasis

In cancer, at the same time as CpG islands become hypermethylated, cancer cell genomes conversely undergo global hypomethylation, losing around 20 percent to 60 percent of their genomic 5-methylcytosine content, compared with their normal counterparts [6, 8, 28]. In the early 1980s, one of the first epigenetic alterations to be found in human cancer was the low level of DNA methylation in tumors relative to their normal-tissue counterparts [29]. This loss arises mainly by hypomethylation of the "body" of the genes involved (the coding region and introns) and through demethylation of repetitive DNA sequences, which make up 20 percent to 30 percent of the human genome [15].

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer (Figure 8.1) [30]. The alterations in overall DNA methylation status can be studied using two complementary approaches: high-performance capillary electrophoresis (HPCE), an analytical technique that provides absolute measures of 5-methylcytosine content [31–33], and immunolocalization of 5-methylcytosine, which provides qualitative information about its nuclear distribution [34]. Importantly, HPCE reveals that there is a continuous loss of 5-methylcytosine during tumor progression [30]. These findings highlight the value of using DNA hypomethylation levels as a biomarker of tumor aggressiveness and confirm that global genomic hypomethylation is a dynamic characteristic, rather than a static feature, of carcinogenesis. This strongly underlines the importance of DNA hypomethylation in malignant transformation leading to metastasis [30].

DNA hypomethylation has been linked to malignant processes and also metastasis through different mechanisms (Figure 8.1). First, loss of DNA methylation

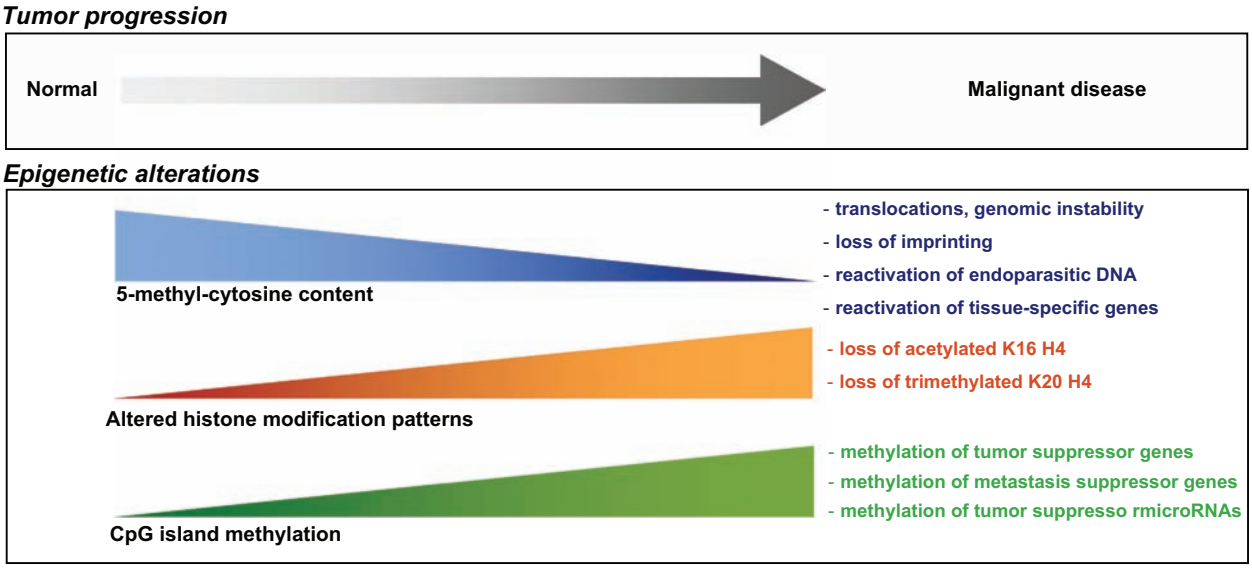

Figure 8.1. Epigenetic contribution to tumor progression. In any tumor progression model, in conjunction with phenotypic cellular changes and the accumulation of genetic defects, there is a progressive loss of total DNA methylation content, an increased frequency of hypermethylated CpG islands, and an increased histone modification imbalance in the development of the disease.

can favor mitotic recombination, leading to deletions and translocations [35], and can promote chromosomal rearrangements. This mechanism was seen when DNA methylation was erased by genetic disruption of DNMTs and caused aneuploidy [36]. Second, loss of imprinting of *IGF2* is a risk factor for colorectal cancer [37, 38] and disrupted genomic imprinting contributes to the development of Wilms tumor [39].

Third, hypomethylation of DNA in malignant cells can reactivate intragenomic endoparasitic DNA, such as LINE-1 (long interspersed nuclear elements), and Alu (recombinogenic sequence) repeats [40]. These demethylated transposons can be transcribed or translocated to other genomic regions, thereby disrupting the genome. Furthermore, a relationship between higher levels of hypomethylation in LINE-1 and Alu elements in neuroendocrine tumors and lymph node metastasis was found [41], and a tendency toward a loss of methylation at these repeats in prostate adenocarcinoma progression. Additionally, a strong correlation has been found between hypomethylation on chromosome 8 and the presence of metastasis in prostate carcinoma [42]. Finally, certain testis-specific genes, such as those that code for melanoma antigens or specific proliferation-linked genes [43], are methylated in a tissue-specific manner and are therefore silent. In contrast, however, these promoter regions undergo demethylation in some cancer cells with the consequence that habitually repressed genes become expressed. Two notable examples of the hypomethylation mechanism are the activation of PAX2 and of the *let-7a-3* miRNA gene, which has been implicated in endometrial and colon cancer [44, 45].

There are other examples of hypomethylated genes in metastasis. For example, hypomethylation of S100A4, a calcium-binding protein previously implicated in metastasis, is associated with gene activation in colon adenocarcinoma cell lines [46], medulloblastoma development [47], poor differentiation and/or higher grade in pancreatic ductal adenocarcinomas [48], and endometrial carcinoma [49]. Finally, for certain genes, such as uPA/PLAU [50] and synuclein gamma (SNCG) [51], in which hypomethylation has been reported in a wide range of cancers with high invasive or metastatic potential, there is an opportunity for genetic and epigenetic approaches to knock down their expression.

Abnormal Histone Modifications Associated with Cancer Progression

Little is known about the patterns of histone modification disruption in human tumors. It has been shown that promoter CpG-island hypermethylation in cancer cells is associated with a particular combination of histone marks: deacetylation of histones H3 and H4, loss of histone H3 lysine K4 (H3K4) trimethylation, and gain of H3K9 methylation and H3K27 trimethylation [52]. It is also recognized that certain genes with tumor-suppressor–like properties, such as p21WAF1, are silent at the transcriptional level in the absence of CpG-island hypermethylation when hypoacetylated and hypermethylated histones H3 and H4 are present [53]. Generally, histone acetylation is associated with transcriptional activation [25, 54], but the effect of histone methylation depends on the type of amino acid and its position in the histone tail [25, 54].

Until recently, a genome-wide profile of histone modifications and their locations had not been available for any transformed cell type, but the posttranslational modifications of histone H4 have now been profiled at a global level in a comprehensive panel of normal human tissues, cancer cell lines, and primary tumors [55]. In this study, cancer cells exhibited a loss of monoacetylated and trimethylated forms of histone H4. Mass spectrometry showed these losses to occur predominantly at the acetylated K16 and trimethylated K20 residues of histone H4 and to be associated with the well-characterized hypomethylation of repetitive DNA sequences. Similar results have been obtained for breast and liver tumorigenesis [56, 57], indicating that the global loss of monoacetylation and trimethylation of histone H4 might be a common feature of human tumor cells, as has now been accepted for global DNA hypomethylation and CpG-island hypermethylation (Figure 8.1).

Interestingly, these changes appear early and accumulate during tumorigenesis (Figure 8.1), as was shown in a mouse model of multistage skin carcinogenesis [30]. In this tumor progression model, chromatin immunoprecipitation (ChIP) assays using two antibodies against anti-acetyl-H4 and anti-dimethyl-K4-H3, which are associated with transcriptional activation, show a drastic loss of acetyl-H4 and dimethyl-K4-H3 in hypermethylated CpG island promoters, whereas the two modifications are significantly enriched in the unmethylated CpG islands [30]. For instance, the MLH1 promoter, which is unmethylated and actively transcribed in all cell lines, has large amounts of both acetyl-H4 and dimethyl-K4-H4 throughout tumoral progression. However, the scenario for E-cadherin and Snail is more dynamic: in the PAM212 cell line, an unmethylated CpG island for E-cadherin is associated with enhanced amounts of acetyl-H4 and dimethyl-K4-H3 and active transcription, whereas in CarB and CarC cells, a dramatic reduction in both histone modifications is associated with CpG island hypermethylation and silencing [30]. Therefore, histone modification patterns arise very early in tumorigenesis and develop during tumor progression, for which reason they are of considerable biological significance.

Promoter DNA Methylation of Candidate Genes in Tumor Progression and Metastasis

Hypermethylation of the CpG islands in the promoter regions of tumor suppressor genes is a major event in the origin of many cancers [3], contributing to all of the typical hallmarks of a cancer cell that result from tumor suppressor inactivation [24].

The first reports of hypermethylation of CpG islands in the promoter regions concerned the retinoblastoma tumor suppressor gene (Rb) [58, 59] and were followed by findings concerning the epigenetic inactivation of the tumor suppressor genes VHL (associated with von Hippel–Lindau disease) [60], p16INK4a [61–63], hMLH1 (a homolog of MutL *Escherichia coli*) [28] and BRCA1 (breast cancer susceptibility gene 1] [28, 64]. Therefore, DNA hypermethylation can affect genes involved in the cell cycle, DNA repair, the metabolism of carcinogens, cell-to-cell interaction, apoptosis, and angiogenesis, all of which may play a part in the development of cancer [13, 28].

The contribution of DNA methylation to metastasis is less well understood than are the expression changes in metastasis-associated genes [4]. On one hand, the attempts to identify the epigenetic contribution have focused on mapping increased DNA methylation within the promoter regions of individual candidate genes [4] (Figure 8.1). These hypermethylation events usually result in the repression of gene expression that may permit tumor cells to metastasize or have a selective advantage at the secondary tissue site [4]. Most reports relate DNA methylation changes in individual genes or in the context of individual cancers. These metastasis-associated genes that can undergo promoter CpG-island hypermethylation are involved with cadherin genes, the heparan sulfate synthesis pathway, tissue inhibitors of proteinases, axon guidance molecules, thrombospondins, and laminins, among others. These are analyzed in depth below (Table 8.1).

The most illustrative example is that of the CDH1 gene (Table 8.1). Briefly, E-cadherin germline mutations are responsible for the inherited form of diffuse gastric cancer and somatic mutations are characteristic of breast cancer, but the major mechanism of E-cadherin loss in human cancer is epigenetic silencing through DNA hypermethylation [65, 66]. Epigenetics is the most convenient Darwinian means of inactivating a metastasis suppressor gene because it can be dynamic and reversible. Following this line of reasoning, it has been shown that in some primary tumors displaying E-cadherin hypermethylation, the corresponding metastases were unmethylated at the E-cadherin gene [67]. These findings are consistent with previous observations of a lack of E-cadherin expression in the original neoplasm, but reexpression of E-cadherin at distant metastatic sites. Thus, demethylation and reexpression of E-cadherin are thought to be necessary for the correct incorporation of the metastatic cell into its new, normal cellular neighborhood. Other epigenetic mechanisms, such as a shift in the histone modification pattern and recruitment of chromatin-remodeling factors with repressive activity, can account for the loss of gene expression. E-cadherin gene inactivation in transformed cells can be mediated in some cases by the action of the transcriptional repressors Snail and Slug, which recruit histone deacetylases to the E-cadherin promoter [68, 69].

TABLE 8.1. A catalog of genes silenced by CpG island promoter hypermethylation in human metastasis.

Gene name	Function	Location	Tumor type
CDH1	E-cadherin, cell adhesion	16q22.1	Breast, stomach, leukemia
CDH13	H-cadherin, cell adhesion	16q24.2-q24.3	Breast, lung
CDH4	R-cadherin, cell adhesion	20q13.3	Colon
FAT	Protocadherin	4q35	Colon
EXT1	Heparan sulfate synthesis	8q24.11-q24.13	Leukemia, skin, sarcoma
GPC3	Heparan sulfate synthesis	Xq26.1	Ovarian
HS3ST2	Heparan sulfate synthesis	16p12	Breast, colon, lung, pancreatic cancer
TIMP2	Tissue inhibitor of metalloproteinase 2	17q25	Different tumor types
TIMP3	Tissue inhibitor of metalloproteinase 3	22q12.3	Different tumor types
TFPI2	Tissue factor pathway inhibitor 2	7q22	Glioma, pancreatic cancer
SEMA3B	Semaphorin 3B, axon guidance	3p21.3	Lung cancer
SLIT1	Slit homolog 1, axon guidance	10q23.3-q24	Different tumor types
SLIT2	Slit homolog 2, axon guidance	4p15.2	Different tumor types
SLIT3	Slit homolog 3, axon guidance	5q35	Different tumor types
THBS1	Thrombospondin 1	15q15	Glioma
THBS2	Thrombospondin 2	6q27	Glioma
LAMA3	Laminins	18q11.2	Different tumor types
LAMB3	Laminins	1q32	Different tumor types
LAMC2	Laminins	1q25-q31	Different tumor types

Other members of the cadherin family undergo hypermethylation in neoplasms. The most studied members are H-cadherin (CDH13), CDH4 (R-cadherin), and protocadherin (FAT) (Table 8.1). Inactivation of CDH13 by promoter hypermethylation is a common finding in most tumor types [70, 71], whereas the aberrant methylation of CDH4 and FAT has so far been described in gastric and colorectal carcinomas [72, 73].

Regarding the genes involved in heparan sulfate synthesis, germline mutations in the exostoses-1 gene (EXT1) are found in hereditary multiple exostoses (HME) syndrome, which is characterized by the formation of osteochondromas and an increased risk of chondrosarcomas and osteosarcomas (Figure 8.2) [74]. The reintroduction of EXT1 into cancer cell lines displaying methylation-dependent silencing of EXT1 induces tumor-suppressor-like features, with reduced colony-formation density and reduced tumor growth in nude mouse xenograft models [74]. EXT1 CpG island hypermethylation is common in leukemia and non-melanoma skin cancer (Table 8.1) [74]. Two other genes in this network also manifest methylation-related silencing in human neoplasms: the glypican-3 (GPC3) (Table 8.1) [75], a membrane-bound heparan sulfate proteoglycan, and the heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) (Table 8.1) [76].

Another important group of genes involved in growth suppression, angiogenesis, invasion, and metastasis is the tissue inhibitor of metalloproteinase (TIMP) family, which antagonizes matrix metalloproteinase activity (77). The best-characterized members of this family are TIMP1, 2, 3, and 4; promoter hypermethylation of TIMP2 and TIMP3 CpG islands has been described in a number of tumor types (Table 8.1) [78, 79, 80]. In leukemias and lymphomas, TIMP-2 promoter hypermethylation is associated with transcriptional repression and a more aggressive phenotype [81]. In prostate tumors and cell lines, TIMP-2 is frequently methylated; this gene can be reexpressed in metastatic prostate cell lines after combined treatment with 5-aza-2′-deoxycytidine and trichostatin A (TSA) (82). Similar behavior may be observed in another broad-range proteinase inhibitor, TFPI2 (Table 8.1), which also displays epigenetic inactivation in glioma and primary pancreatic ductal neoplasms [83, 84] and an association with the progression of the disease.

The semaphorin family of proteins plays a critical role in axonal guidance [85]. SEMA3B and SEMA3F reside at 3p21.3, a hot spot for loss of heterozygosity in human neoplasms, where they play a tumor-suppressive role in tumorigenesis [86]. It seems that SEMA3B mediates its tumor-suppressing effects, at least in part, by blocking VEGF autocrine activity.

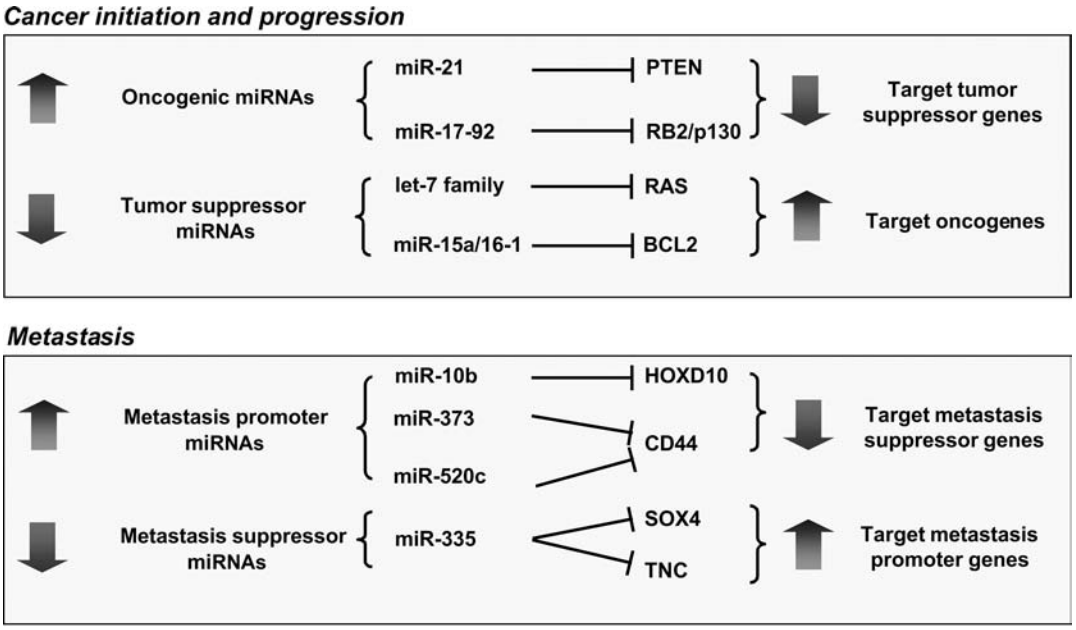

Figure 8.2. MicroRNA contribution to tumor progression. The involvement of microRNAs in tumor initiation, progression, and metastasis is depicted. MicroRNAs act as repressing target genes. MicroRNA levels can be downregulated or upregulated in cancer, leading to changes in the expression levels of the target genes.

Promoter methylation has been observed in the SEMA3B promoter in non–small-cell lung cancer (Table 8.1) [87, 88]. A second family of axon guidance molecules is made up by the Slit genes. Three human Slit gene orthologs that show methylation-associated silencing in various tumor types have so far been characterized: slit-1, slit-2, and slit-3 (Table 8.1) [89].

The thrombospondins (THBSs) are a well-known family of proteins involved in the regulation of tissue genesis and remodeling [90]. In many tumors, downregulation of THBS-1 and THBS-2 appears to be a prerequisite for the acquisition of a proangiogenic phenotype [91]. The normal suppression of angiogenesis by both proteins involves several mechanisms, including direct interaction with VEGF, inhibition of matrix metalloproteinase activation, inhibition of endothelial cell migration, and induction of endothelial cell apoptosis [91]. THBS-1 and THBS-2 undergo hypermethylation-associated silencing in a number of tumor types [92] but are particularly prevalent in gliomas, a neoplasm with a recognized high level of neoangiogenesis (Table 8.1).

Finally, different laminins participate in the induction and maintenance of cell polarity, the establishment of barriers between tissue compartments, the organization of cells into tissues, and the protection of adherent cells from detachment-induced cell death [93]. More than twelve laminin isoforms can currently be differentiated by integrins [93]. However, during tumor invasion, the basal membrane barrier is lost and a discontinuous pattern of laminin staining is observed. CpG island hypermethylation of laminin-5 (LN5)-encoding genes (LAMA3, LAMB3, and LAMC2) has been reported in various human tumors, including those of the breast, lung, prostate, and bladder, in which it occurs mainly in large, advanced-stage tumors (Table 8.1) [94].

In summary, we have described a subset of metastasis-related genes that are inactivated by epigenetic mechanisms in cancer and metastasis. DNA hypermethylation of the CpG islands of the promoter regions of metastasis-related genes is a mechanism that can explain the inactivation of these important genes. Moreover, epigenetic techniques, such as the study of DNA methylation by bisulfite genomic sequencing, can be used to search for new metastasis-related genes, thereby highlighting how the link between metastasis and epigenetics is a promising source of results.

ROLE OF MicroRNAs IN METASTASIS

In recent years, a new class of regulatory genes, the microRNAs (miRNAs), has burst onto the scene. These are small noncoding RNAs, around twenty-two nucleotides long, that negatively regulate gene expression in a variety of eukaryotic organisms [95]. miRNAs are important in numerous cellular processes, such as proliferation, differentiation, apoptosis, and development, in which they control the expression levels of hundreds of genes simultaneously [95].

Recent studies have shown that miRNA expression profiles are altered in cancers, giving rise to a range of malignancies [96]. Some miRNAs are downregulated,

whereas others are overexpressed, suggesting that miRNAs can act as tumor suppressor genes or oncogenes, respectively (Figure 8.2) [96]. Some examples of miRNAs with tumor suppressor properties are miR-15a and miR-16–1 in chronic lymphocytic leukemia (CLL) [97] and the let-7 family in lung cancer [98], which targets the BCL2 [99] and RAS [100] oncogenes, respectively. Even though miRNA downregulation in cancer is more frequent than miRNA upregulation, these single-stranded RNAs can act as oncogenes by targeting tumor suppressor genes [101]. miR-21 was recently found to be overexpressed in human glioblastomas and glioblastoma cell lines relative to normal tissues [102]. The miR-17–92 cluster, also known as OncomiR-1, was the first miRNA to be verified as acting as a mammalian oncogene [103]. Although the cellular function of miR-17–92 has not been completely determined, the pathology of tumors that overexpress this OncomiR indicates low rates of apoptosis contributing to the development of the tumors [103].

A new function mediating tumor metastasis in breast cancer has recently been ascribed to miRNAs, whereby they may promote [104, 105] or suppress [106] this malignant step (Figure 8.2). On one hand, three metastasis-promoting miRNAs have been described [104, 105]. miR-10b promotes cell migration and invasion through homeobox D10 inhibition, resulting in prometastatic RHOC upregulation [104], whereas miR-373 and miR-520c cooperate in tumor invasion and metastasis by suppressing CD44 [105]. On the other hand, miRNAs can act as metastasis suppressors, as is the case of miR-126 and miR-335, which regulate cell proliferation and tumor invasion, respectively [106]. In addition, miR-335 suppresses metastasis and migration by targeting oncogene SOX4 and tenascin C [106]. Taken together, the discovery of new metastasis-related miRNAs is crucial if we are to improve our understanding of the metastatic process.

As some miRNAs can act as tumor suppressor genes and some tumor suppressor genes can be aberrantly hypermethylated in cancer, it was just a matter of time before it was demonstrated that miRNAs with tumor suppressor features could be hypermethylated in cancer [107]. The coupling of epigenetic methods to miRNA expression profiling yielded the discovery of two putative methylated tumor suppressor miRNAs, miR-127 and miR-124a, that negatively regulated the expression of oncogenes BCL6 and CDK6, respectively [108, 109]. Some recent research has demonstrated the epigenetic regulation of other miRNAs in cancer, such as miR-9–1 [110], miR-193a, miR-137 [111], and miR-342 [112].

Therefore, we believe that epigenetic studies can provide information about not only cancer-related miRNAs but also metastasis-related miRNAs. We have adopted a pharmacological approach to explore this possibility [107, 113]. We have measured the miRNA expression levels of three metastatic cell lines before and after treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine using a miRNA expression profiling method. Treatment with this DNA demethylating agent induces a release of the gene silencing associated with CpG-island hypomethylation [113]. We have discovered several hypermethylated miRNAs exhibiting cancer-specific methylation using this approach [114]. The restoration of the expression of two of these methylated miRNAs, miR-148a and miR-34b/c, affects the invasion capacity both in vitro and in vivo. Furthermore, these miRNAs are significantly more methylated in those primary tumors that give rise to metastasis. In conclusion, our findings indicate that epigenetic studies are a suitable technique for the study of metastasis-suppressor miRNAs.

Therefore, miRNAs have an important role in cancer and metastasis and, as the classical tumor- or metastasis-suppressor genes, can be aberrantly methylated in this malignant disease.

EPIGENETIC CONTRIBUTION TO THERAPY

Most of the hypermethylation events that we have described above are molecular markers of progression and dissemination and are therefore associated with poor prognosis. However, not all the news is bad, as these genes are excellent targets for the new epigenetic drugs used in the treatment of cancer.

Unlike genetic changes in cancer, epigenetic changes are potentially reversible. In cultured cancer cell lines, it has been possible for years to reexpress genes that had been silenced by methylation by using DNA-demethylating agents [54]. When given to patients at low doses, these drugs have shown significant antitumoral activity, and the U.S. Food and Drug Administration (FDA) has approved the use of two agents, 5-azacytidine and 5-aza-2′-deoxycytidine, as elective treatments for the preleukemic disease, myelodysplastic syndrome [54].

HDAC inhibitors are another promising group of agents for the epigenetic therapy of cancer [115]. One of the main therapeutic mechanisms of action of HDAC inhibitors is their transcriptional reactivation of "dormant" tumor suppressor genes, such as *p21WAF1*. However, the pleiotropic nature of these inhibitors means that there is a risk of their well-known abilities to induce differentiation, cell-cycle arrest, and apoptosis being accompanied by other, less desirable, outcomes. Despite these concerns, many Phase I clinical trials indicate that HDAC inhibitors are well tolerated, and the first drug of this type, suberoylanilide hydroxamic acid (SAHA), was recently approved by the FDA for the treatment of cutaneous T-cell lymphoma [54].

With respect to metastasis, many studies have identified specific genes that are associated with metastasis and whose expression is altered as a result of epigenetic changes. Whole epigenome signatures of metastasis have yet to be reported, partly because the necessary promoter microarray platforms have only recently become available [116]. Finally, in contrast to strategies for reactivating the expression of silenced genes, there has been a report that the use of S-adenosylmethionine and antisense oligonucleotides (directed at the methyl DNA-binding domain protein MBD2) can inhibit the tumor-promoting genes uPA/PLAU, VEGF, and MMP2, with the concurrent inhibition of tumor-cell invasion in vitro and growth in vivo [117]. Again, in adopting such approaches in a clinical context, it will be important to establish the stability and specificity of the epigenetic reprogramming initiated by these therapeutic compounds.

An effective treatment for the generation of metastasis involving the reactivation of all the hypermethylated genes described is not a pipedream. In future, research needs to aim at discovering new metastasis-related genes and metastasis-related miRNAs that are controlled by epigenetic mechanisms, and developing epigenetic drug-based therapies.

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9 Germline Variation and Other Host Determinants of Metastatic Potential

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CONVENTIONAL MODELS OF METASTATIC PROGRESSION

The somatic mutation theory has long been regarded as the "conventional" model to explain metastasis at the molecular level. Originally postulated by Nowell [1], it states that metastatic tumor cells acquire the necessary characteristics to facilitate colonization and proliferation at distant sites through a sequential accumulation of somatic mutations. Experimental support for this theory was subsequently provided by Fidler and Kripke [2], who demonstrated that clonal variants isolated from bulk tumor tissue had differing metastatic potentials. It was postulated that the origins of this differential metastatic capacity arose from the acquisition of different somatic mutations in individual clonal isolates. Later work demonstrated that these somatic mutations induce hyperactivation of metastasis-promoting genes and silencing of metastasis suppressors, and that individual tumor cells are susceptible to the accumulation of such mutations as a consequence of their inherent "genomic instability" (reviewed in [3]).

Subsequent in vivo experimentation, however, revealed that although somatic evolution is likely a critical determinant of metastatic potential, it cannot entirely explain the molecular basis of metastasis [4–6]. The advent of microarray technology to assay global patterns of gene expression has shed fresh light on the limitations of the somatic evolution theory as a comprehensive mechanism for metastasis at the molecular level (reviewed in [7]). Specifically, many studies have demonstrated that patterns of gene expression (or "signatures") in bulk tumor tissue can be used to predict survival in breast cancer (early examples include [8–10]) as well as many other solid tumors [7]. Indeed, most of these microarray experiments were performed using primary tumor tissue collected prior to the onset of clinically detectable metastasis. This observation is therefore seemingly at odds with the somatic evolution theory: Given that the vast majority of cancer-related deaths are a direct result of metastasis, how is it that gene expression signatures predictive of survival can exist prior to the development of clinically overt metastasis?

There are, of course, many possible answers to this question, the most obvious of which is that clinically undetectable metastases already exist in some individuals deemed to be "premetastatic" at the time of diagnosis. Another explanation is that somatic mutations occurring soon after the onset of tumorigenesis ("founder" mutations), which will be transmitted to successive generations of tumor cells, induce prognostic gene expression signatures [8, 11]. This is without a doubt an attractive hypothesis that not only offers clarification as to why prognostic gene expression signatures exist in early-stage tumors but also proposes an explanation for the existence of unknown primary cancer (UPC) metastatic disease. UPC metastatic disease is defined as an instance in which a patient presents with overt metastatic disease but either no clinically detectable primary tumor or a small, well-differentiated lesion. The existence of this type of disease, which constitutes approximately 5 percent of newly diagnosed cancer cases [12], is seemingly at odds with the somatic evolution theory, as small tumors should not have had sufficient time to develop the necessary mutations to metastasize. However, if metastatic potential is encoded as an early event in tumorigenesis, it is not difficult to comprehend how a small subset of tumors could metastasize early, leading to the phenomenon of UPC metastatic disease.

The founder mutation theory falls short when related to experimental observation, however. The implication of this theory is that metastasis-inducing founder mutations should be present in most cells within primary

tumors prone to dissemination. It is fortunate that this is not the case, as the metastatic efficiencies of primary tumor cells is universally poor, regardless of the gene expression signature exhibited in bulk tumor tissue. These results therefore suggest that although it is not improbable that metastatic potential is influenced by founder mutations, additional factors most likely also influence this process.

CONCEPT OF GERMLINE-ENCODED METASTASIS SUSCEPTIBILITY

An alternative yet complementary hypothesis is that inter-individual differences in metastatic potential and tumor-derived gene expression signatures are influenced by host germline variation. That is, any given individual is "hard wired" in terms of metastatic potential as a consequence of hereditary variation in metastasis "susceptibility" genes, and, to some extent, metastatic potential is determined prior to the onset of tumorigenesis. On its face, this idea seems somewhat outlandish when compared with more traditional mechanisms of metastasis. However, further consideration of such an idea reveals the possibility that germline-encoded metastasis susceptibility complements conventional theories. The natural evolution of any solid tumor is most likely dependent on the sequential acquisition of somatic mutation, and the inherent metastatic capacity of individual tumor cells depends mostly on these mutations. However, host germline variation could influence not only the intrinsic properties of the tumor cell, but also the natures of all other tissues with which it interacts at the primary and secondary sites. Therefore, all steps ranging from initiation of tumorigenesis to proliferation of metastatic tumor cells at the secondary site could potentially be influenced by host germline variation (Figure 9.1).

Many studies have demonstrated that germline variation is involved in breast cancer susceptibility, with multiple low-penetrance alleles modulating vulnerability (reviewed in [13]). The most well-characterized breast cancer susceptibility genes are *BRCA1* and *BRCA2*, with specific germline mutations being associated with various indicators of poor outcome [14–17]. Given that hereditary variation influences breast cancer susceptibility, it is not inconceivable that similar germline variation renders certain individuals more susceptible to metastasis following primary tumor development. Epidemiological studies support this idea, with a recent study by Hartman et al. [18] demonstrating that overall survival in breast cancer is significantly reduced in individuals who have a first-degree relative who died within five years of diagnosis of breast cancer.

Additionally, disease outcome appears to differ among races, with age-adjusted mortality in the United States from breast cancer in white women being 28.3 deaths per 100,000, compared with 36.4 deaths per 100,000 in African American women [19]. A recent detailed epidemiological study demonstrated that basal-like breast tumors occurred at a higher prevalence among premenopausal African American women [20]. The implication of such observations is that polymorphic loci in different races may well play a role in the induction differential susceptibility to disease development and rates of progression. However, the origins of racial differences in cancer progression are likely extremely complex, and factors other than germline polymorphism, including differences in environmental exposures and access to health care facilities, likely play equally important roles.

MOUSE MODELS AND GERMLINE-ENCODED METASTASIS SUSCEPTIBILITY

Although it is an interesting hypothesis, what evidence exists to suggest that germline-encoded variation influences metastatic potential? The most compelling data have been derived using mouse models of mammary tumorigenesis, which have long proven a valuable tool for investigating cancer susceptibility [21, 22]. The value of these models stems from the ability to control variables that confound analysis of disease susceptibility in human populations: genetic variation and environmental exposure. Specifically, low-penetrance (and mostly unknown) cancer susceptibility genes prevalent in the general population and disparate levels of environmental exposure (e.g., carcinogens) hamper the ability of researchers to define cancer susceptibility genes [23].

THE PyMT MOUSE AS A TOOL TO DEFINE HERITABLE FACTORS MODULATING METASTATIC POTENTIAL

The polyoma middle T (PyMT) mouse is a transgenic model that expresses the polyomavirus middle T antigen under the control of the mammary-specific mouse mammary tumor virus promoter (FVB/N-TgN(MMTV-PyMT)^{634Mul}). It has proven particularly useful in studying mammary tumorigenesis because mice develop a highly aggressive, highly metastatic disease with 100 percent penetrance by approximately 60 days of age, and 85 percent to 95 percent of mice develop overt pulmonary metastatic lesions by 100 days of age [24]. The effect of germline polymorphism on metastatic potential was investigated by breeding male PyMT mice to females of a variety of different inbred laboratory strains and quantifying metastatic potential

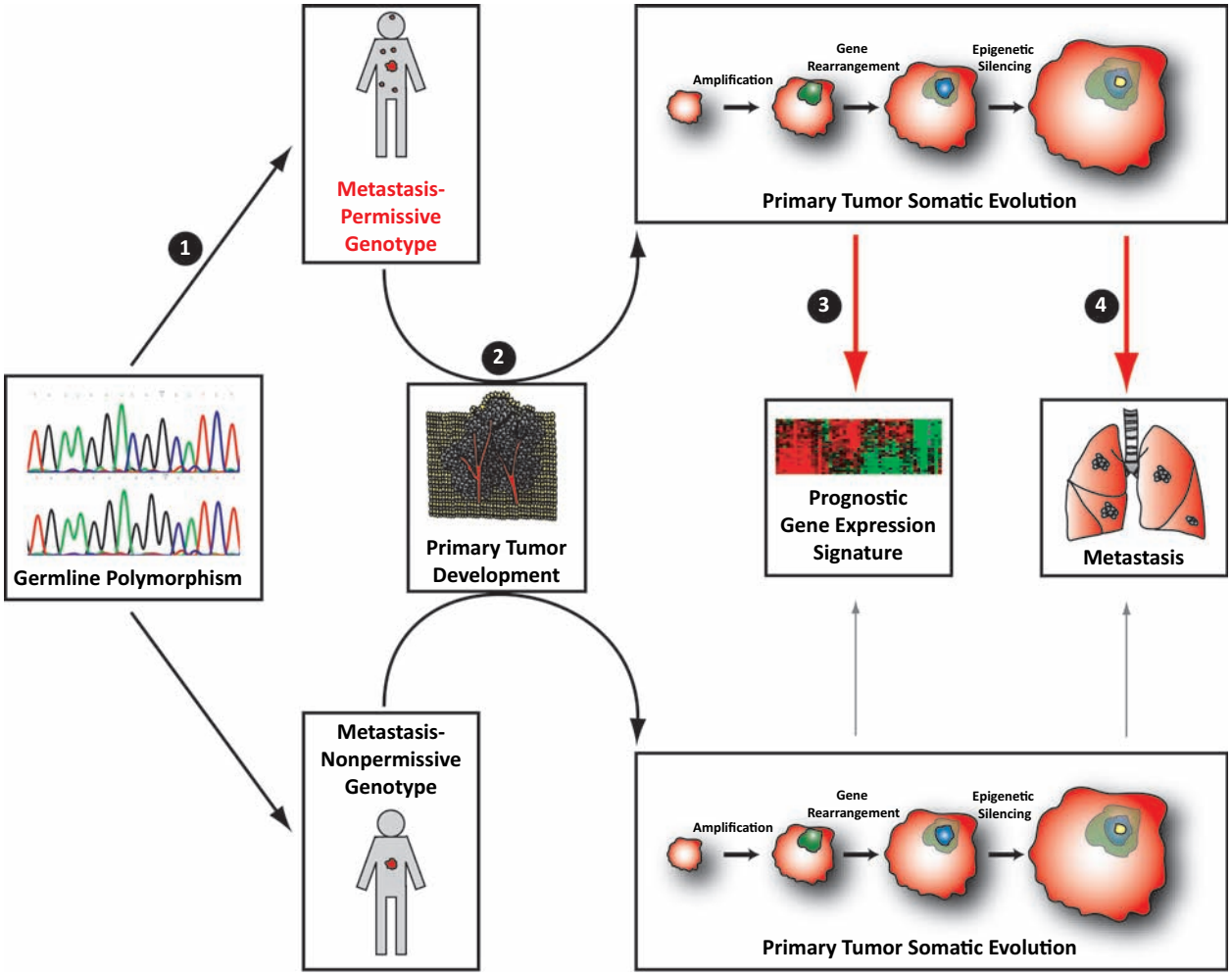

Figure 9.1. Germline modulation of metastatic potential. Recent studies have demonstrated that metastatic potential is modulated by host germline polymorphism. Microarray analysis of expression patterns in normal, non-cancerous mammary tissue derived from inbred mice suggests that metastatic propensity is "programmed" prior to the onset of tumorigenesis [33]. These expression differences presumably result from the combined effects of multiple polymorphic metastasis susceptibility genes. Therefore, even prior to tumor development, any given individual will have a varying susceptibility to developing metastasis as a result of these polymorphic genes (1). However, it is unlikely that these metastasis susceptibility genes influence the initiation of tumorigenesis, and tumors will subsequently evolve through somatic evolution (2). The clinical effects of metastasis susceptibility genes will probably be most prominently manifested following primary tumor development. They likely exert an influence on gene expression within the primary tumor and contribute to the induction of microarray expression signatures indicative of poor outcome in individuals with metastasis "permissive" genotypes (3). Finally, the overall likelihood of metastasis will be influenced by the effects that metastasis susceptibility genes exert not only on the primary tissue but also at sites of secondary tumor implantation and other interacting tissues (4).

in the resulting F_1 progeny. It was observed that F_1 mice had widely varying metastatic efficiencies, with pulmonary metastatic burden ranging from approximately a tenfold decrease to a threefold increase compared with that of the wild-type PyMT mouse [25]. It is important to note that all F_1 progeny acquired the PyMT transgene through breeding. Thus, all animals have the same number of copies of the transgene integrated into the same genomic site. Furthermore, all tumors were initiated by the same oncogenic event (i.e., the PyMT antigen). Given these facts, it seems apparent that the observed differences in metastatic potential of the F_1 progeny are caused by germline variation between the parental strains. Therefore, it follows that different inbred laboratory mice likely carry multiple polymorphic loci modulating metastatic efficiency.

QUANTITATIVE TRAIT LOCUS MAPPING IN THE PyMT MOUSE

Quantitative trait locus (QTL) mapping was performed to define individual loci responsible for the observed differences in metastasis susceptibility in different mouse strains. Briefly, QTLs, or susceptibility loci, are continuous genomic regions harboring large

numbers of polymorphic genes, one or more of which are responsible for susceptibility to a given trait. They are defined by correlating a measurable trait (e.g., metastasis burden) with allelic variation in linked polymorphic genetic markers (e.g., microsatellites or single nucleotide polymorphisms [SNPs]) in a defined population.

Mapping experiments to define metastasis QTLs were performed by breeding the PyMT mouse to inbred strains of differing metastatic potentials followed by a series of backcrosses. Initial results revealed the presence of two metastasis susceptibility loci on chromosomes 6 and 19 [26], with subsequent studies defining loci on chromosomes 7, 9, and 17 [27]. The first of these to be studied in any great detail was the locus on proximal chromosome 19, designated *Mtes1* [26]. Analysis of *Mtes1* used an experimental approach known as "multiple cross mapping," which exploits shared haplotypes among different inbred strains [28]. This allowed for construction of a medium-resolution map of the *Mtes1* locus comprising approximately 10Mbp of mouse chromosome 19. The number of plausible candidate genes within this locus was subsequently narrowed from approximately 500 to 23 by identifying haplotype blocks common to high-metastatic-potential inbred strains [29]. The first known metastasis susceptibility gene was defined using combined experimental approaches, including categorization based on molecular function or known association with the metastatic process, DNA sequence analysis to determine whether the identified polymorphisms had a potential functional relevance, and segregation analysis of genetically linked variable tandem repeats [30]. The responsible gene, which will be discussed in greater detail later, was identified as "signal-induced proliferation-associated gene 1" (*Sipa1*, also known as *Spa1*), which encodes a protein containing a C-terminal leucine zipper motif and an N-terminal GTPase activating protein (GAP) domain homologous to the human RAP1GAP.

MICROARRAY ANALYSIS OF PyMT-INDUCED MAMMARY TUMORS AND NORMAL MAMMARY TISSUE

As discussed earlier, human primary breast tumors more prone to metastasizing display characteristic microarray gene expression signatures [8–10]. With this in mind, it follows that similar gene expression signatures should exist in PyMT-induced tumors in more metastasis-prone mice. Indeed, this is the case, with a high degree of correlation observed between human breast cancer primary tumor expression signatures [8–10] and those present in PyMT-induced mammary tumors on different genetic backgrounds [31, 32].

A pertinent question arising from this is whether these mammary tumor-derived gene expression signatures in mice of differing metastatic potentials are influenced by strain-specific germline polymorphism in addition to differences in patterns of somatic mutation. If germline polymorphism is actually involved not only in the induction of these gene expression signatures but also in metastatic progression as a whole, it should stand to reason that normal, non-neoplastic tissues derived from mice with differing metastatic propensities should carry gene signatures bearing the hallmarks of those present in mammary tumors. To investigate this, patterns of gene expression were investigated in normal mammary tissue derived from the F_1 progeny of PyMT mice crossed with either high- or low-metastatic-capacity mice [33]. However, to investigate the isolated effects of genetic polymorphism rather than those of the PyMT antigen, gene expression signatures were characterized in transgene-negative F_1 mice. Quantitative real-time polymerase chain reaction (PCR) was used to measure the expression of genes described as components of a human breast cancer expression signature described by Ramaswamy et al [8]. It was demonstrated that expression levels of ten of the seventeen genes comprising this metastasis-predictive signature could be used to accurately categorize normal mammary tissue samples as derived from the either high- or low-metastatic-potential genotype mice [33].

These observations therefore confirm the hypothesis that germline polymorphism is a modulator of metastatic potential, as metastasis-predictive changes in gene expression pre-exist the onset of neoplasia. To confirm this observation, a microarray signature was derived from AKXD recombinant inbred (RI) strains [34] bred to PyMT mice [33]. Briefly, RI strains are specialized panels of inbred mice ideally suited to the study of complex, non-Mendelian traits such as metastasis susceptibility. They are bred to contain unique and approximately equal proportions of genetic contributions from two progenitor inbred strains. In the case of AKXD RI mice, the progenitor strains are the high-metastatic-potential AKR/J strain and the low-metastatic-potential DBA/2J strain [34]. RI strains are typically constructed by crossing two inbred strains to produce an F_1 generation, followed by twenty or more consecutive generations of brother × sister mating [35]. Following derivation of an AKXD × PyMT gene expression signature, signature gene expression was quantified in normal mammary tissue derived from high- and low-metastatic-potential transgene-negative F_1 mice. As was the case with human signature genes, characteristic expression changes in AKXD × PyMT signature genes were observed in strains of differential metastatic capacity [33]. Furthermore, these gene expression differences could be used to accurately predict whether tissues were derived from high- or low-metastatic-potential mice.

Therefore, expression changes of genes commonly dysregulated in metastasis are present in normal tissues prior to the onset of tumorigenesis. Given that confounding variables, such as environmental exposure, are tightly controlled in laboratory mice, the most plausible explanation for the observed gene expression differences is that they are dictated by strain-specific germline variation. These observations, as well as those relating to the discovery of metastasis QTLs, strongly suggest that metastasis susceptibility is, to some extent, a heritable trait. However, to further understand the origins of germline-encoded metastasis susceptibility, it is necessary to define individual polymorphic genes responsible for differential metastatic propensity. In the next section, we discuss individual metastasis susceptibility genes and describe how they were discovered.

METASTASIS SUSCEPTIBILITY GENES

Sipa1, the "Original" Metastasis Susceptibility Gene

As mentioned earlier, a multifaceted approach facilitated the discovery of the first known metastasis susceptibility gene, *Sipa1* [30]. *Sipa1* encodes a GAP that is specific for RAP1 and RAP2. Both these factors are members of the Ras family of GTPases and are involved in regulation of cell proliferation, differentiation, and cell adhesion [36]. Concomitant with this, SIPA1 plays a prominent role in regulating of cell adhesion, with transient expression of *Sipa1* in Hela cells inducing cellular rounding up and detachment from the culture surface [37]. The mechanism by which SIPA1 modulates cell adhesion appears to be through modulation of various cellular adhesion molecules, which disrupts cellular interactions with extracellular matrix proteins and other adhesion critical factors [38–40]. Modulation of a class of molecules known as integrins appears central to this, with the RAP1GAP activity of SIPA1 being critical in this respect [39].

Although dysregulation of cell adhesion has been demonstrated to be an important element of metastatic progression, the specific mechanism by which SIPA1 acts in metastasis has yet to be determined. However, a recent study underscored the importance of SIPA1 in germline-encoded metastasis susceptibility [41]. In this study, the frequencies of SNPs within the human *SIPA1* gene were determined in a well described cohort of non-Hispanic Caucasian breast cancer patients from southern California. It was found that specific polymorphic alleles within *SIPA1* were associated with markers of poor outcome in breast cancer, the most notable of which being that certain *SIPA1* SNPs appear to be associated with a higher incidence of distant metastasis [41]. This is a particularly significant result, as it brings the concept of germline-encoded metastasis susceptibility from being a phenomenon observed only in mice into the realms of human metastatic progression. The potential clinical significance of this as well as similar observations with other metastasis susceptibility genes will be discussed later.

Germline-Encoded Modifiers of Extracellular Matrix Gene Expression

As discussed previously, QTL mapping and microarray analysis of tumor gene expression patterns have proven particularly powerful approaches for defining germline elements modulating metastatic potential. A number of recent studies have combined these approaches to uncover novel metastasis-susceptibility genes [42–44]. All these studies stem from the observation that extracellular matrix (ECM) genes are ubiquitous components of metastasis-predictive microarray gene expression signatures exhibited by both human breast cancers [8–10] and mouse mammary tumors [45]. This implies that ECM gene dysregulation is either a causative factor or marker of metastatic potential.

Earlier microarray studies suggest that gene expression in mouse mammary tumors is influenced by host germline variation [33, 45]. To determine whether the differential ECM gene expression levels observed in tumors with differing metastatic potentials are also influenced by germline polymorphism, an experimental approach called expression QTL (eQTL) mapping was performed [43, 44]. Here, global patterns of gene expression were quantified in tumors derived from the F_1 progeny of AKXD RI mice crossed to PyMT mice. ECM eQTLs (i.e., genomic loci controlling the expression of ECM genes described in earlier breast cancer gene expression signatures) were defined using the WebQTL database within the GeneNetwork, an Internet-based analytical package/repository that allows for analysis of RI microarray expression data [46, 47]. ECM eQTLs were subsequently discovered on chromosomes 7, 17, and 18, implying that these genomic regions play a potentially important role in modulation of metastasis-predictive ECM gene expression. It is very interesting to note that the ECM eQTL loci on chromosomes 7 and 17 co-localize with previously described metastasis susceptibility loci [27], giving further strength to the hypothesis that differential ECM gene expression is a marker of metastasis susceptibility.

To identify individual candidate genes within each of the three ECM eQTLs, whole-genome correlation analysis of the microarray data was performed using the WebQTL Trait Correlation function [47]. The aim was to identify candidate genes within the ECM eQTL intervals with expression highly correlated to that of metastasis-predictive ECM genes across the entire AKXD RI panel. This facilitated the identification of

seven highly promising candidate genes whose expression appears highly correlated with that of metastasis-predictive ECM genes in AKXD RI mice [42–44]. Two of the most promising candidates, *Rrp1b* and *Brd4*, will be discussed in further detail in the following sections.

Rrp1b as an ECM eQTL and Metastasis Susceptibility Candidate Gene

Rrp1b, a gene of unknown function, was defined as a potential metastasis susceptibility candidate gene through concurrent and independent experimental approaches [44]. First, using the approach described in the previous section, it was determined to be a gene that was physically located at the peak region of linkage of the chromosome 17 ECM eQTL. Furthermore, microarray data from these eQTL mapping experiments demonstrated that *Rrp1b* expression levels were highly correlated with the expression levels of a number of metastasis-predictive ECM genes. RRP1B was also identified in a second series of seemingly unrelated yeast–two-hybrid experiments performed to define factors interacting with the previously described metastasis efficiency modifier SIPA1. Co-immunoprecipitation not only confirmed this interaction, but also demonstrated that the enzymatic RAP1GAP activity of SIPA1 was reduced by virtue of this interaction.

To further investigate the role of this gene in expression of ECM genes as well as modulation of metastatic potential, *Rrp1b* was ectopically expressed in multiple mouse cell lines [44]. In keeping with ECM eQTL mapping experiments, over-activation of *Rrp1b* induced dysregulation of numerous metastasis-predictive ECM genes. Furthermore, implantation of *Rrp1b*-expressing clonal isolates from the highly metastatic Mvt-1 mouse mammary tumor cell line proved that *Rrp1b* suppressed both tumor growth and pulmonary metastasis.

RRP1B also appears to play a role in modulation of tumor progression at the germline level in humans. Specifically, ectopic expression of *Rrp1b* in the Mvt-1 cell line induced a microarray gene expression signature that can be used to accurately predict survival in a well-characterized human breast cancer microarray dataset [44]. To confirm the significance of *RRP1B* in germline-encoded metastasis susceptibility, the frequency of a non-synonymous coding polymorphism within the human *RRP1B* gene was determined in two breast cancer cohorts, one from southern California and a second from Maryland [44]. These studies demonstrated that the variant *RRP1B* SNP allele is associated with a lower incidence of distant metastasis and a higher overall likelihood of survival. The precise mechanism by which RRP1B modulates metastatic potential at the germline level remains unclear at present, although its interaction with SIPA1 and modulation of ECM composition may well be central to this (Figure 9.2).

Brd4 as an ECM eQTL and Metastasis Susceptibility Candidate Gene

BRD4, a relatively well described bromodomain protein, is a transcriptional regulator and a potent modulator of cell growth [48, 49]. One of the roles of BRD4 is to promote G_2/M transition [48], an effect that appears dependent on the intracellular balance of BRD4 to its binding partner, the previously described germline-encoded metastasis efficiency modifier SIPA1 [50].

As was the case with *Rrp1b*, *Brd4* is also an ECM eQTL candidate gene residing in very close physical proximity to both *Rrp1b* and the peak region of linkage of the chromosome 17 eQTL [42, 43]. Expression levels of *Brd4* in AKXD × PyMT mammary tumors are also highly correlated with those of metastasis-predictive ECM genes. Finally, ectopic expression of this gene in the highly metastatic Mvt-1 cell line not only modulated ECM gene expression but also suppressed tumor growth and metastasis following mammary fat pad implantation [42]. Most significantly, however, microarray analyses of Mvt-1 cells ectopically expressing *Brd4* demonstrated that *Brd4* induces a gene expression signature that can be used to accurately predict survival in five breast cancer microarray datasets [42]. Furthermore, this same signature could be used to sub-classify individuals with estrogen receptor (ER)-positive tumors and lymph node (LN)-negative breast cancer (traditionally believed to be at lower risk of recurrence) to identify individuals at higher risk of relapse following primary tumor resection.

OTHER FACTORS MODULATING METASTASIS SUSCEPTIBILITY

It is well known that exposure to environmental factors such as cigarette smoke and alcohol can increase susceptibility to developing a range of cancers, but do such factors also modulate metastatic potential? This does indeed appear to be the case, with a recent study using PyMT mice demonstrating that high-fat diets increase pulmonary metastasis incidence [51]. Furthermore, this group demonstrated that there is a degree of interaction between these dietary factors and metastasis susceptibility loci. This type of study very much underscores the importance of accurately modeling not only human cancer characteristics in mice, but also the environmental exposures of human populations.

Another factor contributing to metastasis susceptibility is immune competency. For example, surgical excision of the primary tumor has long been suspected to facilitate growth of preexisting micrometastases and

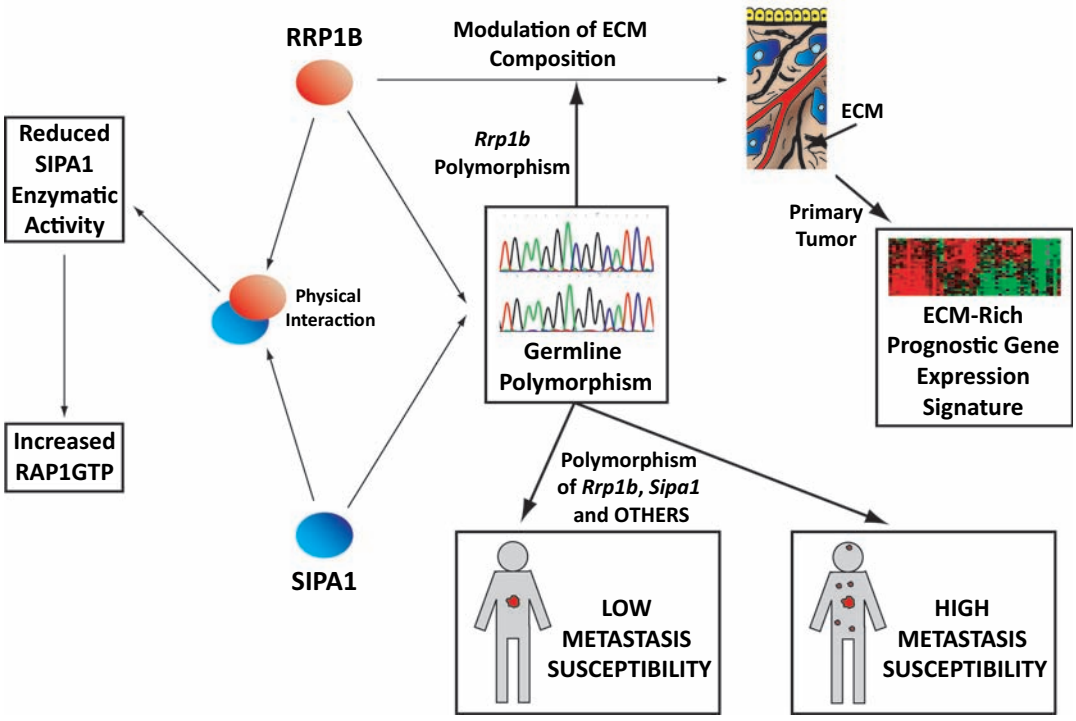

Figure 9.2. *Rrp1b*, *Sipa1*, the ECM, and metastasis. The relationship between metastasis susceptibility genes *Rrp1b* [44] and *Sipa1* [30] appears to be an important element of germline modulation of metastatic potential, although the intricacies of their relationship remain unclear at present. It is known that RRP1B physically interacts with SIPA1 and is able to reduce the RapGAP enzymatic activity of SIPA1 by virtue of this interaction. *Rrp1b* also modulates the expression of ECM genes that are dysregulated in many metastasis-predictive microarray gene expression signatures, implying that such signatures may partially be driven by germline. It is plausible that this germline-driven differential ECM gene expression creates an environment that is either more or less permissive for a primary tumor cell (which has arisen through the process of somatic evolution) to complete the metastatic cascade. Whether this is actually the case is currently under investigation.

dissemination of tumor cells during the perioperative period [52]. A number of possible mechanisms for this have been cited, one of which relates to the suppression of cell-mediated immunity (CMI) characteristic of major surgery [53, 54]. Previous studies have demonstrated that natural killer (NK) cells form a major component of CMI-mediated control over circulating tumor cells and micrometastases (reviewed in [55]). This is a potentially interesting concept, given the accessibility of the immune system to pharmacological intervention. For example, perioperative COX-2 inhibition and β-blockade in rodent models attenuates post-operative reductions in NK cell cytotoxicity [56]. This is a particularly intriguing result, given the importance of NK cell function in metastasis [55], although further work will be necessary to determine the relevance of these observations to human disease.

CONCLUSIONS AND CLINICAL PERSPECTIVES

Mouse models of mammary tumorigenesis demonstrate that germline polymorphism plays an important regulatory role with respect to metastatic potential. The PyMT model of mouse mammary tumorigenesis has been successfully used to define metastasis susceptibility loci that harbor modifier genes responsible for the differential metastatic capacities of inbred mice [26, 27, 29]. Further analyses have revealed the identities of these germline-encoded metastasis susceptibility genes, with the three most promising candidates being *Sipa1*, *Rrp1b*, and *Brd4* [30, 42, 44]. The influence of these genes does not appear limited to metastasis susceptibility in mice, with each gene shown to be a germline-encoded modulator of metastasis in human breast cancer. In vitro activation of *Rrp1b* and *Brd4* induces a gene expression signature that accurately predicts survival in multiple human breast cancer cohorts [42, 44]. Furthermore, epidemiological association studies have identified polymorphisms within human *SIPA1* and *RRP1B* that render carriers of variant SNP alleles differentially susceptible to metastasis [41, 44].

Assessment of prognosis in the clinical setting at present is somewhat of an imprecise science, with current clinical protocols relying on disease characteristics such as tumor grade and stage, as well as expression of various cell surface markers by the primary tumor.

However, microarray-based tumor gene expression profiling has the potential to refine the ability of clinicians to predict prognosis at the time of diagnosis. A number of clinical trials are currently under way to assess the utility of tumor expression profiling as a prognostic tool (e.g., TAILORx, MINDACT [57]). However, tumor gene expression profiling does suffer from a number of limitations, including that assessment of expression patterns requires access to primary tumor tissue. Furthermore, numerous reports have suggested significant lab-to-lab variation in microarray data (reviewed in [58]). Prognostic assays based on tumor gene expression patterns will have to prove that they can overcome such problems to be of true value in the clinical setting.

However, the implications of a germline component in the modulation of metastatic potential are particularly interesting with regard to assessment of breast cancer prognosis. Given that polymorphisms in metastasis susceptibility genes are present in all tissues, it is conceivable that the risk of progression could be assessed by typing polymorphisms in readily accessible tissues such as blood. Generally speaking, there is much less ambiguity in typing SNPs compared with assaying tumor gene expression. Therefore, typing germline polymorphisms in metastasis susceptibility genes such as *SIPA1* [41] and *RRP1B* [44] to assess prognosis offers a number of advantages over analysis of tumor gene expression patterns. Specifically, SNP genotyping uses easily accessible tissue, produces unambiguous results, and is relatively inexpensive to perform.

The main disadvantage of using germline polymorphisms to assess prognosis is that the predictive value of individual SNPs is low. However, a panel of polymorphisms in a variety of metastasis susceptibility genes could offer a test with sufficient predictive ability to be of clinical value. This, of course, necessitates the identification of novel metastasis susceptibility genes that are polymorphic in human populations. Future work will therefore determine whether the assessment of polymorphisms in germline encoded metastasis susceptibility genes are useful in the clinical setting.

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10 The Influence of Aging and Cellular Senescence on Metastasis

Futoshi Okada and Hiroshi Kobayashi

Current research estimates that the proportion of the population aged 75 and older in developed countries will increase to approximately 40 percent by 2050 [1]. This is the result of the full provision of public health, improved medical care, and adequate nutrition. People have shown a remarkable increase in median life expectancy and, in fully developed countries, life expectancy now approaches 80 years [2]. On the other hand, no matter how much the maximum survival (life span) may be prolonged, the span will not exceed 125 years, as no one has surpassed 122 years and five months (the age of Jeanne Calment in France when she died [3]). Although it has been estimated that if cancer and atherosclerosis were eliminated as causes of death, about ten years would be added to the average life span, there would still be no change in the maximum life span [4]. This may indicate that the current life span of humans has reached its plateau.

Aging is not itself a disease, but it accompanies the decline of organ and body functions and indicates a correspondingly increasing susceptibility to diseases such as infection, autoimmunity, and cancer [3]. Recent estimates from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute reveal that the median age of patients with cancer in the United States is around 70, and that death rates from cancer, for almost any site, increase with each additional decade of age [5]. More than 60 percent of all cancers and 69 percent of all cancer deaths occur in the US population aged 65 and older, which is 13% of the population as a whole [6]. The number of patients with cancer is expected to double from 1.3 million in the year 2000 to 2.6 million by 2050, with elderly patients predicted to account for most of this increase [7]. Consequently, in view of a shift in the global population toward a greater proportion of elderly persons, cancer now can be recognized as a kind of tax on life [8].

The frequency of tumor development and tumor cells' acquisition of malignant properties, such as metastasis, are modulated by the host's age-dependent biological condition and social environment. Moreover, the malignancy of arising tumors varies depending on the host's age, even if the tumors are histologically of almost the same type.

How are tumor formation and malignant properties modulated by host age or cellular senescence? We show that age-dependent organ carcinogenesis can be classified into categories, and we discuss several molecules that act as a bridge between senescence and carcinogenesis. Moreover, because some of the clinical data or experimental studies reveal that tumors grown or implanted in aged hosts are less malignant, we hope to shed light on the intrinsic and extrinsic mechanisms in the aged.

AGING AND CANCER

Accelerated Aging Diseases and Cancer

The association between aging and cancer manifests itself by concomitant emergence of spontaneous tumors in clinical premature aging syndromes [6]. Hutchinson-Gilford syndrome is an early-onset disease called *progeria*. Other accelerated aging diseases include Werner syndrome, Cockayne syndrome, or xeroderma pigmentosum. Hutchinson-Gilford syndrome is caused by a *lamin-A/C (LMNA)* gene mutation that encodes the architecture proteins of the nucleus. Other accelerated aging diseases are mostly the result of defective DNA replication and a faulty DNA repair system [7]. The well-known Werner syndrome is caused by mutation in a single *WRN* gene located on human chromosome 8. The *WRN* gene encodes a helicase enzyme, which has a role in DNA recombination, maintaining genomic stability and enabling telomere maintenance. Functional mutations of the *WRN* gene cause premature aging and high susceptibility to carcinogenesis [9]. In human cancers, *WRN* gene

TABLE 10.1. Molecular links among senescence, aging, and carcinogenesis.

Disorder	Molecules	Function		Senescence/ aging	Carcinogenesis	Reference
		Senescence/ aging	Carcinogenesis			
	ATM	DNA repair, cell cycle	Signaling	↑	↑	64
	Bub 1b	Cell cycle, mitotic checkpoint	Tumor-susceptible gene	↑	→	65
	jun	Cell cycle	Oncogene	↑	↑	66
	myc	Cell cycle	Oncogene	↑	↑	67
	ras	Cell cycle	Oncogene	↑	↑	68
	Rb	Cell cycle	Tumor suppressor gene	↑	↑	7
	p53	DNA damage response	Tumor suppressor gene	↑	↑	69
	Sir2	Genome instability	Tumor suppressor gene	↑	↑	70
	BRCA	DNA repair, genome instability	Tumor-susceptible gene	↑	↑	7
Xeroderma pigmentosum A	XPA	DNA repair	Tumor-susceptible gene	↑		71
Xeroderma pigmentosum B	ERCC3	DNA repair	Tumor-susceptible gene	↑		71
Xeroderma pigmentosum C	XPC	DNA repair	Tumor-susceptible gene	↑	↑	72
Xeroderma pigmentosum D	ERCC2	DNA repair	Tumor-susceptible gene	↑	↑	73
Xeroderma pigmentosum E	DDB2	DNA repair	Tumor-susceptible gene	↑	↑	74
Xeroderma pigmentosum F	ERCC4	DNA repair	Tumor-susceptible gene	↑	↑	71
Xeroderma pigmentosum G	ERCC5	DNA repair	Tumor-susceptible gene	↑		71
Xeroderma pigmentosum H	ERCC1	DNA repair	Tumor-susceptible gene	↑	↑	71
	Terc	Telomere maintenance	Tumor-associated gene	↑	↑	75
	Tert	Telomere maintenance	Tumor-associated gene	↑	↑	76
Werner syndrome	Wrn	DNA repair, helicase, genomic instability	Tumor-susceptible gene	↑	↑	10
Cockayne syndrome	ERCC4&8	DNA repair, helicase	Tumor-susceptible gene	↑	→	6
Hutchinson-Gilford syndrome	LMNA	Abnormal gene expression		↑	→	6
Rothmund-Thomson syndrome	ERCQL4	DNA repair		↑	↑	6

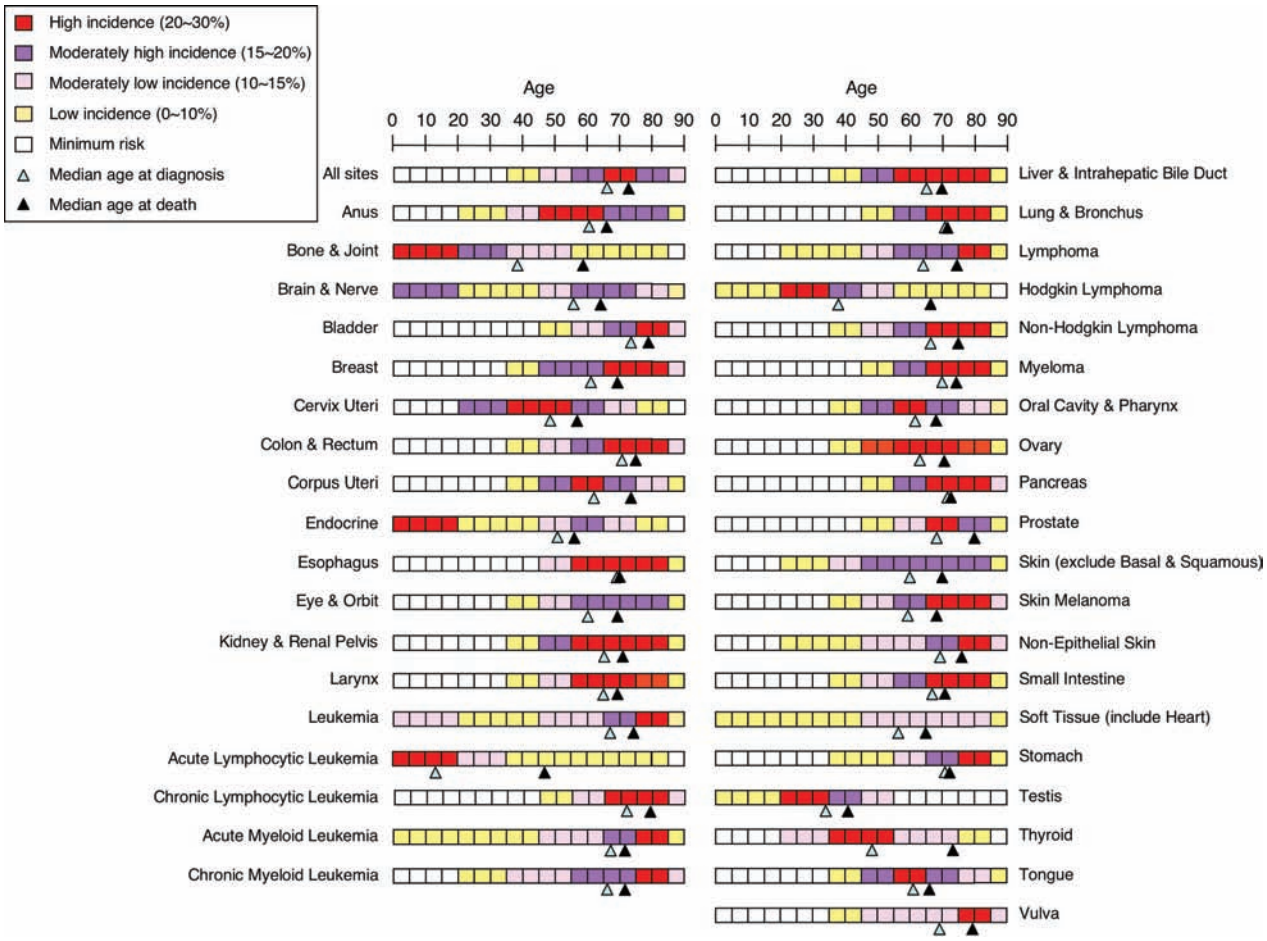

Figure 10.1. Cancer incidences by age and by type (site) of cancer. Data are based on the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute [11].

inactivation through epigenetic mechanisms has also been observed [10]. Table 10.1 summarizes the possible molecules that act as a bridge between aging and the carcinogenic process. Because accurate regulation of concerted DNA replication and DNA repair must be the function that is critical for maintaining the genomic stability of cells, any disturbances in such processes of well-organized regulation will be the fundamental mechanisms responsible for the descent of normal immature cells into senescent or transformed cells [7].

Age- and Organ-Specific Cancer Incidence

Many genetic studies have indicated that carcinogenic processes take time, and it is therefore likely that more tumors will develop in the elderly. A linear increase in the incidence of cancer is thus likely to be favored by the aging of the hosts. Analysis by SEER of cancer incidence from 2001 to 2005 revealed, however, that every type of cancer has its own age-dependent tendency of incidence [11] (Figure 10.1). This analysis provides grounds for classifying organ cancers into six broad categories according to the ages of onset: those preferentially developing (1) in infants and youths (e.g., bone and joint, and ALL); (2) in adolescents (e.g., Hodgkin lymphoma and testicular cancer); (3) in middle age (e.g., anus, cervix uteri, eye, oral cavity and pharynx, tongue, skin [excluding basal and squamous cell] and thyroid); (4) in tumors with organ aging, but seldom in youth (e.g., bladder, breast, colorectal, corpus uteri, esophagus, kidney, larynx, liver, lung, ovary, pancreas, prostate, nonepithelial skin, small intestine, stomach, AML/CML, melanoma, myeloma); (5) with several peaks of tumor incidence (e.g., brain and the endocrine); and (6) in a non–age-dependent manner (e.g., soft tissue).

Although the incidence of cancer generally increases with age, clinical data also reveal that malignancy, especially metastasis, seems to be reduced with age [12]. Extensive autopsy studies of all patients dying of histologically identical cancer revealed that the percentage of distant metastases was greater in younger patients than in the elderly. Also the frequency of hematogenous and lymphogenous metastases declined beyond the age of 60 [12, 13].

We may adduce several reasons for reduced malignancy in the elderly. More opportunities for medical examinations means a better chance for early diagnosis, or survival intervals between diagnosis and metastasis are short in the elderly, compared with those of younger generations. More realistically, age-related changes seem to moderate the aggressive nature of tumor proliferation or metastasis.

Aging: Friend or Foe for Carcinogenesis?

Ershler and his colleagues have revealed that tumor behavior is never uniform at all ages. Some experimental tumors exhibited reduced primary tumor growth and metastatic behavior (Table 10.2). Likewise, clinical cancers – particularly cancers of the breast, stomach, and prostate, and, to a lesser extent, lung and colon – exhibited slower growth, fewer metastases, and longer survival as the patients grew older [13–17] (Table 10.3).

In the very old (those with a 10 percent longer-than-average life span) who died of cancer, antemortem symptoms and autopsy observations suggest that tumors were nonaggressive, extremely slow-growing, and minimally symptomatic. Most died peacefully after having enjoyed long lives (*Tenju*) and in an atmosphere of family celebration that included the patients themselves. Such delayed onset of tumor formation with a least-aggressive phenotype in the very late phase of human life is called "natural-end cancer" (*Tenju-gann*) [8].

Experimental animal models have postulated several explanations for age-associated tumor behavior, involving biological features of tumor cells and host factors [15]. Possible causes of age-related differences include telomere shortening and/or changes in telomerase activity, DNA replication/DNA repair system, and immunogenicity of tumor cells themselves. All these factors link oncology and gerontology [6]. The factors of the host's environment that account for the age-associated changes in tumor incidence and malignancy can be classified into two types: local or humoral (e.g., angiogenesis, wound-healing, extracellular matrix, immune effector cells, hormones, growth factors/cytokines, nutrition, reactive oxygen species [ROS], and a number of others) [5, 15, 18–20]. Intrinsic factors that regulate aging, tumor development, and metastatic spread are depicted in Figure 10.2.

Angiogenesis

Experimental and clinical studies have revealed that tumor vessels are formed and their density is enhanced inversely to the advancement of the host's age [21–23]. Tumor vessel architectures in old or young hosts are very different. In the old, avascularity or decreased blood vessel density and reduced penetration of blood vessels into the tumor mass are more common than in the young. The lumen of large feeding vessels formed in a growing tumor mass in a young host is straight and of uniform diameter and has numerous buds (dense vascular network), whereas those in the tumor-bearing elderly host are twisted, sparse, and irregularly distributed [23]. The feature is sometimes called *blood vessel senescence,* as it resembles the findings in the telomerase-deficient $Terc^{-/-}$ mice [24].

Angiogenesis-stimulating soluble factors change with age, and reduction of responsiveness to angiogenic factors has been considered to be a factor responsible for the age-related decline of tumor growth and spread [22, 25]. Tumor angiogenesis is partly dependent on the immune function. In particular, T cells and macrophages produce angiogenic factors such as lymphokines, lymphocyte-induced angiogenesis factor, and fibroblast growth factor. It appears, therefore, that tumor angiogenesis is likely to be more potent if the body's immune system is intact, as in young hosts, and that the production of angiogenic factors and the body's responsiveness to them decline with age [22].

Fibrous Reaction and Extracellular Matrix

Tumor cells are surrounded by and manipulate extracellular matrices (ECM). Malignancy is physiologically or mechanically affected by ECM. Embryogenesis, maturation, and aging also influence ECM biosynthesis. Tumor encapsulation, or desmoplasia, is constructed mainly by means of fibrous reaction. Generally, tumors in older hosts contain more fibrous tissue [12, 25, 26]. After fibrosis, angiogenesis in old hosts is inhibited [23]. Fibrosis may also reduce invasiveness and metastasis, as dense fibrous formation networks physically inhibit transmigration across basement membranes and reduce susceptibility to proteolytic enzyme degradation [26].

The ECM is composed of four major molecular families: collagens, elastins, proteoglycans, and structural glycoproteins. Collagens represent around 30 percent of all proteins in the organism. Most have been investigated in relation to tumor malignancy. The ECM study has proposed the upregulation of fibrous-reaction–associated collagen synthesis as the mechanism that is responsible for reduced tumor aggressiveness in aged hosts. In fact, tumors in mice treated with collagen synthesis inhibitors are more aggressive growth in old hosts [12, 26].

Immunity

A number of immunologists believe that age-related immune attenuation starts coincidently with the involution of the thymus gland and corresponding T-cell dysfunction, which has been causally linked to

TABLE 10.2. How tumor development and its malignancy are modulated by host aging (experimental study).

Tumor cells	Recipients	Injection site/mode	Primary tumor growth	Metastases	Reference
B16, melanoma	C57BL/6 mice	sc	young $\gg$ old	young $>$ old	22, 75
B16, melanoma	C57BL/6 mice	sc	young $\gg$ old		75
B16, melanoma	C57BL/6 mice	sc, ip, iv	young $<$ old	young $<$ old	78
B16, melanoma	C57BL/6 mice	sc	young $\geq$ old		78
B16, melanoma	C57BL/6 mice	sc	young $\gg$ old		77
B16, melanoma	C57BL/6 mice	iv		young $<$ old	79
B16, melanoma	C57BL/6 mice	iv		young $\leq$ old	78
B16 & B16/Col/R, melanoma	C57BL mice	sc	young $>$ old	young $\gg$ old	80
G3.26, B16 melanoma variant	C57BL/6 mice	sc	young $\gg$ old	young $\ll$ old	81
B16-F1, melanoma	C57BL/6 mice	iv		young $\gg$ old	25
B16-F1, -F10, melanoma	C57BL/6 mice	sc, ip	young $\gg$ old	young $\gg$ old	25
B16-F10, melanoma	C57BL/6 mice	sc	young $\gg$ old		25
B16-F10, melanoma	C57BL/6 mice	sc (outer ear)	young $=$ old	young $\ll$ old	19
B16-F10, melanoma	C57BL/6 mice	sc, iv	young $\gg$ old	young $\gg$ old	82
B16-F10, melanoma	C57BL/6 parabiotic mice, same age	iv	young $\gg$ old	young $\gg$ old	18. 45
B16-F10, melanoma	C57BL/6 parabiotic mice, different age	iv		young $\geq$ old	18, 45
3LL, Lewis lung carcinoma	C57BL/6 mice	sc	young $\gg$ old		25, 30
3LL, Lewis lung carcinoma	C57BL/6 mice	sc	young $=$ old		83
Lung carcinoma	C3H mice	iv		young $<$ old	84
A-755, mammary carcinoma	C57BL/6 mice	sc	young $<$ old		78
Ca-755, mammary carcinoma	C57BL/6 mice	sc	young $\leq$ old		78
SST-2, spontaneous mammary carcinoma	SHR rats	sc	young $=$ old	young $=$ old	40
SST-2, spontaneous mammary carcinoma	SHR rats	iv		young $=$ old	40
Spontaneous mammary carcinoma	Dogs	st	young $=$ old	young $=$ old	85
64pT, non-metastatic mammary carcinoma	BALB/c mice	mpf	young $>$ old	young $\geq$ old	19
4T07, metastatic mammary carcinoma	BALB/c mice	iv		young $\gg$ old	86
4T07cg, metastatic mammary carcinoma	BALB/c mice	mpf	young $\leq$ old	young $<$ old	54
Liver epithelial tumor	rat	ih	young $\ll$ old		44

(Continued)

TABLE 10.2 ***(Continued)***

Tumor cells	Recipients	Injection site/mode	Primary tumor growth	Metastases	Reference
Liver epithelial tumor	rat	sc	young = old		44
Hepatoma-22a	C3HA	sc	young < old		83
BAG2-GN6TF hepatoma	rats	ih	young ≪ old		44
BBN-induced renal pelvic tumor (female)	NON/Shi mice	po	young ≪ old	young = old	87
BBN-induced bladder tumor (female)	NON/Shi mice	po	young ≥ old	young = old	87
OTT6050 teratocarcinoma	129/Sv mice	sc	young ≫ old		20
EHS, Englebreth-Holm-Swarm carcinoma (chondrosarcoma)	C57BL mice	im	young > old		82
La, hemocytoblastosis	C57BL/6 mice	ip	young < old		83
P388, leukemia	DBA/2 mice	ip	young = old		78
L1210, leukemia	DBA/2 hybrid mice	ip	young < old		88
AKR lymphoma	AKR mice	sc	young > old		77
P815, mastocytoma	Balb/c mice	ip	young < old		89
LCP-1, myeloma	mice	ip	young > old		90
Fibrosarcoma	rats	sc	young > old		91
Fibrosarcoma 1023	mice	sc	young < old		92
Sarcoma 180	C57BL/6 mice	sc	young = old		43
1591, UV light–induced fibrosarcoma	C3H/HeN mice	sc	young ≪ old		93
3-methylcholanthrene-induced fibrosarcoma	rats	sc	young ≫ old		94
SP1, 3-methylcholanthrene-induced fibrosarcoma	C57BL/10 mice	sc	young = old		22
RA-2, rhabdomyosarcoma	rats	iv		young < old	95
RA-2, rhabdomyosarcoma	albino rats	iv	young > old	young = old	78

Abbreviations: sc, subcutaneous; iv, intravenous; ip, intraperitoneal; ih, intrahepatic; im, intramuscle; mfp, mammary fatpad implantation; po, peroral administration; st, spontaneous tumor; BBN, N-butyl-N-(4-hydroxybityl) nitrosamine.

age-dependent development of neoplasia [25]. The concept of immune surveillance advocated by Thomas [27], and later by Good [28], is based on age-related immune deficiency, in which the age-associated decline of the immune function is considered to be an important factor for the pathogenesis of tumor development in patients of middle and old age [29]. Supporting this theory, immune dysfunction resulting from immunosuppressive therapy after organ transplantation or acquired immune deficiency syndrome occurs

TABLE 10.3. A survey of how tumor development and its malignancy are modulated by host aging (clinical study).

Tumor origin	Preference of tumor growth	Longer survivals	Preference of metastasis	Reference
Lung	young $\gg$ old		young $\gg$ old	13, 96
Breast	young $\gg$ old		young $\gg$ old	15, 96
Breast (Germany)		young $<$ old		97
Stomach	young $=$ old	young $\gg$ old	young $\geq$ old	98
Stomach (Japan)	young $>$ old	young $>$ old	young $\leq$ old	16
Stomach (intestinal-type)	young $\gg$ old		young $\gg$ old	99
Stomach (diffuse-type)	young $\ll$ old		young $\ll$ old	99
Kidney	young $\gg$ old		young $\gg$ old	96
Prostate gland	young $\gg$ old		young $\gg$ old	17, 96
Colon	young $\gg$ old		young $\gg$ old	96

concomitant with increased tumor incidence. Mice selectively bred for high immune function have generally lower incidence of tumor development than mice with poor immune responsiveness. Paradoxically, immune senescence may contribute to reduced tumor incidence and metastasis in other models.

Reconstitution experiments using immune effector cells from old donor mice have provided direct evidence of the involvement of age-associated immune dysfunction. When thymoectomized, lethally irradiated young mice received bone marrow cells or splenocytes from old donor mice, tumor growth was significantly less [14, 30]. However, attenuated immune function can accompany reduced tumor growth and metastasis in young mice treated with sublethal irradiation, anti-T-cell antibodies, anti-helper T-cell antibodies,

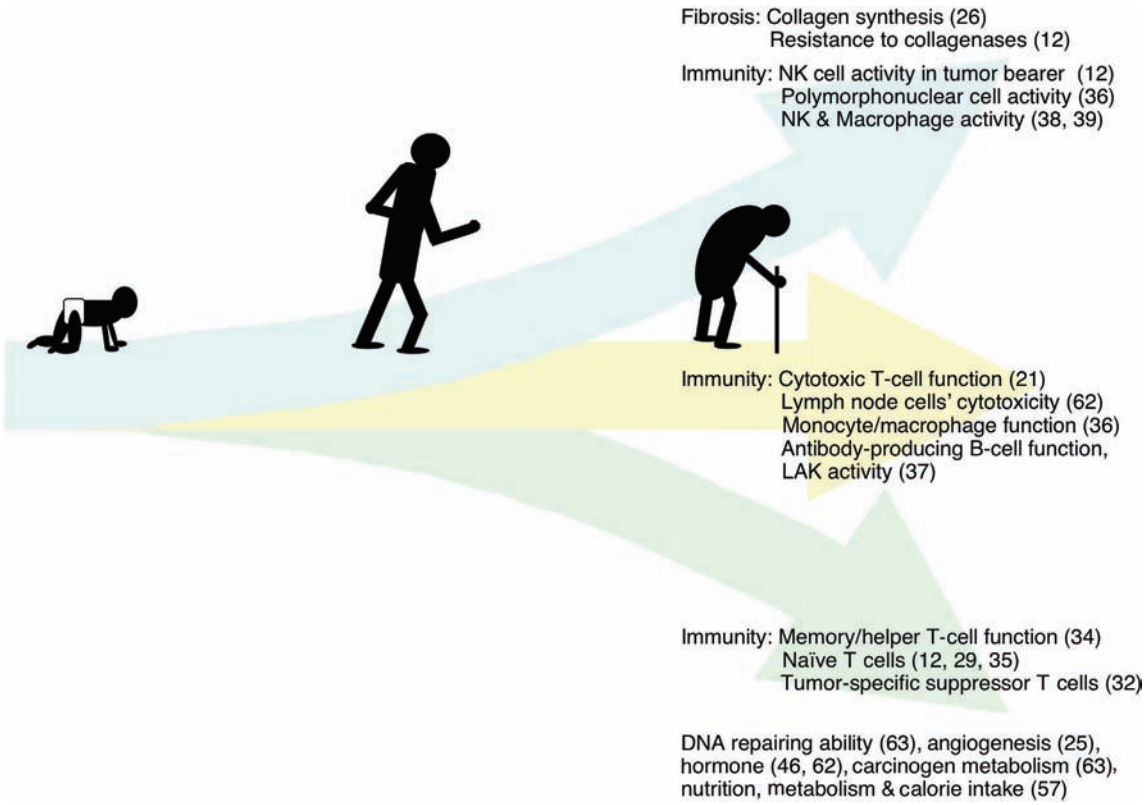

Figure 10.2. Host and cellular factors that may account for age-related reduction of tumor development and metastasis. The corresponding references are in parentheses.

or corticosteroids to suppress immune function [14, 30]. Tumors grow less in congenitally immunocompromised mice and in young mice with T-cell deficiency. These observations are consistent with Prehn's "immune enhancement theory," which posits that host immunity may, under certain circumstances, stimulate tumor growth [31].

A fundamental immune mechanism that can enhance tumor growth is emergence of tumor-specific T-suppressor cells (Ts) [32]. The presence of Ts was identified by passive transfer of T cells obtained from tumor-bearing mice to mice that had previously been immunized with the same tumor cells. A recipient mouse's resistance to tumor cell challenge is abrogated [33]. Thus Ts inhibited the onset of antitumor immunity in otherwise immune competent hosts. The reduced ability of Ts' effectiveness accompanying advancing age may explain tumor restraint in older patients [32]. T-cells' subtypes are differentially affected by aging. $CD8^+$ and B-cell function appear less impaired than $CD4^+$ and naïve T cells [21, 34, 35].

Polymorphonuclear leukocytes, monocytes/macrophages, and natural killer (NK) cells' component of natural immunity are better preserved in later life. The number of NK cells increases during aging, whereas their activity decreases, highlighting the distinction between number and function [12, 36]. Likewise, immune cell responses to regulatory cytokines can be altered in aging tumor-bearing hosts [37].

A spontaneous hypertensive rat model has been used to study relations between age-associated immune activity and metastasis. SHR rats develop aging-associated T-cell dysfunction owing to the appearance of a natural thymocyte autoantibody and declining thymic hormone secretion [38, 39]. Macrophages and NK cells are nonspecifically activated. Mammary adenocarcinoma (SST-2) metastasis decreases in aged rats [38, 40]. As expected, in the young SHR rats in which NK cells and macrophages were activated, metastasis was reduced [41].

The immunogenicity of tumor cells is another key factor in antitumor immunity. If weakly immunogenic or nonimmunogenic tumors are used, the pattern of tumor growth appears to be more influenced by host age than if highly immunogenic tumors are used [6, 15, 42, 43].

Soluble Growth Factor and Responsiveness

When tumor cells are injected orthotopically, age-related microenvironmental factors are apparently present, as there are differences in tumor formation by age, whereas such differences are not observed when they are injected ectopically [44].

Parabiosis (i.e., shared blood flow) between young and old mice revealed the existence of soluble factors. Intravenous injection of tumor cells into young mice forms metastatic colonies tenfold greater than in old mice. In parabiotic mice constructed from young and old mice, the metastases in the old parabionts were at the same levels as in young mice [18]. These results suggest that metastatic potential may depend partly on systemic humoral factors transported by the blood or by the lymphatic stream [45].

Likely candidates include sex hormones [19]. Whereas some tumors – such as cancers of the breast, prostate, kidney, or melanoma or carcinoid tumor – are dependent on hormone for their growth and spread, malignancy of tumors can be influenced by age-associated, fluctuant hormones, such as thymic or glucocorticoid hormones [12, 42, 46]. Some of their effects can modulate immune function. Likewise, cytokine and other growth factors are also involved in tumor formation and metastasis.

Reactive Oxygen Species

The most common and major intrinsic causes of DNA damage involve ROS and byproducts formed by their reaction to nitric oxide (NO). ROS are produced by external substances such as ionizing radiation or genotoxic drugs. They are also intrinsically produced through mitochondrial metabolism, activation of nicotinamide adenine dinucleotide phosphate oxidases, peroxisomes, cytochrome p450 enzymes, nitric oxide synthase uncoupling, and the antibacterial oxidative burst of inflammatory cells. When any of these physical functions generates ROS, they cause approximately 10^4 alterations per cell per day. ROS involve superoxide anion and hydroxyl radicals, which are extremely unstable, whereas other substances – hydrogen peroxide, for example – are freely diffusible and relatively long-lived [47]. ROS exert genotoxic/cytotoxic activity through lipid peroxidation or protein damage, or by causing replication errors, spontaneous chemical changes of single- and double-strand breaks, adducts, and crosslinks.

Harmful ROS are scavenged by several antioxidative defense mechanisms, including superoxide dismutase (SOD), catalase, glutathione peroxidase, peroxiredoxins, and glutathione. In addition, a variety of nonenzymatic, low-molecular-weight antioxidants (e.g., ascorbate, pyruvate, flavonoids, and carotenoids) also scavenge ROS. When the ROS overwhelm cellular antioxidative and antioxidant defenses, the condition is known as *oxidative stress*.

Oxidative stress is of fundamental importance for the emergence of senescent phenotypes. Studies in the 1970s revealed that cells grown in low oxygen tension exhibited a prolonged life span, whereas those grown at high oxygen concentrations had a shorter life span and telomere shortening [48]. As the expression of SOD and

catalase prolongs survival of fruit flies by 30 percent, oxidative stress has been proposed as a crucial factor for aging [49].

ROS-caused biomolecular damage occurs stochastically during normal oxygen consumption (aging). Ironically, maintaining of ROS homeostasis appears to be essential for life. The bactericidal function of inflammatory-cell–derived ROS is a well-recognized host defense mechanism, although inflammation-associated ROS can convert benign tumor cells into metastatic ones [50–52]. Niitsu and colleagues have been investigating how ROS modulates metastasis. ROS activate PKCζ, which in turn phosphorylates RhoGDI-1, which liberates RhoGTPases from RhoGDI-1 and leads to motility [53].

Recently, ROS and NO were pointed out to be specific cellular signaling molecules [47, 54, 55]. Regardless of how or where ROS and NO are generated, intracellular oxidative stress has two potentially significant effects: to damage various cell components and to trigger activation of specific signaling pathways [47]. Both effects can stimulate numerous cellular processes linked to aging, and development of age-related diseases, such as cancer.

Nutrition and Calorie Restriction

Human and experimental studies have demonstrated that dietary restriction may delay aging and the development of cancer and its metastasis [54, 56]. Nutritional requirements change with age, as do nutritional habits [42]. Young mice fed with the same diet as the diet given to old mice (the same content but with reduced calories) lost weight, had slower tumor growth, and survived longer [42]. Dietary habits common among elderly people may account for age-associated changes of the tumor phenotype [42]. Dietary restriction may also significantly reduce tumor incidence and metastasis in cancer-prone, viral- or chemical-treated animals. Dietary restriction may also significantly prolong survival. When mice were fed a calorie-restricted diet of 50 percent less food than usual, age-dependent decline of immune competence was prevented [57]. A more recent study has revealed that caloric restriction reduces the metabolism-associated production of ROS.

Telomeres and Telomerase

Age-dependent chromosomal modification occurs during each mitosis at chromosomal end nucleotide repeats called *telomeres,* which maintain chromosomal stability by protecting chromosome ends during replication [9]. Telomeres are shortened during cell division. As they reach a critical length, cell replication is arrested. Telomere shortening is thus the intrinsic process relevant to the courses of cellular and organism-replicative senescence, aging, and cancer. A relationship of telomeres to metastasis has not yet been formally established. Telomerase reverse transcriptase (TERT) gene expression regulates or is regulated by several oncogenic signaling pathways implicated in metastasis. For example, the induction of the TERT gene is observed when retinoblastoma/E2F1 and Akt pathways are activated. Also, TERT upregulates glycolysis genes and the Met gene, which regulate motility, invasion, angiogenesis, and metastasis.

Mitochondrial DNA Mutations Related to Metabolism

Mitochondrial metabolism and formation of ROS share mechanisms that hasten aging and cancer development [56]. As a byproduct of energy production, ROS are generated by mitochondria and can damage mitochondrial DNA (mtDNA). Some DNA-damaged cells undergo apoptosis. In general, accumulation of mutant mtDNA thus serves as an aging clock [56]. Moreover, reduced tumorigenicity and metastatic ability occur following overexpression of manganese SOD, a mitochondria-localized antioxidative enzyme.

Somatic mutations in mtDNA play a causal role in malignant transformation, whereas it has also been suggested that preferential accumulation of somatic mutations in tumor mtDNAs also contributes to tumor growth. Another theory proposes that the acquisition of metastatic potential in tumor cells is driven by mtDNA mutations. When a cytoplasmic organelle reconstitution (cybrid) technique is used, transfer of highly metastatic tumor-cell–derived mtDNA into weakly metastatic tumor cells increased metastasis, and vice versa [58]. Ishikawa et al. identified that, among the mtDNAs, mutations in the gene encoding NADH dehydrogenase subunit 6 is a causative region for the malignant conversion of tumor cells. The conversion is thought to occur through the overproduction of ROS owing to a deficiency in respiratory complex I activity.

Psychosocial Impact of Aging

In addition to the physiologic changes associated with aging and their impact on metastasis, aging is also accompanied with reduced tolerance to stress and changes in socioeconomic and psychosocial status [7].

Social isolation, divorce, and/or bereavement are reported to increase the risk of recurrence, metastasis, and mortality [59]. Psychological effects on metastasis have been increased because of social isolation stress (individual housing), which saw decreased thymus weight and suppressed immune response (NK and macrophages) [60]. In contrast, reducing the impact of psychosocial isolation stress through social support or intervention, as in relationships, prolongs life span and reduces incidence of metastasis [61].

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11 The Continuum of Epithelial Mesenchymal Transition – Implication of Hybrid States for Migration and Survival in Development and Cancer

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EMT AND CELL MIGRATION – EMBRYONIC NECESSITIES CO-OPTED BY INVASIVE CANCER

The concept of the epithelial–mesenchymal transition (EMT) originated from studies of events in development, particularly those preceding the onset of cell migration [1]. These were initially brought together and popularized by the efforts of the late Elizabeth Hay and colleagues [2, 3]. One of the most intensively studied examples of EMT and cell migration is the generation of migratory neural crest mesenchyme from the neurectodermal epithelium [4] (Figure 11.1). These cells go on to form the autonomic and sensory nervous systems. Even prior to this, the emergence of the primary mesenchyme from the epithelial epiblast during gastrulation (the first EMT) results in the formation of highly motile cells that are critical to the development of the body plan [1] (Figure 11.2A). Further EMTs occur in other epithelia after the neural crest EMT to generate the cells that form muscle, bone, and connective tissues (Figure 11.2B). Such cellular plasticity is fundamental to embryological development and is regulated largely at the transcriptional level. Various transcriptional repressors of E-cadherin (and other cadherins), such as Snail (Snail 1), Slug (Snail 2), Twist, Zeb1 (δEF1), Zeb2 (SIP), and E47/E12, regulate EMT in developmental system (reviewed in [5]).

Commitment to lineage differentiation in normal cells is more pliant than first thought, and cellular transition is emerging as a major mechanism of adult tissue homeostasis [6].

The descriptive similarity of developmental EMT and cell migration to local tissue invasion from a carcinoma is striking, suggesting that the latter is a pathological EMT [7–9]. The similarities have been extended down to the level of molecular expression and gene regulation, although, it must be admitted, mostly using in vitro models employing cancer-derived cell lines [10]. Direct evidence in human or animal cancers has been elusive, fueling a controversy as to the reality of EMT in cancer invasion [11]. However, more recent in vivo approaches employing markers whose expression is driven by regulatory elements of EMT-associated genes have provided stronger "guilt by association" evidence of EMT occurring at sites of invasion in real tissues (reviewed in [12, 13]). This has been supported functionally; when cell suicide was linked to EMT gene expression it resulted in reduced metastasis in animal models [12].

Evidence of EMT-like traits in cancer cell lines has been endorsed by recent genome-wide Affymetrix profiling studies of large collections of human breast cancer cell lines comprising thirty-four [14] and fifty-one [15] cell lines (reviewed in [16]). At the same time, the concept has been raised of cancer stem cells (rather than the bulk of the cancer cells) as a disproportionately important factor in cancer growth and dissemination. This is consistent with early reports of migrating cancer stem cell attributes in cells exhibiting EMT properties at the invasive front of gastric cancers [17]. The stem cell concept has become confluent with that of cancer EMT, with commonality between the EMT and the breast cancer stem cell (BCSC) transcriptional makeup derived by serial analysis of gene expression [18]. Indeed, BCSC-like attributes can be seen in human mammary epithelial cells after EMT induction [19]. From an etiological point of view, processes that occur in a small fraction of cancer cells may play a key role in the phenotype of the overall disease. Metastatic disease may be established from single cells recurrently, and EMT may play a key role in metastatic dissemination of these cells, with only a minute fraction of the carcinoma cells in the primary site exhibiting EMT markers. It is for this reason that BCSC biology draws significant attention in recent research [20].

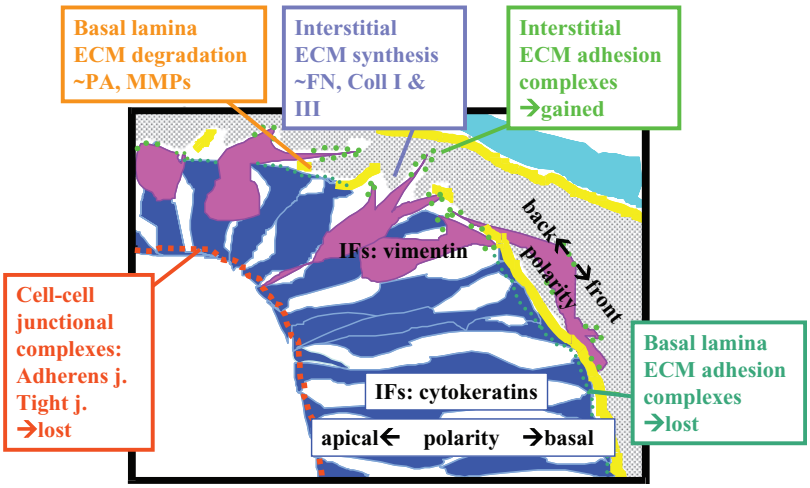

Figure 11.1. Neural crest EMT. Characteristic changes in EMT shown in a diagrammatic transverse section of the neural crest example. Neural epithelial cells (blue) convert to mesenchymal neural crest cells (pink) with changes in polarity and intermediate filaments (IF). Cell–cell adhesions are reduced, and cell–ECM adhesions are modulated. In addition, proteases such as plasminogen activator (PA) and MMPs are upregulated, and ECM is altered by both degradation and synthesis.

THE EMT SPECTRUM – EXTREME TO PARTIAL

Extreme examples of any biological phenomenon lend themselves to study; this is true of the study of EMT and its immediate aftermath, cell migration/invasion. From these studies, the defining features of EMT and migration (see Figure 11.1) include:

1. Downregulation of cell–cell adhesion, particularly due to loss of classic cadherin-mediated junctions. This enables the new mesenchymal cells to separate from former epithelial neighbors.
2. Reorganization of the cytoskeleton (exemplified by F-actin changes) leading to loss of apicobasal polarity typical of epithelial cells and gain of locomotor (front–back) polarity. This leads to degeneration of the highly structured epithelial pattern and, potentially, to cell migration away from the site.
3. Altered expression of genes for molecules of the extracellular matrix (ECM), and for the cell surface adhesion molecules (particularly the integrins) that mediate cell–ECM adhesion. This allows cells to rearrange with respect to basal lamina versus interstitial ECM, and potentially to gain traction on the latter.
4. Upregulation of genes encoding extracellular proteases, such as plasminogen activators and diverse matrix metalloproteases (MMPs), potentially allows cells to remove cell–cell adhesive molecules, enables cells to pass through basal lamina ECM, and facilitates penetration of dense interstitial ECM.

In examples such as gastrulation or neural crest migration in mammalian or avian embryos, and in certain cancer cells in vitro, these features are carried through to a high degree, and the mesenchymal cells are regarded as behaving individualistically. However, when even extreme EMT and migration events such as avian neural crest cell migration have been studied dynamically by time-lapse imaging in real tissues, the cells are seen to have strong social interactions that govern their behavior. Neural crest cells, when migrating, are almost always in contact with other neural crest cells and form "head-to-tail" chains. The contacts between individual cells are measured in minutes, and neighbor exchange is frequent. When deprived of neighbors, however, neural crest cells cease directionally persistent locomotion, and hence show minimal real migration or invasion [21, 22].

Much less extreme situations also exist, in which some EMT features occur but not others, or the level of the feature is less than extreme. A developmental example of this is gastrulation in frogs. This process is clearly homologous to gastrulation in mammals and birds, but instead of the gastrulating cells migrating as an apparently disorderly mob of individualistic cells, the cells spread as a sheet with the advancing edge cells showing front–back polarity and motile specializations, but maintaining intimate epithelial-like cell–cell adhesions to the cells behind the front. This kind of sheet like spreading resembles wound healing in epithelia. Many other examples of movement of groups of cells, or collective cell movement, are known [23]. These include the extension of cells as coherent cords in angiogenesis; tubulogenesis in nephric, mammary, and lung morphogenesis; the movement of essentially a ball of border cells in *Drosophila*; movement of cell clusters in early vertebrate heart morphogenesis; and the behavior of many cancer cells in various in vitro assays (as summarized later). The terminology applied to these diverse cases in not yet agreed on, but they clearly share many features of EMT; such "epithelial" cells have been termed "relaxed," "activated," "hybrid," "metaplastic," or "metastable" with a "partial EMT."

MET – BACK TO THE FUTURE

Developmental EMT and cell migration is often followed by a later phase whereby the migrating cells cease migrating and aggregate. When cells in some lineages are followed through time, cycles of EMT and mesenchymal–epithelial transition (MET) have been revealed. The classic developmental example of this is the epithelial epiblast lineage that generates the

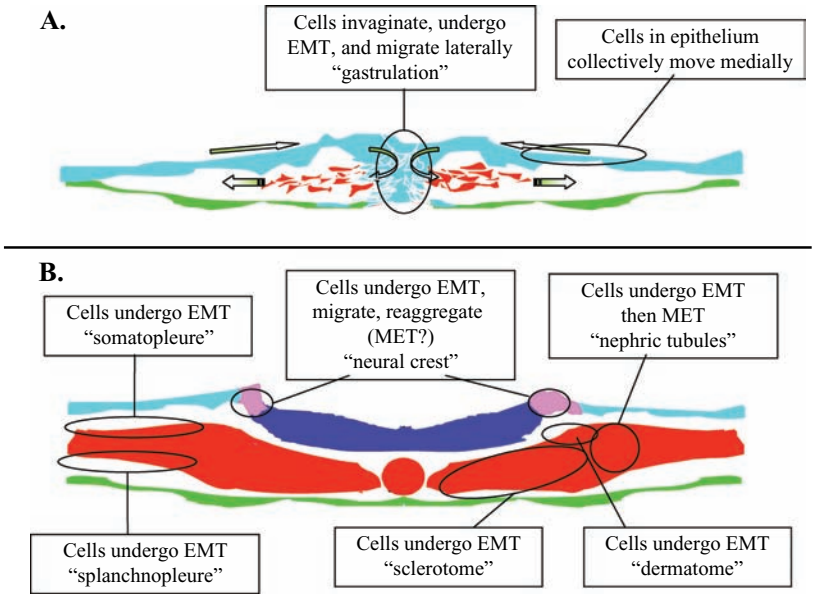

Figure 11.2. Developmental EMTs. **A.** The first EMT in metazoans is gastrulation, shown in transverse section. The upper epiblast epithelium cells collectively move medially, then invaginate and undergo EMT at the midline. The resulting mesenchymal cells (red) form a new and apparently disorderly middle layer, with the cells moving individualistically laterally. **B.** After gastrulation, the mesenchyme cells condense to form new epithelia (mesoderm), which undergo complex patterns of further EMTs and METs. Several stages of development are represented in this one diagram.

primary mesenchyme (via an EMT) during gastrulation (Figure 11.2A). This primary mesenchyme then forms epithelial layers termed the *segmental plate* of presomitic, intermediate, and lateral plate mesoderms (via a MET). The segmental plate mesoderm, after reorganizing into rosette-shaped epithelial somites, forms the mesenchymal sclerotome (another EMT). The lateral plate mesoderm (termed *somatopleure* and *splanchnopleure*) also disperses to mesenchyme (EMT), whereas the intermediate mesoderm disperses (an EMT) but goes on to later reepithelialize to form nephric tubules (MET) (Figure 11.2B). The notion that cells after EMT might not be irreversibly bound to this state, and indeed might revert via MET, has been embraced in the field of metastasis (see below).

CANCER AND THE EMT CONCEPT

Developments in gene and molecular expression provide depth to the enormous increase in studies documenting EMT and associated functional changes in culture systems (for recent reviews, see [24–28]) and the increasing number of studies attributing prognostic significance to EMT indicators in the primary tumor. These include specific studies of EMT markers, such as vimentin, which is typically expressed by cells of mesenchymal origin, although some controversy still exists in respect to its prognostic significance in breast cancer [29]. Recently, a collection of EMT-like markers, including vimentin, were linked to the more aggressive "basal" subtype of breast cancer [30]. Other studies have shown prognostic significance to EMT drivers, such as the E-cadherin repressors mentioned above. These include Snail (Snail 1), Slug (Snail 2), Zeb1 (δEF1), and Twist [31–40].

Such studies are not limited to breast cancer. Baumgart et al. [41] investigated the prognostic significance of several markers known to be linked with EMT in clinical samples of varying stage and grade of bladder tumors. It was found that a loss of E-cadherin, accompanied by a reduction and relocalization to the cytoplasm of β-catenin and plakoglobin, were associated with late tumor stage and grade. This study also determined an upregulation of N-cadherin and vimentin expression in some of the tumor samples examined. In addition, several more specific studies investigating E-cadherin expression in particular, along with a range of physically, functionally, and mechanistically associated molecules (such as moesin, zyxin, α-catenin, p53, RB, and INK4A), were performed [42–47]. Some of these studies investigated E-cadherin expression in clinical samples with respect to its possible prognostic value; however, these studies provided variable results. Thus, despite the large amount of data implicating some of the EMT-related processes in tumor progression, clinical data remain somewhat problematic and the definitive EMT marker has not yet emerged. Many laboratories, including our own, are pursuing markers that may better distinguish breast and other cancers that have a propensity for EMT.

Several studies link the process of EMT, and presumably also plasticity around the EMT axis, to the actual metastatic process. Extraordinary demands are placed on epithelial-derived carcinoma cells to successfully metastasize, including separation from the epithelial collective, degradation of the surrounding matrix, migration and invasion through the basement membrane, intravasation and survival in the circulation, extravasation at a secondary site, survival as micrometastases, and finally growth into overt metastases [48]. To complete these complex steps, cancer cells exhibit both mesenchymal- and epithelial-like properties at different times, or even at the same time [3, 26, 28]. EMT regulators do alter the cell cycle machinery of cells, and through these means may allow the prolonged survival of residual cancer cells [49]. Indeed, the EMT regulator Snail 1 was implicated in the emergence

of residual disease into local recurrence after oncogene silencing [50].

Mesenchymal derivatives of carcinoma cells show a number of attributes that would favor metastasis, such as separation from the collective as individual cells, increased migratory and invasive potential, increased survival in suspension, and resistance to apoptosis in response to chemotherapy. Sustained expression of mesenchymal traits would assist in extravasation at the metastatic site and possibly also survival at that site. Some anecdotal evidence has accrued on the likelihood that circulating tumor cells (CTCs) and micrometastases show EMT characteristics. It has long been recognized that CTCs show reduced expression of specific cytokeratins [15], which are regarded as epithelial hallmarks, and it was recently shown that cell lines derived from breast cancer micrometastases stably express the mesenchymal marker vimentin [51].

In addition to translocation during metastasis, EMT biology has grown to encompass resistance to anoikis [52], enhanced survival [49], genomic instability [53], and resistance to chemotherapies [54], and thus represents a potentially comprehensive target in cancer biology. By commandeering developmental EMT pathways, sessile epithelial carcinoma cells are transformed into cells with migratory and invasive capability, metastatic potential, and a resistance to anoikis and chemotherapy [55].

CANCER AND THE MET CONCEPT

The concept of the MET has also found application the cancer field, in which the establishment of metastases has been suggested to involve a MET-like reversion of the EMT that enabled the initial escape from the primary tumor site. This is consistent with the well-established similarity of primary and secondary tumors [56–60]. In one of the clearest examples, metastatic colorectal carcinoma, metastases distant from the primary site may adopt a morphogenesis and differentiation pattern closely resembling colonic epithelium [17]. Studies in our own laboratory showed that variants of the metastatic T24/TSU-Pr1 bladder carcinoma line selected for metastatic potential express more epithelial markers (cadherins and keratins) than their less metastatic counterparts [59, 61]. Furthermore, in prostate cancer metastasis to the liver, upregulation of the epithelial marker E-cadherin was observed on cancer cells; this increase in E-cadherin could be modeled by the co-culture of prostate cancer cells with hepatocytes [62]. In addition, fluorescent markers driven by epithelial and mesenchymal-related FGF receptor isoforms in prostate cancer cells have highlighted the epithelial–mesenchymal plasticity both in the primary site and in lung metastases. This is in accord with the notion that the ability to convert between the epithelial and mesenchymal states is most effective in allowing cells to both leave the primary tumor and to establish a distant metastasis (Figure 11.3). Indeed, careful analysis of EMT-derived populations suggests that the "hybrid" or metastable phenotype is more prevalent than pure mesenchymal derivatives. We have found coexpression of both epithelial and mesenchymal markers in the PMC42LA human breast cancer system of epidermal growth factor (EGF)-induced EMT (discussed later). These cells exhibit coexpression of vimentin and epithelial cytokeratins [63], as well as EpCAM (unpublished data).

This hybrid phenotype reflecting epithelial–mesenchymal plasticity has become well-recognized recently in cancer systems [25], and has been referred to as a *metastable phenotype* [64] or activated epithelium [65]. Others have also reported this hybrid state [66]; this could partly explain the difficulty of observing EMT in clinical samples [11]. Acquisition of mesenchymal characteristics may be transitory, may occur on a background of epithelial gene expression, and may be reversed during metastasis.

MOLECULES OF EMT AND MET

The most reliable piece of evidence that establishes a continuum between complete EMTs and the partial or less obvious forms is the expression of genes and molecular functions in common between development and cancer. EMT is a culmination of changes affecting adhesion components and their associated signaling pathways in a manner that promotes the migration of cells in settings such as gastrulation during development or metastasis in cancer [67]. An example would be the downregulation of E-cadherin, which may occur as a result of receptor tyrosine kinase (RTK) activation upregulating MAPK or Wnt signaling pathway activity, which in turn blocks the ability of GSK3β to repress MAPK activity [26, 28]. Both pathways allow an increase in Snail 1 and Snail 2 activity, blocking E-cadherin transcription [68–70]. Other changes as part of EMT include RTK activity in reducing the assembly and stability of adherens-junction components, as well as the breakdown of tight junctions through transforming growth factor β (TGFβ)-induced depolymerization of cytoskeletal elements [28]. A summary of the signaling components common to cancer and developmental EMT is shown in Table 11.1.

THE SHOP FLOOR OF EMT–CELL ADHESION AND CYTOSKELETAL MOLECULES

The archetypical epithelial cadherin (E-cadherin) effects homotypic adhesion between most adult epithelia, through the adherens junctions [71]. Regulation of the various types of cadherin and their expression

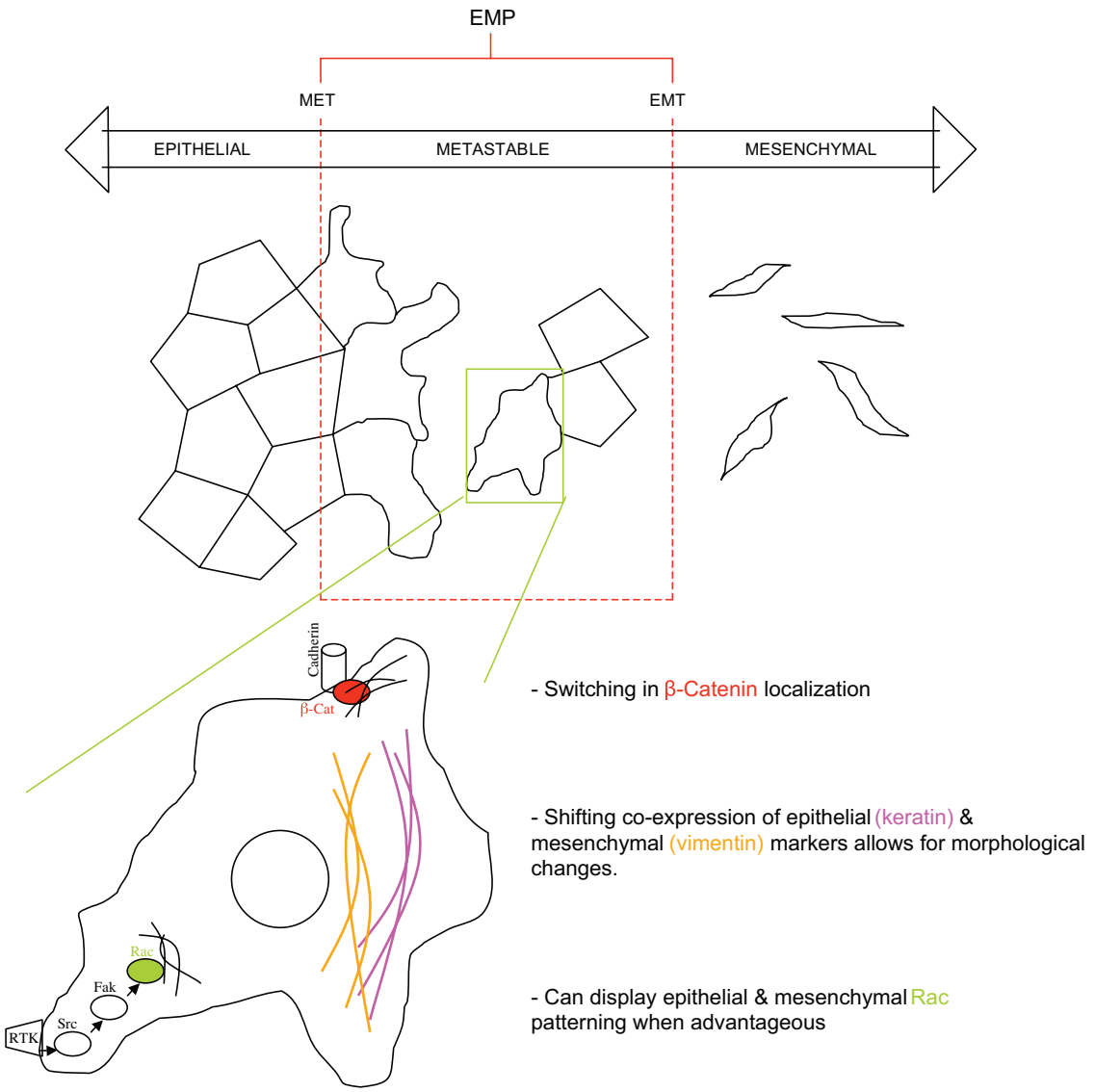

Figure 11.3. The hybrid/metastable phenotype. The "metastable" cell, as indicated in the lower panel, may embrace features that allow the cell to readily switch between epithelial and mesenchymal phenotype. This subpopulation of cells may be key in allowing the various steps of metastasis to be accomplished. Three aspects that the metastable cell could give it a selective advantage include (i) shifts in Rac patterning; Rac is involved in cytoskeletal and focal adhesion rearrangement; (ii) β-catenin localisation; β-catenin is important in strengthening cell–cell adhesion by stabilizing the intracellular tail of cadherins to cytoskeletal elements; (iii) coexpression of epithelial and mesenchymal markers, such as vimentin, a well-known mesenchymal intracellular filament, and epithelial keratins. Readily shifting the scale of these (among others) would allow for a more epithelial or mesenchymal phenotype when required. EMP: epithelial-mesenchymal-plasticity, MET: mesenchymal-epithelial transition, EMT: epithelial-mesenchymal transition.

levels profoundly influences cell properties. For example, several cadherins are expressed during kidney organogenesis, including E-cadherin (cadherin-1), N-cadherin (cadherin-2), R-cadherin (cadherin-4), and K-cadherin (cadherin-6). Interestingly, E-cadherin is first detected after induction of the metanephric mesenchyme, and blocking antibodies against E-cadherin do not affect MET [72]. These findings suggest that an alternative cadherin (or other type of adhesive molecule) is responsible for the induction of the epithelial phenotype during kidney organogenesis. Notably, both K-cadherin$^{-/-}$ and R-cadherin$^{-/-}$ mice display defects in MET [73]. N-cadherin can stimulate the FGF receptor, which in turn upregulates MMP activity [74]. This has a number of effects, including cleavage of the extracellular domain of other cadherin molecules. For example, MMP-3 or MMP-9 can cleave the extracellular domain of E-cadherin [75]. Cleaved fragments

TABLE 11.1. Molecular parallels between cancer and normal EMTs*

Molecule	Cancer-related function	Normal function
Adhesion Molecules		
Cadherins	E-cadherin suppression causes EMT	Cell–cell adhesion
Integrins	Disruption of cell–matrix adhesion and signal	Cell–matrix adhesion
Extracellular Factors (and Receptors)		
IGF	Can activate Ras/Raf, PBK/Akt via RTK	Growth
HGF	Can activate PBK/Akt via RTK	Growth, motility, morphogenesis
EGF	Can activate Ras/Raf, NF-$\kappa\beta$ activity via RTK	Cell division
FGF	Can activate Src/Rac, Ras/Raf, PBK/Akt via RTK	Growth, morphogenesis, division, tissue repair, embryonic development
MMP	Break down and remodel tumor microenvironment to allow for growth and invasion	ECM breakdown, tissue remodeling
BMP	Can activate Smad signaling pathways	Cell fate, induces neural crest and other EMTs, stimulates ectopic bone growth
Jagged	Binds to and activates Notch	See Notch.
Wnt	Can block GSK3β activity	Cell fate, patterning during embryogenesis
TGFβ	Interacts with the bulk of proteins listed below	Proliferation, differentiation, has immune function
Signaling Proteins		
Smad	Can activate LEF1	Transcriptional regulators
Rho GTPase	Can activate PβK, ROCK (involved in actin stress fiber formation)	Small G protein, regulates intracellular actin dynamics
Ras/Raf	Activates MEK-ERK pathway, causes transcription of EMT promoting genes; activates PβK, MAPK	Regulating adherens junctions, focal adhesions, myosin phosphorylation, actin stress fiber formation
Src/Rac/ROS	Activate Snail	Embryonic development, cell growth
PI3K	Interact with Ras, Akt, ILK	Embryo implantation in the uterus
Notch	Activate H/Espl transcriptional regulators (e.g., Heyl/Hey2/Hesl/Hes5)	Fate determination during development
GSK3β	Block MAPK and NF-Kβ	Metabolism, neuronal cell development, body pattern formation
NF-$\kappa\beta$	Promote Snail	Important in regulating immune response to infection
MAPK	Promote Snail/Slug, Jun/Fos, Ets	Gene expression, mitosis, differentiation, apoptosis/survival
ILK	Activate Snail	Important for integrin function during development
Nuclear Regulators		
Snail/Slug	Suppress cadherin transcription including E- and N-cadherin. Protein binds E-cadherin gene regulatory E-box.	Promotes EMT in multiple systems. Associated with function of signal protein, which governs this nuclear regulator
Id	Inhibit E2A	Differentiation inhibitor. Growth, angiogenesis, and apoptosis regulation
Twist	Inhibit HOXD10 and RHOC, increasing motility. Suppress E-cadherin transcription (see Snail).	Cell identity. Promotes EMT. Mesoderm differentiation and neural crest cell migration
H/Espl (Heyl/ Hey2/Hesl/ Hes5)	TGF-β induced H/Espl = positive regulation of Snail, repress VE-cadherin; targets of H/Espl in EMT still need further identification	Embryonic segmentation, cardiac development
δEF1/ZEB1	Suppress cadherin transcription (see Snail). Target of mIR200 suppression.	Promote EMT in multiple systems. Segmental patterning and morphogenesis of many systems
SIP1/ZEB2	Suppress E-cadherin transcription	as δEF1/ZEB1
Fos	Suppress E-cadherin transcription	Regulation of angiogenesis, cell proliferation and apoptosis
LEF1	Promote vimentin, MMP	Mesenchymal gene program (co-trancription factor with β-catenin
E2A	Repress E-cadherin	Control angiogenesis, cell proliferation, and apoptosis in many cell systems.

* Classes of molecules implicated in both development and cancer EMTs are summarized. Much of the described signaling is associated with modulation of cell–cell adhesion components that allow for EMT. However, their ability to regulate other aspects of the cell (e.g., intracellular framework, ECM adhesion, activation/inhibition of other EMT molecules) can occur in concert with one another as well as with the functions listed above. The list of molecules is not complete, nor are the described signaling pathways with which they interact exclusive. There is significant crosstalk among many of the pathways described above and the molecules associated with them, as well as with a plethora of others not described here. Additionally, cellular context and the nature of signaling pathway activation/inhibition and expression will determine which one of the many functions a single molecule may have.

compete with intact cadherins for binding to adjacent cell cadherins, reducing cell–cell adhesion and promoting EMT. In neurogenesis, this is important in rearranging cells to form developing structures. In cancer, this may be a mechanism to promote metastasis, facilitating tumor cell migration away from the tumor mass. Catenins are key molecules involved in cadherin function. They modulate cadherin clustering and strength of the cadherin–actin cytoskeletal connection. In addition, $p120^{ctn}$ regulates cadherin turnover by promoting cadherin stability [76].

Although the majority of studies investigating the role of cadherins have been EMT-directed, it is likely that cadherins are involved in the development of the cohesive tumor mass at the secondary site. Cellular and tissue context plays an important role in determining the action of any given cadherin. N-cadherin has been observed to cause vigorous cell adhesion in multiple cell types, including cardiac muscle [77]. Conversely, it has been found to promote cell motility and scattering in other cell types, such as endothelial cells in blood vessels undergoing angiogenesis [78]. N-cadherin generates migratory signals through distinct pathways [79].

EMT IMPLICATIONS FOR CELL LOCOMOTION

A typical epithelium is a sheet of cells, polarized at apical and basal surfaces [10]. Mesenchymal cells generally exhibit neither regimented structure nor tight intercellular adhesion but do show some leading-edge polarity when motile. Mesenchymal migration is mechanistically different from epithelial movement, in which cells move as a sheet en bloc. Mesenchymal migration is considerably more dynamic and, at first sight, unruly. During EMT, otherwise sessile collectives of epithelial cells downregulate cell junctional machinery (adherens junctions, tight junctions, and desmosomes) and gain motility.

MESENCHYMAL AND AMOEBOID MIGRATION AND INTERCONVERTIBILITY BETWEEN MIGRATION STATES

Individual cancer cell movements have been described as either mesenchymal or amoeboid [80]. The transition between the different states is thought to occur from epithelial to mesenchymal to amoeboid [81]. These states are, to some extent, interconvertible, depending on the molecular expression of genes and the microenvironment of the cell. In any given cancer or cancer-derived cell line, there is some degree of heterogeneity, a characteristic that is beginning to be recognized and explored. When investigated at the single-cell level, it is clear that cell lines are composed of mesenchymal-like and amoeboid-like cell types [82]. It remains unclear what the relative contribution of each subpopulation is to cancer development and what the resulting implications are for cancer treatment.

During mesenchymal cell migration, a cell protrusion extends anteriorly and draws collagen fibers posteriorly, a process that couples tightly with adhesion and cell contraction in the movement cycle. Mesenchymal migration is dependent on Rac activity and protease secretion but not on ROCK-mediated actomyosin contraction. The activation of Rac by NEDD9 and DOCK3, a GEF for Rac, promotes mesenchymal-type migration. In concert with its downstream effector WAVE2, Rac also suppresses amoeboid movement by reducing actomyosin contractility [83]. In the presence of protease inhibitors, mesenchymal cells can convert to amoeboid migration and continue to invade into the matrix.

Amoeboid migration, on the other hand, requires actomyosin contractility regulated by Rho and ROCK, but proteases are dispensable owing to the ability of the cells to move through the matrix sieve by cell contraction and hydrogel propulsion [84]. Amoeboid cells suppress mesenchymal movement through Rho-kinase-mediated activation of ARH GAP22, a Rac GAP that causes Rac inactivation [83]. It is possible to convert amoeboid cells to a mesenchymal migration phenotype through expression of an activated form of cdc42 [85]. These cells now assume a more elongated morphology and require proteinases for invasion.

Although cdc42 is thought to be necessary for both amoeboid and mesenchymal migration, its downstream effectors have more specific roles. For example, DOC10, a GEF for cdc42, is necessary for amoeboid migration. Abrogation of DOC10 results in conversion from amoeboid to mesenchymal migration with reduced myosin light chain phosphorylation and increased Rac activation [85]. Its effectors NWASP and PAK also serve to maintain the amoeboid phenotype. However, blocking cdc42 suppressed the mesenchymal phenotype suggesting that although cdc42 is important for both amoeboid and mesenchymal migration, different effectors may functionally distinguish the two migration modes.

Amoeboid movement is represented by either a blebbing, contraction-mediated mode or a protrusion-centred movement found in leukocytes. Blebs occurring during migration are small, hemispherical membrane protrusions present at the cell periphery (Figure 11.4). Blebbing has been described to occur in cortical actin regions that are susceptible to breakages, allowing the outflow of cytoplasm and the extension of plasma membrane (PM) to form blebs [84, 86]. These structures are roughly 2 μm in diameter and are transient, existing for about one minute before retracting into the PM. In stimulated cells, extracelluar activation initially causes the destabilization or depolymerization of actin at a region of the PM and hydrostatic pressure, then drives cellular cytoplasm and the PM to form a bleb. The expansion of

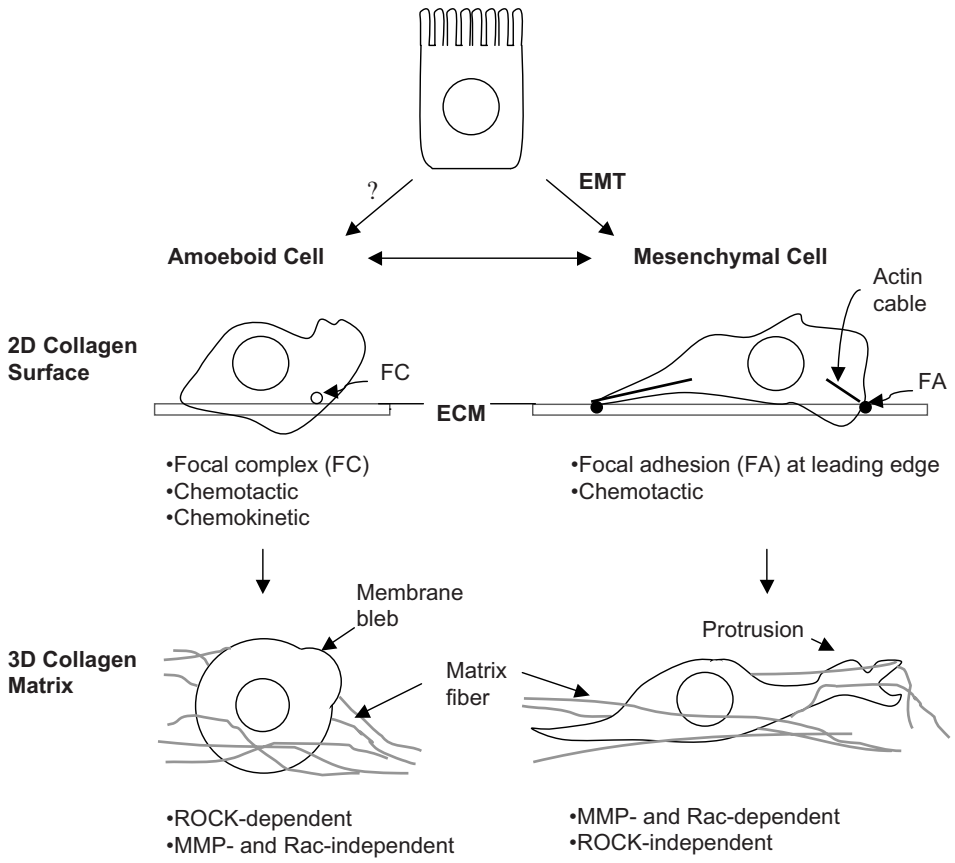

Figure 11.4. Modes of migration. Cell transformations following EMT produce amoeboid- and mesenchymal-like tumor cells that move in characteristic manners on two-dimensional and within three-dimensional environments. Amoeboid cells express transient and weak focal complexes that lie within the lamellipodium, whereas mesenchymal-like cells produce focal adhesions that make strong contact with the substratum. Amoeboid cells are efficient at both chemotaxis and chemokinesis, but mesenchymal cells move efficiently under gradient conditions only (as tested in Boyden chambers). In time-lapse imaging, amoeboid cells tend to move in random directions owing to a propensity to produce a new lamellipodium in different directions. Mesenchymal cells, on the other hand, are more persistent in moving in a single direction according to the polarity of the cell. When seeded in three-dimensional matrices and in tumors, amoeboid cells move through matrix pores by contraction of the cortical actin, a process dependent on ROCK. MMPs and Rac are not required for amoeboid three-dimensional migration. Mesenchymal cells produce MMPs that are used to digest a path through the matrix for cell movement, but cell contractility is not required.

blebs is restricted by subsequent actin polymerization. The transport of myosin to the region followed by Rho-ROCK contraction causes the retraction of blebs [86]. Although physiologically relevant in cell migration, the function of blebs is not very clear and will probably be elucidated when more is known about how blebs are formed and inhibited.

When migration of neural crest cells was first studied in vitro, it was noted that the initial movements involved vigorous surface blebbing that later resolved into a more rapid mesenchymal mode of locomotion. In vivo, blebbing migration is less evidenced but it has been described by Trinkaus in the epiboly movement in early development of the fish *Fundulus* [87].

Leukocytes demonstrate a version of amoeboid migration that differs from the blebbing, contractility-focused movement of cancer cells. Cell contraction is used to propel the nucleus forward, but when the mechanism is impaired through myosin inhibition, the cells continue to navigate through collagen matrices, albeit at slower rates. When the collagen gel concentration is lowered to increase the matrix pore size, the myosin-inhibited cells are able reach the same instantaneous velocity peak value as control cells. In contrast, the actin inhibitor latrunculin B, which interferes with the formation of cell protrusions, significantly reduces the speed of movement irrespective of matrix concentrations. This suggests that the primary mechanism

that determines migration speed is protrusive movement, but under restrictive conditions, in which the cells are unable to push through collagen by protrusive forces alone, cell contraction supplements migration [88]. Furthermore, this movement occurs independently of integrins in three-dimensional gels – this again differs from mesenchymal migration of fibroblasts and mesenchymal cancer cells that depend on protrusion-mediated mechanisms. In leukocytes, the cell protrusions project forward without exerting pulling forces on the matrix, and the cell contractions at the rear cell body occur in irregular patterns. During cell protrusion, the cell body is passively pulled forward, and migration appears to consist of phases of protrusion and contraction that are temporally and spatially unsynchronized [88].

MESENCHYMAL AND AMOEBOID MIGRATION AND HETEROGENEITY OF CANCER CELLS

During EMT, autocrine signals in cells can cause chemokinetic behavior (increased random movement), resulting in the movement of tumor cells away from the primary tumor [89]. Once in the circulation, tumor cells can disperse to specific organs, a process known as *homing*, in which chemotaxis (directional movement) plays an important role [90]. *Chemokinesis* is defined as cell motility in the presence of globally distributed soluble factors involving a change in speed or in the frequency or magnitude of turning behavior. *Chemotaxis* is the motility of cells or organisms in which the direction of movement is determined by the gradient of diffusible factors. Kohn et al. [91] have shown that cancer cells demonstrate both chemotaxis and chemokinesis in response to growth factors.

Cell migration is generally evaluated using two methods. The first uses Boyden chambers that allow endpoint assessment of motility, and the second uses live-cell imaging to record the dynamic movement of cells over time [92–95]. In Boyden chambers, cells are seeded into a top well separated by a porous membrane from a bottom well, which contains growth factors. A gradient of growth factors is established across the membrane that stimulates cell migration through the pores from the top to the bottom well (chemotaxis). The pore size is set to require active migration (8–12 μm). When there is a lack of a gradient across the membrane – for example, when growth factors occur in equal concentrations – the migration of cells into the lower chamber is by chemokinetic means.

In heterogenous cancer cells, two subpopulations demonstrating different migration properties were isolated using Boyden chambers set up for either chemotaxis (with a gradient) or chemokinesis (without a gradient) [82]. Cells that migrated into the lower chambers were collected and propagated. When tested for migration, interestingly, cells isolated under chemokinetic conditions (KINE cells) demonstrated both chemokinetic and chemotactic abilities to the same efficiency as the original cell population. The cells isolated under chemotactic conditions (Con cells) were only chemotactic and not chemokinetic. In invasion assays, KINE cells were found to be significantly more invasive than Con cells.

The properties of the subpopulations were further characterised using live-cell imaging assays. When globally stimulated with serum, the KINE cells moved randomly, whereas the Con cells moved more persistently in one direction. These observations are consistent with ideas that random-moving cells are less polarized and that directionally moving cells have a more polarized front-to-back architecture. To understand the polarity of the cell in terms of the relationship between the internal actin cytoarchitecture and the external adhesion sites, co-staining experiments for actin and for adhesion sites using phalloidin conjugates and paxillin antibodies were performed. Actin staining in the KINE cells demonstrated a more rounded morphology, with extensive membrane ruffling at the edge of cells with few stress fibers. Con cells appeared more polarized, with distinctive front and trailing ends. Adhesion sites were not prominent in KINE cells and had the appearance of more transient focal complexes that occur inside the lamellipodium. Con cells, however, were anchored by focal adhesions at the edge of the cells; these adhesion sites were also found where actin stress fibers terminate (Figure 11.4).

The results collectively suggest that the two subpopulations of lung cancer cells known to be chemokinetic and chemotactic also, respectively, demonstrate amoeboid- and mesenchymal-like characteristics in terms of cell polarity, adhesion, morphology, and two-dimensional migration (Figure 11.4). For the first time, data from two different methods of studying cell migration – Boyden chambers and live-cell imaging – can be reconciled. More important, this recognition of the two migration modes has provided important insight into how the variable nature of cells might impact on the weight given to the different methods of study.

There are also important implications in terms of cancer etiology and treatment. For example, in heterogenous populations, treatment with MMP inhibitors will render mesenchymal cells more amoeboid-like in their behavior. Inhibition with ROCK inhibitors will block amoeboid cell migration but not mesenchymal migration. For most tumors, the cellular composition is heterogeneous. This factor is important when selecting the appropriate treatment regime to counter migration and metastasis and suggests that we need to inhibit both modes of migration simultaneously.

CURRENT STUDY SYSTEMS FOR EMT AND MIGRATION

Neural Crest Migration

The molecular control analysis of neural crest EMT and migration was initiated when it was demonstrated that many of the principal in vivo features were replicated in vitro in two-dimensional cultures (methodological review [96]). From these cultures, it was found that EMT and cell locomotion could be triggered by transient cadherin inactivation and also by cytoskeletal manipulation via inhibition of aPKC or Rho and ROCK. The dependency of migration paths on particular adhesively favorable substrates, especially the ECM molecule fibronectin, was also demonstrated. A battery of integrin receptors mediated this, and the ECM adhesive repertoire was seen to be diverse and complex, leading to the idea that the cells were equipped to make sophisticated migratory changes in response to a variegated ECM microenvironment. The "stripes" assay was developed to test this multisubstrate choice paradigm. In addition, the converse guidance mechanism of repulsion via exclusion zones was realized as a plank now underlying all developmental cell and axon migration studies, and the first ECM repulsive molecule was identified. The driving of locomotion by cell–cell contact (the obverse of "contact inhibition of locomotion") was also described for neural crest cells. Simultaneously, the growth factor signaling and transcription factor expressions that make a neuroepithelial cell into a neural crest cell, determine its EMT, and guide its migration have been revealed. Many of these observations in vitro have now been confirmed in vivo with modern imaging systems and by molecular genetic perturbation techniques (e.g., [97]), and the complexity of molecular controls is being revealed in vivo (see [98, 99]).

The 13762NF System

To examine the metastasis mechanism of amoeboid and mesenchymal cells in culture, we and others have used cells lines that have been isolated from a primary 13762NF mammary adenocarinoma (MTC, mesenchymal) and its lung metastasis (MTLn3, amoeboid) in the same animal [100]. MTLn3 cells retain a high metastatic potential, whereas MTC cells have low metastatic potential. When injected into the mammary fat pads of Fisher 344 rats, MTLn3 cells produced metastases in all axillary lymph nodes and the lungs within four weeks, whereas MTC injection showed metastasis after five weeks in the ipsilateral lymph nodes [101]. The metastatic differential is not caused by the doubling time, as both cell lines have identical doubling time of fourteen hours in culture. The differences in metastatic levels are more likely the result of the different expression levels of EGF receptors. MTLn3 cells express 55,000 receptors per cell, whereas MTC cells do not express EGF receptors [102]. MTC cells engineered to express the same number of (human) EGF receptors as in MTLn3 cells were more chemotactic and showed a higher lung-colonizing ability than parental MTC cells [103]. Thus, amoeboid MTLn3 cells and mesenchymal MTC cells derived from the same primary tumor exhibit very different behaviors in terms of metastatic potential and represent an important study model both in vitro and in vivo. The epithelial/mesenchymal status of these cells has not been reported and is under study in our laboratories.

The PMC42 Model

We have developed a novel model system for EGF-induced EMT studies in the human breast cancer cell line PMC42 [26, 63, 104, 105]. Parental PMC42 cells were shown to have stem like qualities when first characterized [106–109]. They appear mesenchymal (100% vimentin-positive) and respond to EGF with more extreme EMT [26]. An epithelial subline developed by Leigh Ackland of Deakin University in Melbourne forms acini-like structures in three-dimensional Matrigel culture; these structures produce milk proteins in response to lactogenic hormone [110] and elaborate myoepithelial markers in peripheral cells when grown as three-dimensional clusters in Matrigel [104, 105]. Stimulation of PMC42-LA cells with EGF also leads to EMT marker expression [26], and three-dimensional Matrigel cultures of PMC42-LA show increased expression of these markers when treated with factors selectively secreted by breast carcinoma-associated fibroblasts over normal mammary fibroblasts [104]. Thus, the PMC42 system provides a spectrum of EMT progression stages on a background of BCSC behavior. We have examined the PMC42 parental cells and found the presence of mesenchymal- and amoeboid-like subpopulations, the latter being in the minority, as expected [16, 26]. Both morphological types invaded efficiently into a three-dimensional collagen matrix, albeit at faster rates for the rounded (ameboid) cells. Within the same experimental culture, the mesenchymal-like and amoeboid cells invaded in a collective and individual manner, respectively (unpublished data).

The MDA-MB-468 Model

MDA-MB-468 cells have a "Basal A" phenotype, suggesting mixed luminal/basal attributes [34], and were recently reported to undergo EGF-regulated EMT [111]. Although predominantly epithelial and E-cadherin-positive [35], a low percentage of cells in culture show vimentin positivity (~5%), and the cells display intermediate invasiveness [37, 38]. Spontaneous

lung micrometastases have been reported from MDA-MB-468 (Figure 11.1 and [112]), and GFP-tagged MDA-MB-468 cells isolated from a lung micrometastasis (468LN cells) showed evidence of EMT (spindle-like morphology, increased growth) [112], although their in vivo morphology as primary tumors resemble the parental MDA-468-GFP cells [112]. DNA methylation changes in genes related to EMT and cell migration were recently demonstrated in the 468LN cells [113].

The TSU-Pr1/T24 Model

We have developed an increasingly metastatic bladder cancer progression series using the human TSU-Pr1 (T24) bladder carcinoma cell line. TSU-Pr1 sublines were selected for enhanced bone metastases following intracardiac inoculation and have been previously described [61]. There is a significant increase in the formation of metastatic deposits in a number of tissues from the parental cell line (TSU-Pr1) to TSU-Pr1-B1 (B1) through to the most aggressive cell line TSU-Pr1-B2 (B2). Increased metastasis following systemic inoculation was associated with both phenotypic (decreased in vitro migration, invasion, colony formation) and molecular (increased epithelial cytokeratins, cadherins and membrane-associated β-catenin, decreased vimentin, actin cytoskeleton) – hallmarks of a MET.

CONCLUSION

In early work following the identification of EMT as a transforming event in carcinogenesis, EMT was studied and described as an endpoint function. As we probed further, it has become apparent that the transitions are neither as complete nor as permanent as first thought. During development, examples abound of fluidity in transitional states that are part of a cell's normal repertoire of responses to its microenvironment.

In cancer biology, what is currently deemed as unusual, such as the hybrid cells exhibiting both epithelial and mesenchymal markers, is perhaps quite the norm. Heterogeneity in cancer cell populations in vivo and in vitro is a reality that can be partly explained by the presence of cancer stem cells, the penultimate super cells that continually produce a variety of cell types ranging from epithelial- to mesenchymal- and amoeboid-like. When transplanted from two-dimensional to three-dimensional environments or in animal models, these cells can maintain their respective states, but under some circumstances, they switch from mesenchymal to epithelial (MET) or from mesenchymal- to amoeboid-like (MAT). During epithelial sheet migration or cohort migration in three-dimensional matrices, the cells at the leading edge adopt mesenchymal-like characteristics, whereas the attached cells trailing behind remain epithelial-like.

What these paradigms mean to cancer development and treatment is a new appreciation of the chameleonic nature of cancer cells that will drive the derivation of new ways to target this plasticity. The mechanisms facilitating progression from benign to invasive, and finally to metastatic carcinoma, remain largely elusive. A greater understanding of these mechanisms holds much therapeutic and diagnostic promise

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12 Apoptosis, Anoikis, and Senescence

Wen Liu and Kounosuke Watabe

APOPTOSIS: A CRITICAL PLAYER IN TUMOR PROGRESSION

In the early 1970s, pioneering work of Fidler and colleagues demonstrated that tumor metastasis is an extremely inefficient process, and fewer than 0.01 percent of tumor cells shed in the circulation system are able to survive for the following metastatic colonization at distant organs [1]. The majority of tumor cells that depart from the primary cancer mass die by encountering the body's natural defense barriers, which include induction of apoptosis and senescence and immunosurveillance. Even the tumor cells that survive and reach the secondary organ often become dormant or senescent, and their growth is significantly limited owing to the condition of the microenvironment at the organ sites.

Apoptosis is the most common form of programmed cell death in vertebrates and has been extensively studied over the past decade; it is commonly considered as an important mechanism that negatively regulates cancer development. Recent evidence strongly supports the notion that the apoptosis mechanism also serves as a safeguard system that prevents the dissemination of malignant cells and metastasis. Inbal and his group, by using lung carcinoma clones, have shown that the inhibition of the expression of DAPK, a positive mediator of apoptosis, favored the metastatic process [2]. On the other hand, Del Bufalo et al. demonstrated that overexpression of the antiapoptotic oncoprotein, Bcl-2, significantly promoted the metastatic potential of human breast cancer cells [3]. Furthermore, elegant work done by Glinsky et al. demonstrated an inverse correlation between apoptotic propensity and the metastatic potential of the cells in a murine model [4]. Therefore, the metastatic potential of tumors is associated with an increased resistance to apoptosis, and the key anti- or proapoptotic factors have significant impact on the overall metastatic efficiency of tumor cells.

It has been well established that apoptosis is one of the critical determinant factors that regulate metastasis efficiency mainly at three crucial steps in the metastasis cascade (Figure 12.1). First, when primary cancer cells detach from the extracellular matrix (ECM), a specialized form of apoptosis called *anoikis* is triggered. During this process, another form of apoptosis, amorphosis, is also activated when detachment of the tumor cell disrupts the cell–cell anchors, which eventually leads to the loss of cytoskeletal architecture. Both these events are naturally designed to restrict the correct anatomical distribution of multicellular organisms by eliminating those hostile invading cells, which on some level are like the metastatic cancer cells. Therefore, metastatic cells must be resistant to anoikis and amorphosis to accomplish the initial steps of metastasis.

Second, as tumor cells enter into the bloodstream via the process of intravasation, the death of solitary cells occurs with high frequency owing to host immune surveillance or destruction of tumor cells by mechanical stresses. However, tumor cells develop several strategies to resist the immunosurveillance-induced apoptosis, including overexpression of anti-apoptotic molecules (Bcl-2, $FLIP_{L/S,}$ survivin) and direct interference with the perforin/granzyme cytotoxic pathway.

Finally, the survival at the secondary organs for tumor cells via the formation of micrometastases called *colonization* is the most difficult step of all for the tumor cells; they are particularly vulnerable to apoptosis at this stage. Successful colonization requires cooperation between tumor cells and the host tissue stroma. The tumor cells at the secondary site induce permissive signals that might modify the host stroma cells, which in turn secrete factors (growth factors, chemokine, and protease) favorable to tumor cell survival and growth. Therefore, this mutual interaction of stromal–tumor cells plays a determined role in the fate of metastatic tumor cells to die or to progress into colonization.

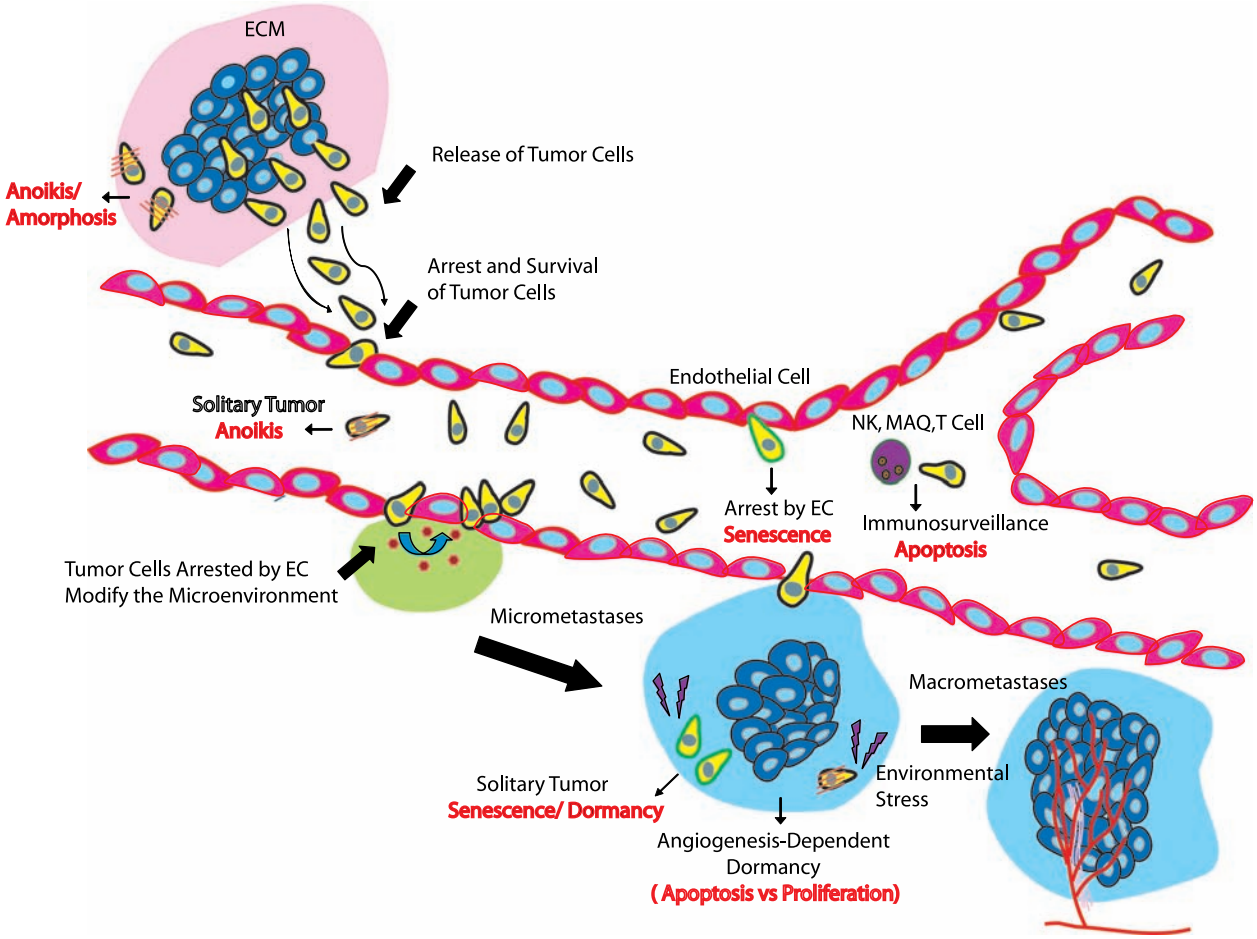

Figure 12.1. Apoptosis, anoikis, and senescence in the metastasis cascade.

Another obstacle to obtain a favorable microenvironment for tumor cells is an acquisition of angiogenic ability. A failure to induce sufficient angiogenesis leads to cell senescence or a formation of dormancy because of the balance between apoptosis and proliferation.

PRO- AND ANTIAPOPTOTIC FACTORS INVOLVED IN METASTATIC PROCESS

Apoptosis holds such important physiological roles in tissue homeostasis and development that the apoptotic process ought to be tightly regulated [6]. Usually, cell death by apoptosis is controlled by a series of factors that monitor both the external and internal cell environments, which eventually lead to the activation of a specific family of cysteine proteases called *caspases* [7]. Two major and separated pathways have been described so far: the extrinsic (death receptor) pathway initiated by the death receptors CD95 (also known as Fas) or members of the tumor necrosis factor (TNF) receptors superfamily, and the intrinsic (mitochondria) pathway that responds to cell stress induced by nutrient deprivation, loss of cell–cell contact, and DNA damage caused by chemotherapy or radiation treatment (Figure 12.2). Although the two pathways are initiated by different stimuli, and proceed via different initial activators, there is crosstalk between the extrinsic and intrinsic pathways. Many regulators, including Bcl-2 family proteins, p53, Ras, and inhibitor of apoptosis proteins (IAPs), are involved in the two caspase-dependent apoptosis pathways. Mutations, loss, or alterations in these apoptosis regulators have been shown to affect tumor progression and metastasis.

Bcl-2 family proteins play a privotal role in the regulation of the intrinsic pathway by regulating outer mitochondrial membrane permeabilization in both antiapoptotic (Bcl-2, Bcl-xL, and Bcl-w) and proapoptotic (Bax and BH3-only) manners [8, 9]. Several lines of evidence indicate that higher resistance to apoptosis of metastatic cancer cells is associated with more mutations or dysfunction of these apoptosis regulators. Increased expression of Bcl-2, and particularly Bcl-xL, as well as decreased level of the proapoptotic gene, Bax, are associated with apoptosis resistance of

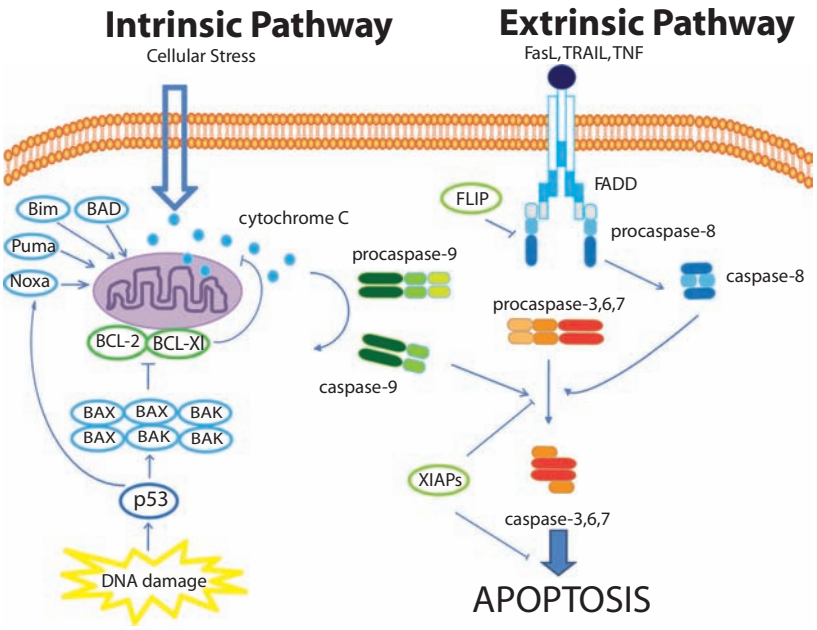

Figure 12.2. The intrinsic and extrinsic apoptotic pathways.

highly metastatic MDA-MB-435 human carcinoma cells compared with the poorly metastatic MDA-MB-468 cell line [10].

Furthermore, endogenous Bcl-2 overexpression has been shown to be correlated with the progression of several human and murine cancer cells to a metastatic phenotype [11, 12]. Whether the imbalance of expression of these apoptosis regulatory proteins also accounts for the increased resistance in apoptosis for other highly metastatic cancer cells still needs to be clarified; however, an intriguing question is whether expression of these apoptosis inhibitors decreases sensitivity to apoptotic stimuli in the metastatic process. It has been suggested that a decrease in the apoptotic susceptibility would benefit the metastatic tumor cells to overcome those "vulnerable" points (as will be discussed later). Indeed, several studies have shown that experimental modulation of Bcl-2s influenced overall metastatic efficiency [13, 14]. For example, ectopic expression of Bcl-2 in breast cancer cells induced a significantly increased number of lung metastases when they were injected either intravenously or intramuscularly into nude mice [3].

Interestingly, Martin et al. have shown that overexpression of Bcl-2 could rescue immortalized mammary epithelial cells from anoikis and amorphosis, thus enhancing the metastatic ability without affecting primary tumor growth, cell motility, or invasiveness [15, 16]. In line with these observations, this group also identified Bcl-xl as the main suppressor for cytoskeleton-dependent cell death in amorphosis-resistant tumor cells, and an enhancer of metastasis [17]. Moreover, it has been found that overexpression of Bcl-xL correlates with increased nodal involvement and a more aggressive tumor in patients with breast cancer [18]. Furthermore, Bcl-xL has been shown to increase the formation of distant metastasis without affecting primary tumor formation [19]. On the other hand, maspin, a member of the serpin family with unique metastasis-suppressing activity, has recently been found to act as an inhibitor against the growth of primary tumor and metastasis by modulating Bcl-2 family proteins [20, 21]. Therefore, Bcl-2s may serve as a potential therapeutic target for metastatic disease, although the exact molecular mechanism by which Bcl-2s block metastasis still needs to be clarified.

The tumor suppressor p53 can stimulate the transcription of specific members of the Bcl-2 family, such as BAX and the BH3-only proteins BAD (Bcl-2 antagonist of cell death), Noxa, and Puma, which results in the release of apoptosis inducers such as cytochrome C and Smac [22–24]. Although mutation or loss of p53 is common in more than 60 percent of human primary tumors, to what extent p53 mutations play a role in progression toward a metastatic phenotype is still undetermined [25]. However, because p53 serves as both apoptotic regulator and tumor suppressor, loss of p53 gives advantage to increase the probability of metastasis through its overall protection from apoptosis. Clinically, loss of p53 function has been found to correspond with increased metastasis, recurrent disease, and poor survival [26, 27]. In a recent study of 1196 gastric cancer samples, it was found that early gastric cancers that exhibited low levels of apoptosis, and increased levels of Bcl-2 and p53 mutation, were more likely to metastasize [28]. Furthermore, Nikiforov and colleagues have shown that p53 inactivation facilitated experimental metastasis by promoting survival of tumor cells within the circulation [29]. The release of nitric oxide (NO) is believed to be a natural defense against the formation of metastases as it clears metastatic tumor cells from the circulation by induction of apoptosis [30–32]. However, tumor cells harboring p53 mutations have been shown to be resistant to NO [33–35], although it is still unclear how exactly p53 induces apoptosis of tumor cells in the circulation system.

IAPs are a newly discovered class of proteins that directly inhibit caspase 3, the final effector caspase for both intrinsic and extrinsic apoptotic pathways, and thus are considered to have unique potential as therapeutic targets [36, 37]. IAPs include the caspase

inhibitors X-linked inhibitor of apoptosis (XIAP) and survivin (SVV), both of which are overexpressed in many types of cancers. SVV is particularly correlated with aggressive cancers, poor prognosis, frequent recurrence rates, and increased resistance to therapies, suggesting that it may play a role in metastasis [38–40]. An increase in IAP expression appears to facilitate metastasis by decreasing the susceptibility of tumor cells to apoptotic stimuli, which may favor not only survival but also other events, such as invasion and angiogenesis, in the metastatic process. It has been recently shown that overexpression of XIAP contributes to anoikis resistance of the metastatic human prostate carcinoma cells in the circulation [41]. Similarly, several recent studies demonstrated that SVV provides invading tumor cells with an enhanced survival capability in response to the cytokine/growth factors, adhesion molecules, and proteinases [42]. Interestingly, the expressions of XIAP and SVV have also been found to be increased by vascular endothelial growth factor (VEGF), which in turn facilitates metastasis through induction of angiogenesis [43, 44]. Therefore, these factors are implicated in angiogenesis and may serve as potential therapeutic targets for metastatic diseases.

Ras is a small GTP-binding protein that plays important roles in signal transduction for proliferation, apoptosis, cytoskeletal organization, and other key biological processes. Members of the Ras family include H-ras, N-ras, and K-ras, and their viral homologs (v-ras) [45]. Generally, members of the Ras family promote cell survival by inhibiting apoptosis through the PI3K/Akt pathway and by downregulating JNK and p38 [46]. How Ras modulates apoptosis in metastasis is not completely understood; however, one possibility is by increasing the overall apoptosis resistance of metastatic tumor cells. It was also speculated that Ras may function at the stage of micrometastasis formation by affecting the proliferation and apoptosis signalings. Varghese et al. have demonstrated that, as expected, mutant H-ras led to increased metastatic potential by shifting the balance of apoptosis/proliferation in favor of proliferation during early micrometastasis development [47]. This idea was further confirmed by Liao and colleagues, who showed that H-ras antisense therapy led to decrease in lung metastasis and increase in the rate of circulating apoptotic cancer cell death. Similarly, a dominant negative form of c-K-ras effectively reduced the number of metastatic colonies in pancreatic liver metastasis [48, 49]. Ras is also known to block the ability of TGF-β in suppressing tumor growth [50, 51]. On the other hand, activated Ras and TGF-β act cooperatively to cause metastasis in a polarized H-ras transformed mammary epithelial cell model [52]. Therefore, it appears that Ras can increase metastatic efficiency by directly modulating apoptotic pathway and also by influencing other factors, such as TGF-β.

APOPTOSIS-SENSITIVE STEPS DETERMINE VULNERABILITY OF METASTATIC CELLS

Metastasis is an "all-or-nothing" process at the cellular level; the metastatic efficiency depends on the ability of individual cancer cells to proceed successfully through each of the sequential steps in the metastatic process. However, most cancer cells, even in a highly metastatic population, fail to complete the metastatic process in the clinical setting, and apoptosis is the major mechanism contributing to the elimination of cancer cells at various steps of the metastatic process. Therefore, gaining survival advantages by decreasing sensitivity to apoptotic stimuli is the common strategy by which cancer cells secure themselves once they escape from the primary site and enter into the metastasis cascade.

Years of quantitative studies on the efficiency of the metastatic process using in vivo videomicroscopy and "cell accounting" techniques have gradually revealed that the "rate-limiting" steps of the metastasis process are indeed vulnerable to apoptotic cell loss. Surprisingly, a series of experimental studies by Ann Chambers's group have demonstrated that the initial steps in hematogenous metastasis, including shedding cancer cells into the vascular system, survival in the circulation, and survival of initial arrest in the microcirculation, are completed with reasonable efficiency and that the majority of cells are not lost in these early steps of the metastatic process [53–55]. For example, it was found that more than 85 percent of B16F1 melanoma cells that were injected via the mesenteric vein were present in the liver ninety minutes after the injection, and more than 80 percent of the cells were still present in the liver even three days after the injection [53]. Similar results were obtained later by Cameron et al. using B16F10 melanoma cells. They demonstrated that 98 percent of the cells injected iv were present hour after injection, and 83 percent and 73 percent of the cells were still present in the lungs after one and three days of injection, respectively [54].

Similar high rates of initial survival of tumor cells were also observed in a mammary carcinoma model, with more than 80 percent of injected cells surviving at three days after injection, for both the highly metastatic D2A1 cell line and the related poorly metastatic D2.0R cell line [55]. Evidence from in vivo videomicroscopy also indicates that the interaction between cancer cells and endothelial cells of the microcirculation is an important determinant of metastatic growth and that apoptosis associated with the release of reactive oxygen species (NO, O_2^-, and H_2O_2) by endothelial cells contributes to the elimination of metastatic cancer cell at an early stage [56, 57].

It has been shown that the arrest of cancer cells in the pulmonary microcirculation induced local release of NO and caused the cancer cells to undergo apoptosis

followed by inhibition of pulmonary metastases. However, because the majority of cells are not lost at this stage, the metastatic burden seems to be determined by the proportion of cells that are subsequently lost at secondary sites. In the same study mentioned earlier, Luzzi et al. found that fewer than 20 percent of B16F1 melanoma cells targeted to the liver were lost within three days of injection, whereas two-thirds of the originally injected cells were lost by day 13 [53]. Furthermore, it was found that only one in forty solitary B16F1 cells in the liver began to form micrometastases, and that one in 100 of these micrometastases successfully forms macroscopic metastases. Apoptosis of tumor cells during these stages of metastasis was also found to be responsible for the loss of a large proportion of cells.

In another study, it was found that the peak of apoptosis occurs at times of loss of solitary metastatic tumor cells and loss of micrometastasis [55]. Metastatic progression from micrometastasis to macroscopic metastasis depends on two factors: cancer cell growth and blood supply formation via the critical angiogenic process. Failure to meet either of these requirements leads to a formation of "dormant" micrometastasis, in which apoptosis and proliferation are balanced following no net growth of tumor. Therefore, activation of the "dormant tumor" is likely to be linked to the mechanism that breaks the balance of apoptosis and proliferation in the tumor cell population. Questions then arise, such as: What is the smarter choice for dormant tumor cells, enhancing proliferation or decreasing apoptosis? Several studies have shown that cells exiting from dormancy and progressing to grow may do so by decreasing in apoptosis rather than increasing in proliferation [58–60]. Evidence from the study on angiogenesis inhibitors that control metastatic growth also indicates the critical role of increasing apoptosis at this stage. Taken together, the apoptotic cell loss contributes to overall metastatic inefficiency by controlling the vulnerable steps of metastases, including arrest in the microcirculation and interaction with endothelial cells, survival of solitary cells at secondary sites, initiation of micrometastatic growth, and the completion of macrometastatic growth.

ANOIKIS PLAYS A MAJOR ROLE IN TUMOR METASTASIS

ECM is a dynamic and complex network of collagens, laminin, fibronectin, and proteoglycans, which provides cells with anchorage and signaling for cell proliferation, migration, differentiation, and survival [61] (Figure 12.3). Disruption of the cell–matrix interactions can trigger anoikis, also termed ECM-dependent apoptosis, both in vitro and in vivo [62, 63]. Therefore, metastatic tumor cells must overcome this crucial step to survive in the absence of normal ECM components, because tumor cells intravasated into the circulation and extravasated to secondary organs are either deprived of matrix or exposed to foreign matrix components.

The results of a series of experiments indicate that anoikis resistance of tumor cells results in an increase in their metastatic potential. Epithelial–mesenchymal transition (EMT) is considered to be an initial step of tumor metastasis; the primary tumor cells readjust their interactions with the ECM and acquire the ability to evade anoikis. During this process, many genes related to anoikis become deregulated in the tumor cells. These genes include growth factors such as TGF-β and insulin-like growth factor (IGF)-1, transcription factors such as Smads and Snail, cell adhesion molecules to the ECM (integrins, CD44, focal adhesion kinase [FAK] and ECM proteins), cell-to-cell adhesion molecules (E-cadherin), and extracellular protease matrix metalloproteinases (MMPs) and caveolin. In particular, neurotrophic receptor TrkB, galectin-3, and caveolin are key suppressive mediators of anoikis; therefore, they are considered to be potential therapeutic targets for tumor metastasis [64–68].

TrkB receptor tyrosine kinase and its ligand, brain-derived neurotrophic factor (BDNF), are essential for the development of the nervous system and activate critical signaling for proliferation, differentiation, and survival [69]. TrkB was initially found to be overexpressed in neuroblastoma, the most common solid tumor in childhood, and was considered to contribute to the metastatic phenotype. Overexpression of TrkB has been shown to protect neuroblastoma cells from antitumor agent–induced apoptosis in vitro and promote tumor cells to disseminate and invade [70–75].

The high level of TrkB expression has also been observed in many other human malignancies and is often associated with increased metastatic potential [76]. Recent elegant genetic studies revealed that TrkB can confer anoikis resistance and subsequent survival advantage on tumor cells in the circulation and at distant sites [77, 78]. Therefore, TrkB is considered to be a key suppressor of anoikis for metastatic cells, and several preclinical studies with Trk inhibitors suggest their negative effects on tumor growth and metastasis [79]. TrkB can thus be a potential therapeutic target for metastatic disease. However, several key questions still remain to be answered as to whether TrkB overexpression is sufficient to drive metastatic tumor cells survival in the circulation and whether TrkB activity is essential to maintain the metastatic phenotype.

Galectin-3, a member of the β-galactoside–binding proteins, has recently been found to be broadly overexpressed in malignant epithelial and tumor-associated stromal cells [80]. Circulating levels of galectin-3 have also been correlated with metastatic potential in many

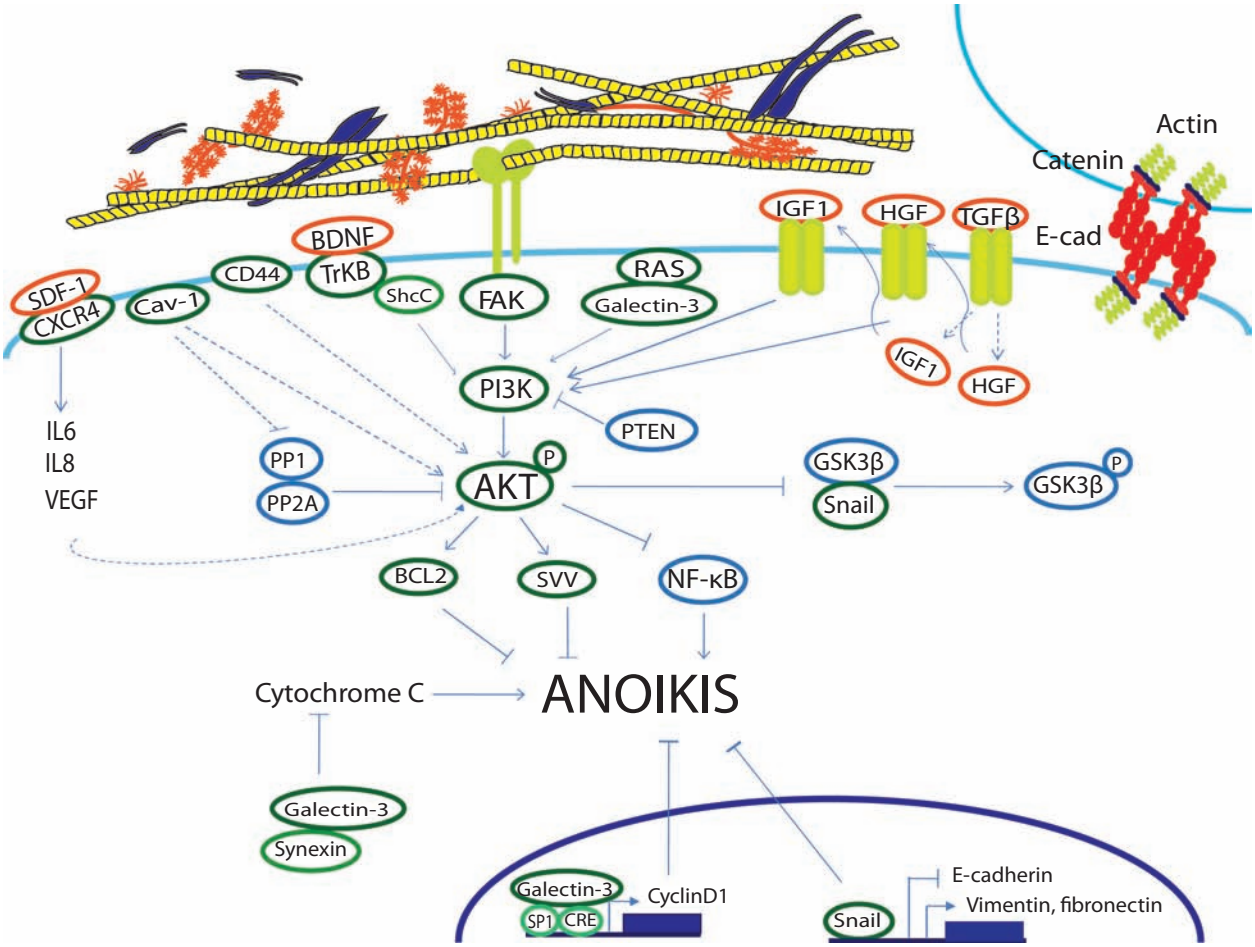

Figure 12.3. Mechanistic link of anoikis resistance to tumor cell metastasis.

malignancies, including breast, gastrointestinal, lung, ovarian cancer, melanoma, and Hodgkin lymphoma [81]. Notably, overexpression of galectin-3 protects tumor cells from anoikis and other apoptotic stimuli such as NO, which is believed to confer advantage to tumor cells during dissemination through circulation [82].

How galectin-3 promotes the anoikis resistance of tumor cells is not well understood. However, the similarity of galectin-3 to lectin leads us to speculate that galectin-3-expressing tumor cells may have some advantage to survive during metastatic process by regulating cell–matrix interaction to "restore" the anchorage between tumor cells and ECM. Indeed, overexpression of galectin-3 in breast cancer cell lines increased cell adhesion to laminin, fibronectin, and vitronectin. Galectin-3 was also shown to be able to increase the expression of some integrins such as $\alpha 6\beta 1$, which is known to be associated with tumor invasion. However, how the galactin-3–mediated cell–ECM interaction modulates cellular signal is yet to be clarified [83, 84].

In addition to the enhancement of anoikis resistance, galectin-3 has been shown to promote metastatic potential in several other steps. For example, the ability of tumor cells to aggregate and form the embolus in microcapillaries helps their survival and arrest by endothelium followed by extravasation at secondary sites [85, 86]. Galectin-3 on tumor cell surfaces has been shown to induce the embolism of metastatic tumor cells through the formation of homotypic aggregation [87, 88]. On the other hand, galectin-3 is also expressed on endothelial cell surfaces and has been suggested to play a role in docking cancer cells onto endothelium and following extravasation [89–91].

Interestingly, the function of galectin-3 appears to be dependent on its localization. Cytoplasmic galectin-3 strongly suppresses anoikis, whereas the nuclear galectin-3 can exert proapoptotic properties [92]. The intracellular galectin-3 generally functions as an antiapoptotic factor; however, the role of extracellular galectin-3 is more complicated, as it works as both an antiapoptotic and a proapoptotic factor. Consistently, several clinical studies have also shown decreased levels of galectin-3 in breast, ovarian, and prostate cancers, suggesting that galectin-3 may have different functions depending on the stage of tumor and cellular context.

Notably, galectin-3 null mice have shown to have no particular phenotype and they are relatively healthy, suggesting that galectin-3 inhibitors may be therapeutically valuable without causing severe side effects.

Caveolin (Cav-1) is an essential component of cellular membrane vesicular structure caveolae and has been implicated in a variety of cellular processes via regulation of multiple signal transductions [92]. Because of the genomic location of Cav-1 on chromosome 7q31.1 (a suspected tumor suppressor region), it was originally identified as a tumor suppressor, and extensive studies in mammary tumor mice models support this notion [92, 93]. Cav-1 has been shown to have proapoptotic activity in various cell types, although it works as an antiapoptotic factor in prostate cancer cells. Recent evidence linked Cav-1 to prostate cancer metastasis and suggested that Cav-1 is able to suppress anoikis by activating the Akt pathway and also by blocking two serine/threonine protein phosphatases, PP1 and PP2A [94]. On the other hand, Cav-1 is also known to be involved in insulin and IGF-1 signaling, which mediates matrix-independent cell survival, a well-studied mechanism for anoiksis resistance [95]. Clinically, Cav-1 has been shown to be expressed at high levels in several types of cancer including prostate, bladder, and esophageal cancers, T-cell leukemia, and multiple myeloma, whereas it is expressed at a lower level in some other types of tumors, including breast, cervical, and ovarian cancers and small-cell lung cancer (SCLC) [92].

What is the role of Cav-1 in tumor development and progression? Is it a tumor suppressor or oncogene? Is it possible that it works as a negative factor in metastatic diseases while it functions as a promoter for primary tumor growth? Answers to these questions may not be straightforward, because the negative role of Cav-1 in metastasis is also challenged by the finding that downregulation of Cav-1 decreased the expression of E-cadherin and increased Snail and β-catenin expression, which resulted in promoting EMT and increasing invasiveness of neoplastic cells [96]. Because of the heterogeneity of Cav-1 expression in different tumors, targeting Cav-1 for therapeutic purpose still needs further clarification of its specific role in each tumor type.

CELL SENESCENCE SUPPRESSES TUMOR METASTASIS

Cellular senescence limits the proliferative capacity of damaged cells and thereby acts as an intrinsic mechanism of tumor suppression. It is known that the majority of intravasated tumor cells die in circulation and only a fraction of those cells that survive reach the secondary organs. Some cells successfully colonize and start to grow; however, other cells enter into senescence and become nonproliferative dormant cells that are clinically undetectable and are associated with cancer recurrence. Whereas the clinical evidence for existence of dormant tumor cell populations in patients continues to grow, the underlying mechanisms that are involved in the induction, maintenance, and escape from senescence or dormancy are not well characterized. Why are tumor cells that are equipped with the proper genetic and epigenetic alterations for initial tumor growth unable to resume growth at secondary sites? What environmental factors affect the decision for disseminated tumor cells to proliferate or to become senescent? What is the signal switch to determine the fate of the disseminated tumor cell? Recent studies are gradually shedding new light on these intriguing aspects of tumor metastasis.

P38 is known to be involved in oncogene-induced senescence (OIS), which is characterized as a telomere length–independent senescence induced by activated oncogenes such as *ras* and *erbB2* in normal nontransformed cells. It is considered to serve as an antitumorigenic defense mechanism. Ras-induced OIS has been shown to be mediated through the MEK–ERK pathway via the activation of MKK3 and MKK6 kinases, followed by upregulation of p38 activity [97]. Interestingly, recent evidence suggests that the microenvironment at the secondary sites has a strong influence on tumor cells to induce senescence by establishing an imbalance that favors p38 over ERK signaling (Figure 12.4). Stress signals, such as hypoxia and inappropriate extracellular matrix, can activate p38 and subsequently inhibit proliferation of tumor cells by blocking the ERK signal and uPAR expression and also by activating G_0/G_1 cell cycle inhibitors such as p53, p27, and Cdc25, which eventually leads to tumor dormancy. In this regard, it should be noted that two metastasis suppressor genes, MKK4/ JNKK1 and RKIP, are demonstrated to inhibit MEK-ERK and promote JNK and p38 signaling [98] (Figure 12.5). Therefore, these genes may suppress metastasis by inducing p38-mediated senescence. Notably, MKK4/JNKK1 was found to be exclusively activated at the secondary site but not in the primary tumor, suggesting the prominent yet puzzling role of the environment of the metastatic site in the behavior of tumor cells. On the other hand, p38 also appears to promote cell survival, which supports the notion that dormancy of tumor cells may be the outcome of a selective adaptive response that allows disseminated tumor cells to pause the growth and cope with stress signaling until growth can be restored. The critical role of microenvironment in mediating tumor cell senescence is also supported by a recently identified mechanism of autocrine motility factor (AMF), which induces cellular senescence and p21 expression in tumor cells that are exposed to an environment of oxidative stress [99].

Tumor cells that survived in the circulation system are either “trapped” in the capillaries or more actively

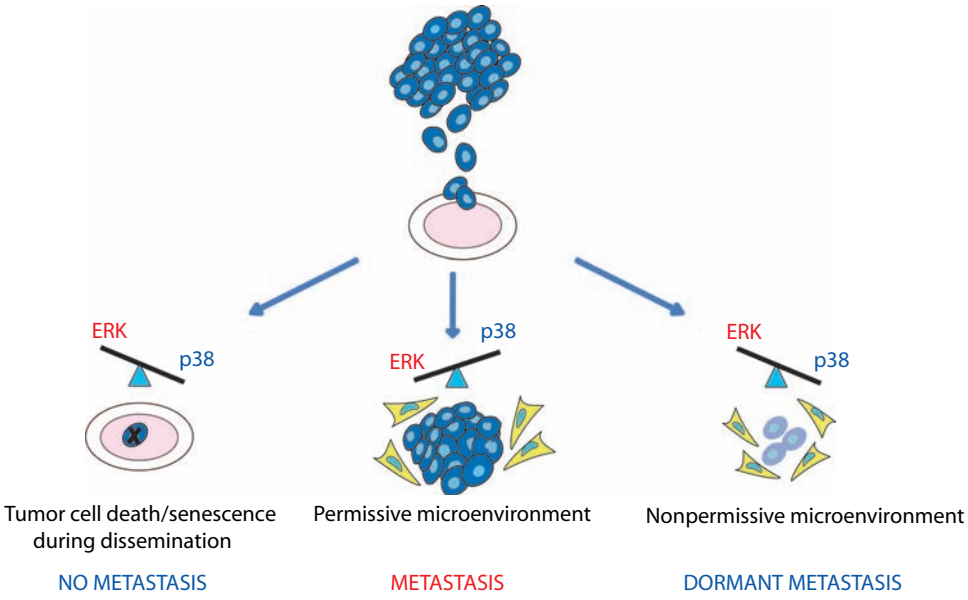

Figure 12.4. ERK/p38 ratio as a determinant of senescence/dormancy in metastasis.

adhere to the endothelium of the blood vessels before they "come out" (extravasate) to the distant sites. This adhesion and extravasation process appears to mimic the infiltration of leukocytes at the inflammation site. Recent evidence suggests that the interaction of cancer cells with the endothelium induces senescence of tumor cells; this is considered to be one of the defense mechanisms to tumor metastasis.

KAI1 has been long recognized as a strong suppressor of tumor metastasis; the expression of this gene is significantly downregulated in various types of metastatic tumors. The KAI1 molecule on the surface of cancer cells can bind to DARC on the endothelial cells when the tumor cells adhere to endothelium (Figure 12.6). This engagement of KAI1 to DARC triggers a signal to the tumor cell to induce cell senescence,

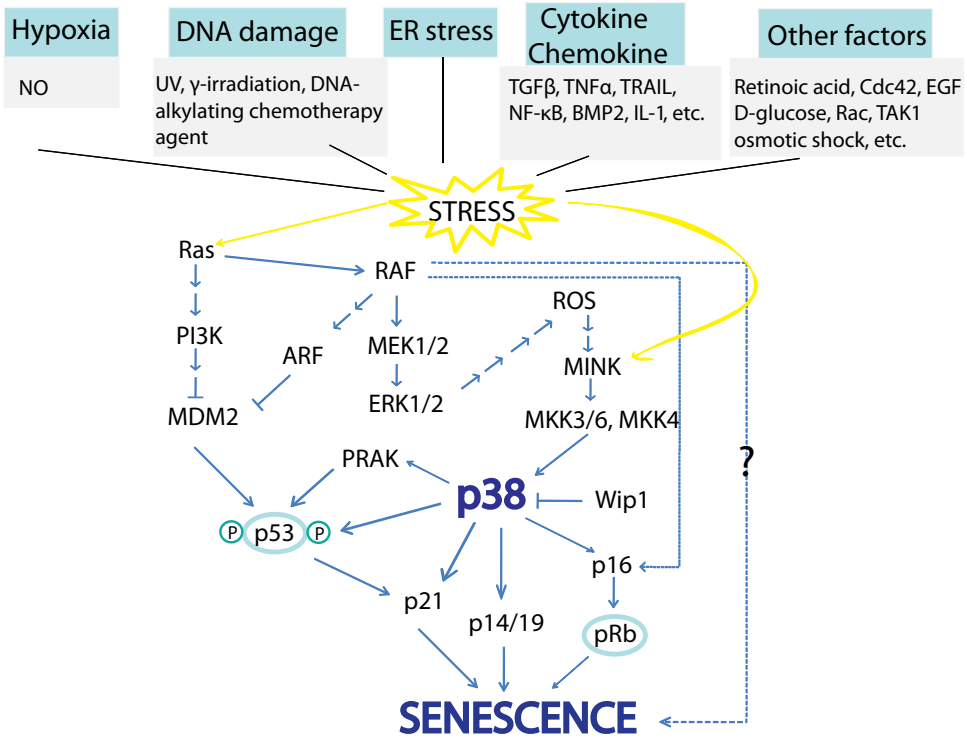

Figure 12.5. Stress signal and p38 in mediated senescence.

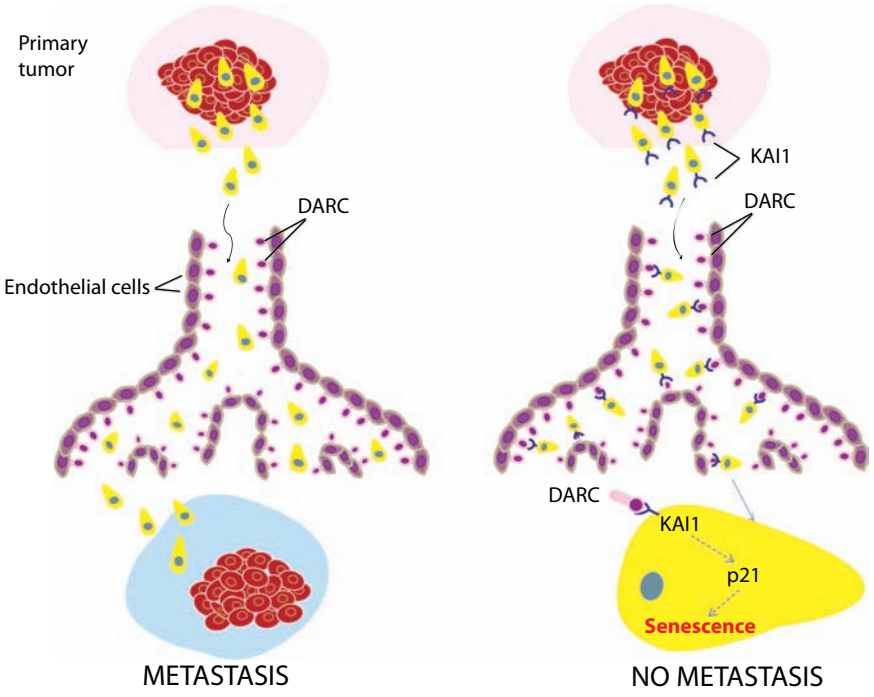

Figure 12.6. Model for KAI1-mediated metastasis suppression.

which eventually blocks the tumor metastasis. However, tumor cells that have already lost KAI1 escape this natural defense system and successfully extravasate at the distant organ and establish colonization. This observation opens a possibility of targeting KAI1/DARC pathway for antimetastatic therapy.

EFFECTS OF STROMAL CELL AND MICROENVIRONMENT ON TUMOR METASTASIS

It is now well recognized that metastatic potential is not only an inherent trait of cancer cells but also is substantially modified by the microenvironment. The ECM that potentiates survival of metastatic cancer cells either endows the advantage of apoptosis resistance to the metastatic tumor cells or provides a positive selection of particular metastatic tumor clones with apoptotic resistance. The "selected" metastatic tumor cells have usually been endowed with a propensity of metastasizing to specific organs. In other words, crosstalking between the distant organ microenvironment (soil) and the cancer cell (seed) will determine whether the cancer cell successfully metastasizes to the specific organ. On the other hand, as mentioned previously, metastatic tumor cells have the ability of resisting ECM-dependent apoptosis in both primary tissue and secondary sites, which may initially harbor hostile factors and defense mechanisms.

It is believed that tumor cells can themselves induce a permissive environment or precondition the future metastatic site by providing selective pressure to the "soil." It has been proposed that the tumor cell grows and invades through the basement membrane into the stromal compartment, which causes a stromal response that generates a new stromal microenvironment. This change, in turn, provides a highly favorable environment for the invading tumor cells. The changed stromal cell, called *reactive stroma*, can promote survival and aggressiveness of tumor cells by ECM remodeling, elevating protease activity, growth factor bioavailability, angiogenesis, and influx of inflammatory cells.

Among the various other factors in microenvironment that drive the selection of the metastatic tumor cells, hypoxia has been intensively studied as a critical factor for promoting each step of the metastatic cascade. Hypoxia facilitates EMT and disruption of tissue integrity through the repression of E-cadherin with a concomitant gain of N-cadherin expression via NF-κB activity, which allows cells to escape anoikis. In addition, hypoxia upregulates the urokinase-type plasminogen activator receptor (uPAR) gene and hence enhances proteolytic activity at the invasive front. This alters the interactions between integrins and components of the ECM, thereby enabling tumor cell invasion through the basement membrane. On the other hand, the elevated uPA/uPAR signal crosstalks with other pathways, including integrins, growth factor receptors, and FAK signaling, which provides a proliferation signal and potently blocks the p38-dependent cellular senescence.

Hypoxia also induces the HGF-cMet signaling, resulting in tumor cell migration toward the blood or

lymphatic vessels. In addition, HGF has multiple roles in tumor progression and functions as a prosurvival and proapoptotic as well as an antisenescent factor. Most importantly, hypoxia induces VEGF, which plays a critical role in the dynamic tumor–stromal interactions that are required for the subsequent stages of metastasis, including extravasation, angiogenesis, and lymphangiogenesis.

Fibroblasts and myofibroblasts represent the majority of the stromal cell compartments within various types of human carcinomas [101, 102]. In particular, large numbers of myofibroblasts, characterized by their production of α-smooth muscle actin (α-SMA), have been observed repeatedly in the stroma of a majority of invasive human breast cancers and are often portrayed as "activated fibroblasts" or "carcinoma-associated fibroblasts" (CAF) [103, 104].

The striking observations that CAF extracted from human carcinomas can promote the growth of admixed epithelial carcinoma cells or otherwise nontumorigenic epithelial cells in immunodeficient mice clearly distinguished the CAF functionally from normal fibroblasts and provoked many interesting questions: How does CAF promote tumor development and progression? What cells are the precursors of CAFs? Is CAF evolved from normal fibroblasts during the tumor progression, or, alternatively, is CAF derived from a carcinoma cell via EMT? Do carcinoma cells drive normal fibroblasts to become CAFs?

Recent extensive works have gradually revealed the intriguing aspect of CAF and shed new light on tumor microenvironments. First, CAF seems unlikely to be derived from carcinoma cells via EMTs, because CAF itself is not tumorigenic nor does it have detectable carcinomalike properties, including karyotypic alterations and anchorage-independent growth [105]. On the other hand, CAFs are found to be heterogeneous with different types of cells, including bone-marrow–derived progenitors, smooth muscle cells, and preadiopocytes in addition to fibroblasts and myofibroblasts, suggesting the various distinct cells of origin responsible for generating the complex of CAFs [106]. Among them, the "fibroblast-to-myofibroblast conversion" appears to be one sure origin of CAFs, which is supported by the observation of Weinberg's group that normal fibroblasts can be recruited to tumor masses and they are then forced to "transform" into myofibroblasts to support tumor growth and angiogenesis [107].

Interestingly, some unique subpopulations expressing fibroblast-specific protein (FSP)-1 are distinct from the α-SMA$^+$ myofibroblasts and promote tumor metastasis [108]. Several lines of evidence have also suggested that CAFs have a capability to promote tumor metastasis by enhancing the survival of carcinoma cells through activation of MAPK, Akt, and Cox-2 [109, 110]. Notably, even though the initial acquisition of myofibroblastic phenotype of CAFs appears to be dependent on the influence of carcinoma cells, once it is acquired, the CAFs display this trait in the absence of further signaling from the carcinoma cells. Considering the essential role of CAFs in reactive stroma, this observation implicates the stability of reactive stroma once it is successfully transformed from normal stroma [107]. Because of the genetic instability of the primary tumor, the tumor microenvironment is forced to face the challenge to be exposed to successive tumor subpopulations that may exhibit different phenotypes. However, once the microenvironment has undergone an appropriate switch induced by the antecedent tumor cells, it can maintain this advantageous condition to take care of the following tumor subclones. This represents a similar paradigm to the "premetastatic niche" theory that the host-organ microenvironment may be conditioned by certain preceding circulating cells to promote the establishment of metastasis by other cancer cells.

The existence of a premetastatic niche is still controversial; however, early alterations at the premetastatic niche are indeed observed in tissues before overt appearance of evidence of carcinogenesis, including early and persistent inflammatory responses, matrix remodeling, increases in ROS, and other bioactive oncogenic molecules such as VEGFR1 and MMPs, suggesting that the changes in local tissue environment are critical components of metastasis [111–113].

Furthermore, the premetastatic-niche theory can partly explain the long-time thought-consuming question as to why metastases are organ-specific. It seems that cancer cells are homing to specific organs most likely because of naturally favorable growth conditions that exist in particular organs that also can be preconditioned. For example, in the osteolytic bone metastasis of breast cancer, osteoblasts themselves in the bone naturally produce cytokines that are chemoattractants for metastatic breast cancer cells. Meanwhile, osteoblasts can be directed by the metastatic breast cancer cells to produce inflammatory cytokines such as interleukin (IL)-6 and IL-8 that have been implicated in osteoclast activation as well as breast cancer cell migration and survival [114–116].

Recent studies on tumor-associated stroma have emphasized the advantage to target tumor-associated stroma, although this concept needs to be further strengthened by more clinical observations to show that significant numbers of stromal fibroblasts and myofibroblasts are correlated with poor prognosis of human carcinoma patients. Considering the critical role of stromal cells in the tumor growth and metastasis, and the genetic stability of the stromal cells in contrast with carcinoma cells harboring accumulated adaptive mutations during the course of chemotherapy, more studies on stromal cells may aid the development of selective antimetastatic treatments and the identification of

crucial points on multiple survival–signal pathways that might represent a useful target for antitumor drugs.

REMAINING QUESTIONS AND CLINICAL IMPLICATIONS

Recent progress in the field of metastasis research has begun to reveal the critical roles of apoptosis and senescence in tumor progression. The new signal pathways of cell death and survival specific to the metastatic process and their balance through crosstalking networks have been discovered. However, many issues still remain to be answered.

For cancer cells, acquiring resistance to apoptosis and senescence by genetic and epigenetic mutations is an essential step to becoming metastatic. When and how they acquire these mutations are important questions. Are these mutations occurring at the primary tumor site, or are they "selected" during the metastasis process? What signals are altered in these tumor cells? In this regard, we need to clearly understand the emerging concept of tumor metastasis stem cells. As the importance of cancer stem cells in tumor initiation has been firmly established in a variety of cancer types, the role of cancer stem cells with respect to metastasis is still at a stage of hypothesis. If "metastasis stem cells" exist, do they have a distinct mechanism for apoptosis resistance? When and where do they acquire such capability? Answering these questions may significantly affect treatment options for metastatic disease in the future.

Tumor metastasis is generally known as a cellular process with both "active" and "passive" aspects, in that it is able to be driven by its intrinsic genetic alteration to gain proliferation, anchorage-independent survival, invasiveness, homing in a preferential host organ, extravasation, and colonization at secondary sites. On the other hand, metastasis efficiency is passively controlled by the stress from extrinsic signals and environment, including accessing to blood vessels, blood flow pressure, and passive trapping of cancer cells in capillaries.

Years of studies on metastasis have been focusing on elucidating the genetic alteration of tumor cells, but it has become increasingly clear that the tumor microenvironment plays a pivotal role in cancer development and progression. We have also learned that the tumor microenvironment, either at primary sites or at distant organs, has crucial roles in determining the balance between apoptosis and survival of metastatic cells. Tumor cells appear to be able to modify the stroma, which itself can provide either positive or negative signals for tumors to survive, grow, and progress. These cancer stromal cells, reactive stroma, have distinct genetic profiles and express various factors to support tumor cell survival. It is therefore crucial to understand when and how "normal" stromal cells gain the ability to become active stroma.

In this context, it is particularly interesting to examine the stromal cells at the "invasive front," which consists of a unique subset of tumor cells interfacing with organ-specific supportive cells, "activated fibroblasts" or CAFs, because it represents a similar paradigm to the premetastatic sites in which tumor cells are entering a new microenvironment. It is also equally important to understand the reciprocal interactions of the reactive stroma and metastatic cells in terms of activating antiapoptotic signaling pathways. These are currently under active investigation.

As discussed earlier, it is believed that tumor cells homing to specific metastatic sites are the result of the favorable growth conditions in the target organ. However, expression of a particular X-metastatic gene signature (X refers to the specific organ) by tumor cells has also been identified in various cancers and linked to the "homing" propensity of metastatic tumor cells. For example, genomic profiling of the bone-metastasis–derived subpopulations of MDA-MB-231 breast cancer cell lines revealed expression of osteopontin, CTGF, FGF5, IL-11, CXCR4, MMP1, and ADAMTS1. Also, a bone-metastasis gene signature, including TCF4, PRKD3, SUSD5 and MCAM, was recently identified for lung cancer [115].

Is there any necessary link between the expression of these X-metastatic gene signatures by tumor cells and the unique growth conditions of a particular organ? In other words, are these genes expressed in an inducible manner by exclusive secondary sites, or do the microenvironments select the metastatic tumor cells expressing the particular gene signature? More studies on the function, as well as temporal and spatial expression, of the metastatic gene signature may eventually reveal the molecular mechanism of organ-specific homing of metastatic tumor cells.

Finally, the most important question is how we can translate the information obtained in this research field into clinical application. Understanding the death and survival pathways and identifying their key factors may lead to a design of a molecular "signature" profile to accurately predict patient outcome in regard to metastasis status and survival. Elucidating the intrinsic survival capabilities of cancer cells that probably determine the organ-specific lodging of metastatic cells, and identifying the mechanism and factors involved in the apoptosis resistance during the process of metastatic cascade, may also implicate the future therapeutic targets for metastatic disease (Table 12.1).

Identifying the role of metastasis stem cell and mechanism of apoptosis resistance may totally change the course of treatment for metastasis disease. The most daunting aspect of cancer treatment is the recurrence of tumor many years after treatment. However, if we are

TABLE 12.1. Apoptosis factors involved in metastasis

	Factors	Categories	Functions	Steps	Expression in cancer	Inhibitors/drugs
Factors promoting metastasis	HGF/ c-Met	Growth factor	Modulates cell–cell adhesion and cell–ECM junctions, hypoxia-inducible gene, inhibits apoptosis/anoikis, controls cell proliferation, angiogenesis	1, 2, 3	Breast, colorectal, ovarian, hepatocellular cancers, NSCLC, head and neck squamous cell carcinoma	NK4, AMG102, PHA-665752, SU11274, K252a
	AMF	Cytokine	Inhibits apoptosis, promotes cell motility and migration, involved in p21-mediated cell senescence, angiogenesis	1, 2, 3	Metastatic colorectal, lung, kidney, breast, and gastrointestinal cancers	Carbohydrate phosphate (E4P, M6P, 5PA) Herceptin
	TGF-β	Cytokine	Promotes EMT via activating related transcription factors (Snail, Slug, and LEF1) and disrupting cell–cell adhesion, evasion of host-immune cells, angiogenesis	1, 2, 3	Potential prognostic biomarker Metastatic breast, colon, liver, lung, prostate, and stomach cancers	AP12009, Fc:TβRII, β-glycan, SD-208, SD-093, SB-431542, A-83–01, LY2109761, 2G7
	IGF1/ IGF1R	Growth factor	Promote cell growth, inhibit apoptosis/anoikis	1	Bladder, ovarian, and endometrial cancer, breast, lung, gastric, pancreatic, prostatic, esophageal cancers, head and neck cancer, salivary gland cancer	NVP-AEW541 NVP-ADW742
	VEGF/ VEGFR	Growth factor	Increase vascular permeability; stimulate autocrine/paracrine growth factors release	1, 3	Breast, lung, thyroid, kidney, bladder, ovarian, uterine cervix, colorectal, esophageal, and gastric cancers	Avastin (bevacizumab) Vandetanib (oral) DC101(VEGFR2 Ab) IMC-1121B
	SDF1/ CXCR4	cytokine	Directly stimulates tumor growth and survival, recruits endothelial progenitor cells, angiogenesis	2, 3	CXCR4-metastatic breast, prostate, and ovarian cancers, melanoma, renal cell carcinoma. SDF-1 highly expressed in organs to which cancer cells preferentially metastasizes.	Anti-CXCR4, AMD3100
	FAK	Kinase	Promotes cell migration, enhances anoikis resistance	1.2	Metastatic breast, prostate, liver, colon, and thyroid cancers	PF-00562271 PF-573228 NVP-TAE226 FRNK
	TrkB	Kinase	Enhances anoikis resistance	1, 2	Neuroblastoma, pancreatic, prostate cancers, Hodgkin lymphomas, and myeloma	CEP-751
	uPA/ uPAR	Protease	ECM proteolysis and remodeling, cell migration, hypoxia-inducible gene, promote cell survival and proliferation, angiogenesis	1, 3	Metastatic colon, breast, ovary, lung, kidney, liver, stomach, bladder, and endometrial cancers	WX-UK1, WX-671, 231 Bi-PAI2, Bikunin, DX-1000, UK-356, UK202

TABLE 12.1 (Continued)

Factors	Categories	Functions	Steps	Expression in cancer	Inhibitors/drugs
PI3K/ AKT	Kinase	Major cell survival pathway, mediates EMT, cell motility	1, 2, 3	Breast, prostate, endometrial, lung, thyroid, pancreatic, and gastrointestinal cancers	LY294002 Wortmannin Perifosine
ERK1/2	Kinase	Promotes tumor growth, survival, cell motility and invasion, ECM degradation	1, 3	Over expression in metastatic breast cancer	PD98059 UO126
NF-κB	Transcription factor	Cell migration, organ-specific metastasis, apoptosis resistance, chemoresistance, angiogenesis	1, 2, 3	Breast, prostate, gastric, pancreatic cancers, myeloma, Hodgkin lymphomas, head and neck squamous cell carcinoma	PS-1145 IMD-0354
Twist-1	Transcription factor	Inhibits apoptosis, mediates EMT	1	Invasive lobular breast carcinomas and metastatic gastric cancers	None
Snail	Transcription factor	Promotes invasiveness, inhibits apoptosis, mediates EMT	1	Invasive ductal breast carcinomas and metastatic gastric cancers, melanoma	None
Galectin-3	β-galactoside-binding protein	Enhances anoikis resistance, restores cell–cell or cell–ECM adhesion, promotes tumor cell aggregation and docking onto endothelium in circulation	1, 2	Metastatic breast, gastrointestinal, lung and ovarian cancers, melanoma, Hodgkin lymphoma	MCP (modified citrus pectin)
Osteopontin (OPG)	Phosphor-protein	Inhibits apoptosis, ECM proteolysis and remodeling, cell migration, evasion of host-immune cells, neovascularization	1, 2, 3	Prognostic biomarker Metastatic breast, prostate, gastric cancers, NSCLC, uveal melanoma, head and neck squamous cell cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, renal cell carcinoma, esophageal squamous cell cancer	None
Bcl-2, Bcl-xl, IAPs	Proapoptosis factor	Induce ECM-independent survival (anoikis resistance)	1, 2	B-cell lymphoma, breast, bladder, prostate, ovary and lung cancers	GENTA IDUN GEMIN-X Agera
MMPs	Proteinases	Modulate microenvironment by ECM proteolysis, inhibit apoptosis, angiogenesis, impair host immunological surveillance, proteolytically activate other factors (TGFβ, SDF-1, IGFBPs)	1, 2, 3	MMP1, 2, 3, 7, 9, 13, 14 overexpression positively associated with tumor progression and metastasis	Marimastat Prinomastat Tanomastat Neovastat Bisphosphonates

(Continued)

TABLE 12.1 (Continued)

	Factors	Categories	Functions	Steps	Expression in cancer	Inhibitors/drugs
	CD44	Adhesion receptor	Mediates cell–matrix and cell–cell interactions – anoikis resistance	1, 2	Metastatic breast cancer, non–small-cell lung cancer	Anti-CD44 mAb, CD44-Ig fusion proteins
	Caveolin-1	Membrane transporter protein	Enhances anoikis resistance	1, 2	Prostate, bladder, esophagus cancers, T-cell leukemia, multiple myeloma, low-expressing in breast, cervical, SCLC, and ovarian cancers	None
Factors suppressing metastasis	P53	Tumor suppressor	Mutation facilitates invasiveness, survival of metastatic tumor cells, and angiogenesis	1, 2	Mutation of loss in >60% human primary tumors, correlated with increased metastasis of breast, colorectal, NSCL cancers	INGN201 ONYX-015
	E-cadherin	Metastasis suppressor	Key cell–cell adhesion factor, controls EMT	1	Prognostic biomarker Decreased expression associated with poor prognosis in cancer patients	Pyrazolo [3,4-d] pyrimidines (PP1) PP2
	MKK4	Metastasis suppressor	Activated by stressful environment at secondary sites and mediates p38/JNK pathway to impair colonization	3	Express inversely correlated with Gleason score and tumor progression of prostate and ovarian cancer	Anti-death receptor antibody (2E12, TRA-18) Bisindolylmaleimide VIII
	KAI1	Metastasis suppressor	Integrin interactions, EGFR desensitization, binds DARC on endothelial cells	2	Prostate, breast cancer	None
	RKIP	Metastasis suppressor	Promotes apoptosis, inhibits cell invasion and angiogenesis	3	Prostate, breast cancer	None
	P38	kinase	Oncogene/stress-induced senescence/apoptosis	3		None
	DAPK	kinase	Increase apoptosis sensitivity	1, 2	Frequently lost or mutated in human B-cell lymphoma, NSCLC, head and neck cancer, thyroid lymphoma, colon, and breast cancers	None
	Maspin	Serine protease inhibitor (serpin)	Intracellular maspin increases cellular apoptosis sensitization; extracellular maspin blocks tumor-induced ECM degradation, cell motility and invasion	1, 2, 3	Prognostic biomarker Expression predicts a better prognosis for breast, prostate, colon, and oral squamous cancers	None

Metastasis cascade: 1 – initial steps of metastasis, including detachment of epithelial cells from the ECM and disruption of the actin skeleton; 2 – intravasation, circulation, and extravasation; 3 – survival and colonization at the secondary site.

able to understand the precise mechanism of tumor cell senescence and dormancy, we may be able to develop a reagent to keep the tumor cell "dormant" in the future.

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13 Metastatic Inefficiency and Tumor Dormancy

Ann F. Chambers

METASTASIS – THE CLINICAL PROBLEM

It is well recognized that metastases – not primary tumors – are responsible for most cancer deaths. Fortunately, however, the metastatic process is highly inefficient. Both clinically and in experimental models, large numbers of cancer cells may leave the primary tumor and be detected in the circulation or in distant organs, yet very few of these disseminated cancer cells go on to form overt, clinically relevant metastases. This was dramatically demonstrated clinically years ago, in a study of patients who received peritoneovenous shunts as palliative treatment for pain associated with malignant ascites. Large numbers of viable, clonogenic tumor cells were detected in the blood of these patients [1, 2]. However, these patients appeared to have no worse outcome, and at autopsy no evidence of increased macroscopic metastatic burden was identified [1, 2].

Consistent with these clinical findings are numerous reports from experimental animal models of metastasis. Large numbers of cancer cells may be detected in (or injected into) the circulation of experimental mice, and only a small fraction of these cells produce progressively growing metastases (early examples include [3–6]). For example, Fidler showed that when 50,000 B16F1 melanoma cells were injected intravenously via the tail vein, only 0.12 percent of the cells produced lung metastases, whereas the B16F11 cells – which had been selected for increased metastatic ability – had an increased metastatic efficiency of 0.74 percent [7]. Thus, even in a cell line selected for its ability to metastasize, the metastatic process is extremely inefficient, with most cells in a population failing to produce metastases. Hill et al. provided additional insight into possible differences between cells of high versus low metastatic ability when they showed that B16F10 cells had a fivefold increased rate of genetic or epigenetic instability that produced cells able to metastasize [6].

Detailed analyses of the fate of circulating cancer cells in experimental animals has revealed key steps that contribute to the overall inefficiency of the metastatic process, including numbers of cells capable of leaving a primary tumor and entering into the circulation, coupled with failure of the majority of cells that arrive in distant sites to initiate growth, as well as the failure of many of these nascent micrometastases to persist in growth [8–15]. Luzzi et al. [9] quantified metastatic inefficiency of B16F1 melanoma cells injected into the mesenteric vein to target mouse liver, and reported that macroscopic metastases arose from only 0.02 percent of the originally injected cells. In contrast, a larger proportion (~2%) began to form micrometastases, but very few of these persisted, and more than 36 percent of the original inoculum persisted as solitary dormant cells at the end of the experiment, coexisting with the progressively growing macroscopic metastases in the livers [9].

Metastatic inefficiency has been shown to have organ specificity; different cancers may grow better in some organs than in others, depending on the tumor type as well as other factors [16, 17]. Large numbers of dormant, solitary cells have been detected in many experimental models [1, 8–12, 18–21] and such cells also have been detected in patients [22–25]. The fate of the majority of circulating tumor cells, and the reasons responsible for metastatic inefficiency, are important areas of continuing research. A better understanding of factors that contribute to metastatic inefficiency may offer new therapeutic strategies to either prevent metastatic disease or to treat it better.

TUMOR DORMANCY – THE CLINICAL PROBLEM

Current cancer therapy for most solid tumor types generally involves removal of the primary tumor with surgery, often coupled with local radiation to remove any remaining cancer cells at the primary tumor site.

If prognostic factors suggest that the tumor is unlikely to have seeded metastatic cells to distant organs by the time of primary treatment, the patient may be considered to be cured, and no further therapy may be needed. However, prognostic factors such as large tumor size and lymph node positivity may suggest a high probability of undetected distant spread, in which case adjuvant therapy (chemotherapy, hormone therapy, molecular targeted therapy) may be offered to prevent subsequent outgrowth of presumed micrometastatic disease.

Treatment for diagnosed (macro-)metastatic disease – that is, where metastases are known to be present – may also include chemotherapy, hormone therapy, and/or molecular targeted therapy, as well as radiation in some cases, but for many cancers these may not be expected to be curative. Thus, a better understanding of the biology of both presumed micrometastatic disease, as well as diagnosed metastatic disease, is needed to improve survival rates from cancer. Here we will consider current ideas about the process of metastasis – biologically, molecularly, and physically – gleaned from experimental studies on metastasis and from clinical considerations.

Adding to the complexity of cancer treatment is the clinical fact that cancer can recur many years after apparently successful treatment for the primary disease. This is particularly true for cancers such as breast cancer, melanoma, and renal cancer, for which metastatic recurrences have been reported years or even decades after primary treatment [26–31]. Additional support for the concept of tumor dormancy comes from reports of occult metastatic disease being transplanted along with a donor organ, and the organ recipient – routinely treated with immunosuppressive therapy – subsequently developing cancer in the transplanted organ [27], as well as reports of patients being treated with immunosuppressive therapy developing recurrent, metastatic cancer from their own previously removed primary tumor [26].

The fact that disseminated tumor cells can go into a dormant state introduces uncertainty for both patients and their physicians, and makes treatment decisions difficult. In weighing the potential benefits versus risks of adjuvant therapy, for example, physicians and patients rely on historical probability data that suggest the degree of risk of micrometastatic disease being present, based on features present at the time of cancer diagnosis, such as tumor size, lymph node status, and so forth [32].

Prognostic factors and biomarkers may be helpful in assessing risk of future recurrence, as well as likelihood of response to given therapies [33–38]. However, this information is population based, and does not necessarily predict the clinical outcome or response for an individual patient. Even following adjuvant treatment, some patients will have disease recurrence. What is the difference between a patient who is "cured" and a patient who has undiagnosed micrometastatic disease and is in a state of "tumor dormancy"? Much remains to be learned about tumor dormancy, factors that lead to dormancy, and factors that lead to the reawakening of dormant tumor cells. Experimental models have helped to shed some light on the metastatic process and the state of dormant cancer cells.

STEPS IN METASTASIS – EXPERIMENTAL STUDIES

Experimental studies have helped to clarify the steps involved in the metastatic process [39]. Metastasis involves a series of sequential steps, beginning with local invasion at the primary tumor site [40]. Cancer cells then may enter into the blood or lymphatic circulation (intravasation), and leave the primary site [14, 15]. Once in the circulation, cells travel to distant organs, where they are filtered out quite efficiently from the blood (or lymph) at the first-pass capillary bed, owing to the difference in size of solid tumor cells (large, e.g., 15–25 μm) versus capillaries in the new organ (small, e.g., 5–10 μm) [12, 41].

Following arrival and retention in the new site, many cells may escape from the vasculature into the tissue (extravasation). Some cells then initiate growth to form preangiogenic micrometastases, and a proportion of these may persist to form vascularized, progressively growing metastases. In detailed experimental "fate analysis" studies, it has been shown that a large proportion of cancer cells that arrive in a new organ remain as solitary, dormant cells, and that metastases arise from only a very small subset of the total cells delivered to that organ [9–11, 21, 42]. These dormant cells have been shown to be resistant to cytotoxic chemotherapy that targets actively dividing cells, and these cells may be responsible for late-developing metastases following apparently successful adjuvant therapy [43].

It is well recognized that metastasis of many cancers can have an organ-specific pattern, with, for example, breast tumors commonly metastasizing to the lung, liver, bone, and brain, whereas colon cancer may commonly spread to the liver (see [44] for the centennial republication of Stephen Paget's seminal 1889 paper, originally published in the *Lancet*). In an analysis of published autopsy studies, Weiss compared organ-specific metastases detected at autopsy with known blood flow patterns, from a series of pairs of primary tumor sites and secondary organs [17]. He found that in two-thirds of these organ pairs, the numbers of metastases detected were in proportion to known blood flow patterns; in contrast, in one-third of these pairs, either more or fewer metastases were detected than could be explained by blood flow alone. Included among these "discordant" pairs were breast and prostate

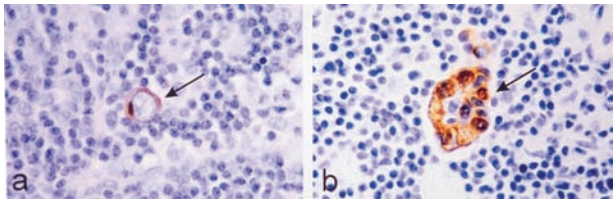


Figure 13.1. Micrometastastic disease in sentinel lymph nodes. Shown are (**a**) an isolated solitary breast tumor cell (arrow) and (**b**) a small micrometastasis (arrow), in the sentinel lymph nodes of two different breast cancer patients. Tumor cells were detected by immunohistochemical staining with anticytokeratin antibodies. The clinical significance of these micrometastatic foci remains unclear. 400X. Photograph courtesy of Dr. Alan B. Tuck. Republished from [79].

cancer metastases to bone, where more metastases were detected at autopsy than could be explained by blood flow patterns alone. Thus, it appears that cancer cells are delivered to secondary sites in proportion to the blood flow patterns from the primary organ, where most are efficiently "filtered" out from the blood in the first capillary bed they encounter, and that molecular and microenviromental factors in the secondary site, coupled with the survival and growth requirements of the tumor cells, then determine the success of the delivered cells in forming overt metastases [12].

The concept of site-specific growth regulation is illustrated nicely by a series of studies by Tarin and colleagues, in which tumor cells labeled with a heritable green fluorescent protein were injected to form a primary tumor in mice [19, 20]. It was noted that metastases formed in some but not all organs, reflecting organ-specific metastasis patterns. However, large numbers of solitary green-fluorescent cells were detected throughout the body of the mice, as dormant cells that persisted but failed to grow in apparently non-supportive growth environments. These cells, when isolated, retained their tumorigenic and metastatic abilities when reinjected [20].

Clinically, distant micrometastatic disease also has been detected, most commonly in studies that have examined bone marrow, or in some cases blood, of cancer patients [22–25, 45], as well as in lymph nodes [46–48]. The presence of distant, disseminated tumor cells is believed to be an indicator of poor prognosis, whereas micrometastatic disease in lymph nodes has been suggested to confer no greater risk of recurrence. However, the clinical significance of micrometastatic disease, either locally or distantly, remains poorly understood.

Figure 13.1 shows examples of breast cancer cells detected in sentinel lymph nodes of breast cancer patients; in both cases shown, the prognostic significance of these cells remains uncertain. Lymph node positivity (as defined by the presence of tumor deposits $\geq$2 mm) is a strong negative prognostic indicator [32]. However, the prognostic significance of small foci of metastastic disease (<2 mm), either isolated tumor cells in distant organs or as micrometastatic foci in lymph nodes, as shown in Figure 13.1, is not clear; studies (e.g., [46–48]) have suggested that prognosis for breast cancer patients with lymph node positivity for micrometastatic disease is not different from that of patients with no micrometastases. Although such tumor cells have clearly been shed from the primary tumor, the probability of their progression to growing metastases at distant, vital organs remains to be clarified, and appears to be generally quite inefficient (e.g., [1, 2, 46–48]). This suggestion is indeed consistent with the reports on peritoneovenous shunts described earlier, in which large numbers of disseminated cells may have low probability of progressing to form macroscopic metastases [1, 2].

Further support for the organ-specific regulation of viable but dormant cells comes from the occasional reports of transmission of cancer from organ donation from apparently cured cancer patients, who have died of other causes, with the recipient individual then developing metastatic cancer in the donated organ (e.g., [27]). In these cases, dormant cells may have resided, undetected, in the donor's organ, only to reawaken in the new host, who will have received immunosuppressive therapy to support acceptance of the donated organ. These studies suggest that immune surveillance is likely one factor that can prevent outgrowth of disseminated tumor cells, although the nature of immune system effects on dormancy are likely to be complex [49, 50].

Ongoing work assessing whether specific subpopulations of disseminated tumor cells, positive for aggressive features such as specific biomarkers or cancer stem cell properties, have a higher probability of subsequent metastatic growth, may help to clarify this dilemma [22, 24, 45]. The important issue is whether cancer cells that have been seeded to distant sites have the potential to grow to life-threatening metastases, and if so, what is the probability of their doing so.

TUMOR DORMANCY

Two distinct classes of tumor dormancy have been described, both experimentally and in cancer patients. First, solitary disseminated tumor cells have been detected. These cells appear to be in a quiescent state, neither dividing nor undergoing apoptosis, and experimental evidence suggests that these cells may be unaffected by cytotoxic chemotherapy that targets actively dividing cells [11, 43, 51]. Second, "dormant" but active preangiogenic micrometastases have been described, in which cell division is balanced by apoptosis, resulting in no net increase in size [13, 52]. These two states of dormancy would present very different clinical therapeutic targets [13, 23, 53, 54]. Many questions remain

about factors that lead to tumor dormancy and subsequent reawakening of dormant tumor cells.

Recent experimental models are beginning to provide tools for studying molecular and biological factors that may influence the transition from dormancy to progressive growth [55–57]. However, factors that regulate entry into a dormant state, and reemergence to active growth, are very poorly understood [18, 23, 25, 51, 58, 59]. Barkan et al. have developed an in vitro cell culture system that appears to predict in vivo dormancy kinetics; using this model, they have identified interactions of components of the cell cytoskeleton with the extracellular matrix as being important regulators of the dormant state [55]. The transition from dormancy to cell proliferation required signaling through integrin $\beta 1$, resulting in phosphorylation of myosin light chain kinase and cytoskeletal reorganization [55]. Aguirre-Ghiso and colleagues have identified signaling pathways, in some cases associated with stress responses and interactions with the extracellular matrix, which may lead to dormancy and survival of cells in a new organ [57, 59–62].

Additional evidence comes from information about the class of metastasis suppressor genes, which have many molecular functions but may all result functionally in induction of tumor dormancy and inhibition of growth in secondary sites [63–66]. Much remains to be learned about factors that can induce dormancy or lead to reawakening of cells; the challenge will be to use this information to devise therapies to induce or maintain dormancy or to kill dormant cells [23, 51, 67]. However, clinical and experimental data suggest that, once dormancy has been broken, cells may resume rapid proliferation [68, 69], which may make treatment difficult at this stage.

CLINICAL IMPLICATIONS OF EXPERIMENTAL STUDIES OF METASTASIS AND TUMOR DORMANCY

In developing therapeutic strategies for both the prevention of future metastasis development and the treatment of diagnosed metastatic disease, a key consideration is whether there is a therapeutic time window available. Figure 13.2 lists steps in the metastatic process and indicates which steps might be more or less amenable to treatment. If a step in the metastatic process has already occurred prior to the initial diagnosis of cancer, this step is no longer available for therapeutic intervention.

Unfortunately, many of the early steps in metastasis may well have occurred long before the cancer is detected. Recent evidence supports the idea that some cells may have been shed from breast tumors early in the progression of disease [70]. Thus, therapeutic strategies designed to target early steps in the metastasis process – including intravasation from the primary tumor, survival of cells in the circulation, arrest in a secondary site, and extravasation into the tissue at that site – are unlikely to be effective, as one cannot assume that they have not occurred prior to cancer diagnosis. However, a broad temporal therapeutic window may exist for later steps in the process.

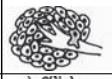
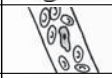
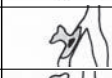
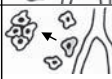
Steps in metastatic process		Appropriate clinical target?
Intravasation		Unlikely
Survival in the circulation		Unlikely
Arrest in secondary site		Unlikely
Extravasation		Unlikely
Dormancy of solitary cells		Perhaps
'Dormancy' of micrometastases		Yes
Growth of vascularized metastases		Yes

Figure 13.2. Diagram of steps in the metastatic process, and whether each step may be an appropriate clinical target. Steps that may have occurred prior to diagnosis of the primary tumor are unlikely to be good targets for therapy. In contrast, steps that are ongoing after tumor diagnosis are appropriate and may offer a broad temporal window for treatment. Treatment of solitary dormant cells is a potentially promising target, because of the large temporal window available. However, new strategies to target this phase of the metastatic process are needed. Modified from [79].

Current adjuvant therapies most likely target disseminated cells that are actively dividing but have not progressed to form detectable metastases. In Figure 13.2, micrometastases in a dormant but preangiogenic state, made up of cells that are actively dividing but also undergoing apoptosis, represent an appropriate target for cytotoxic therapies that target actively diving cells. These micrometastases also might be appropriate targets for antiangiogenic therapies, to keep them in a preangiogenic, and thus small, state. Similarly, actively growing metastases, either large enough to be clinically detected or subclinical but vascularized smaller tumors, would be appropriate targets for both cytotoxic and antiangiogenic therapies.

Cells in both states would also be appropriate for molecular targeted therapies that limit growth of populations of cells bearing the necessary molecular target. Unfortunately, many cancer therapies used currently have limited effectiveness against clinically detected metastases. However, adjuvant therapy has had

excellent success for many tumor types [71]. Evidence suggests that long-term (e.g., 5–10 years) hormonal therapy may have a continuing benefit [72–77], supporting the idea that adjuvant therapy may target a very broad "temporal window." The challenge with long-term therapy, however, is that the side effects must be sufficiently small to warrant such therapy. Ongoing clinical trials are exploring this concept further [78].

In contrast, the potential for targeting solitary dormant cells, which are quiescent and not apparently affected by cytotoxic treatment [43], remains relatively unexplored, and strategies to target these cells have yet to be devised [23, 24, 67]. Nonetheless, compelling evidence suggests that many apparently "cured" cancer patients may harbor dormant tumor cells for years or even decades, and these cells may awaken in response to poorly understood stimuli.

CONCLUSIONS AND REMAINING QUESTIONS

Much has been learned about the process of metastasis and steps involved, from both clinical and experimental studies. Significant advances are being made at improved adjuvant treatment for cancer, leading to long-term survival for many cancers. However, tumor dormancy is a real clinical problem, for which we have no ready answers. More work is needed to better understand tumor dormancy, both mechanistically and as a potential target for treatment, for clinical approaches to be developed to address this significant aspect of cancer progression. Improvements in our knowledge about the nature of tumor dormancy, leading to identification of treatment strategies to target these cells, will be important to address this difficult problem in cancer treatment.

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14 Role of Inflammation in Metastatic Progression

Sunhwa Kim and Michael Karin

EXTRINSIC VERSUS INTRINSIC MEDIATORS OF INFLAMMATION

A link between chronic inflammation and cancer has been suspected since the nineteenth century, when Rudolf Virchow first noted that malignant tumors arise at regions of chronic inflammation and contain inflammatory infiltrates [1–5]. However, the sources of inflammation in tumors that are not associated with a chronic inflammation remain incompletely understood. Recently, it became apparent that inflammation can be evoked not only by extrinsic mediators but also by intrinsic mediators (endogenous molecules) (Figure 14.1). For instance, it has been established that necrotic cell death results in the release of molecules normally stored within cells, such as high-mobility group box 1 (HMGB1) and interleukin (IL)-1α, that act as potent inflammatory mediators [6, 7]. Such molecules may responsible for the triggering of tumor-associated inflammation [7].

Toll-like receptors (TLRs), the mammalian homologs of the *Drosophila* Toll protein, play a crucial role in activation of inflammatory responses and innate host defenses against invading microorganisms by their ability to recognize conserved molecular motifs of microbial origin, also known as pathogen-associated molecular patterns (PAMs) [8–10]. A number of intrinsic mediators have been shown to be capable of engaging TLR family members and other innate immune receptors and thereby trigger the activation of myeloid and lymphoid cells as well as stimulate the maturation of dendritic cells (DCs) [6, 11–13].

The first mammalian TLR to be identified, TLR4, is the receptor for lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria [10]. Since then, different TLRs were found to recognize a wide range of microbial components: TLR1 (in association with TLR2) is activated by tri-acyl lipopeptides [14]; TLR2 is activated by lipoproteins and peptidoglycans [15]; TLR3 is a receptor for double-stranded RNA [16]; TLR5 recognizes flagellin [17]; TLR6 (in association with TLR2) is activated by di-acyl lipopeptides [18]; TLR7 and TLR8 are receptors for single-stranded RNA [19]; and TLR9 is a receptor for nonmethylated CpG DNA [20]. No ligands have been identified as yet for TLR10 and TLR11. Upon binding of their cognate ligands, the TLRs trigger a variety of intracellular signal transduction pathways that culminate in induction of proinflammatory cytokines, chemokines, and interferons (IFNs) [9, 21, 22]. However, in addition to the classical ligands mentioned above, the TLRs may be activated by normal cellular proteins and nucleic acids released during necrotic cell death.

Heat shock proteins (HSPs) have been known as potent activators of the innate immune system [23, 24]. HSPs of mammalian origin, such as Hsp60, Hsp70, and Hsp90, can induce the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, and IL-12, and the release of nitric oxide (NO) and C-C chemokines by monocytes, macrophages, and DCs via TLR-dependent mechanisms [25]. HSPs also induce the maturation of DCs, as demonstrated by upregulation of major histocompatibility complex (MHC) class I and II molecules, and costimulatory molecules, such as CD80 and CD86 [23, 24]. Similar proinflammatory effects have also been reported for other molecules of mammalian origin, including fibrinogen [26], surfactant protein A [27], domain A of fibronectin [28, 29], heparan sulfate [30], short hyaluronan (HA) fragments (soluble HA) [31], β-defensin 2-lymphoma antigen idiotype sFv fusion protein [32], HMGB1 protein [6], tRNA synthase [33], and versican [34, 35]. There is growing evidence that these intrinsic TLR activators may be released during tumor progression, both by dying and living carcinoma cells, and are responsible for persistent low-grade inflammation achieved through activation of TLRs and other receptors [36]. However, the roles of most of these intrinsic mediators in

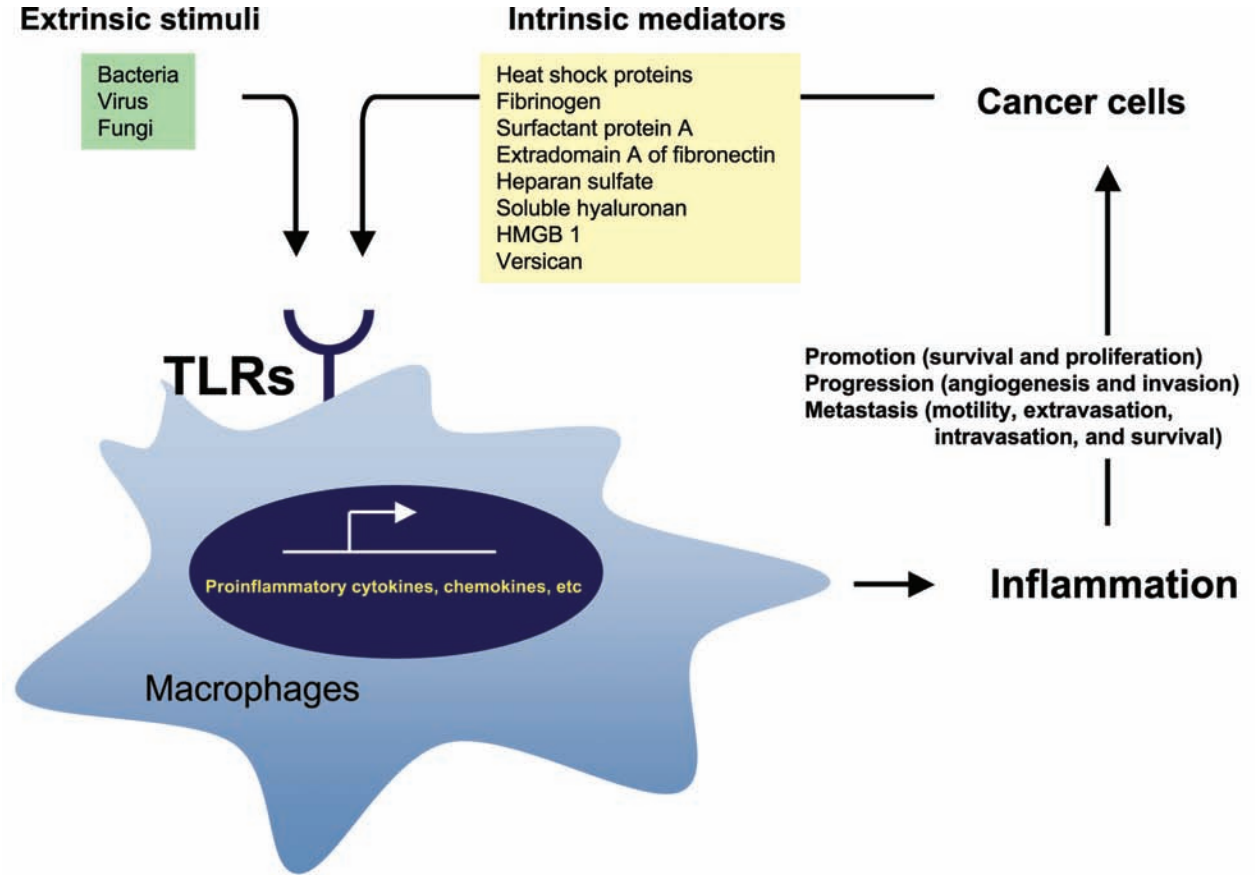

Figure 14.1. Roles of extrinsic and intrinsic inflammatory mediators in cancer progression and metastasis. Endogenous molecules derived from injured and necrotic cells might activate TLRs expressed on hematopoietic cells, including macrophages, and these activated cells release inflammatory cytokines and chemokines, which lead to tumor progression and metastasis.

tumor progression and metastasis have not yet been addressed.

INFLAMMATION AND CANCER

Role of Inflammation in Early Tumor Promotion

Cancer is a chronic disease that is caused by defective genome-surveillance and aberrant signal-transduction mechanisms [37]. If infection and inflammation enhance tumor development, they may act through signal transduction mechanisms that influence factors involved in either malignant conversion or genomic surveillance. Chronic inflammation has been proposed to act as an initiating factor in malignancy through the generation of reactive oxygen and nitrogen species (ROS and RNS) and subsequent DNA damage [38]. During chronic inflammation, there is excessive and prolonged generation of ROS and RNS by resident and infiltrating inflammatory cells, which may increase mutational load [38]. One of the enzymes involved in free radical generation is the inducible form of nitric oxide synthetase, iNOS, which is frequently expressed not only in inflamed tissues, but also in premalignant lesions and tumor tissues [39, 40]. However, there is little genetic evidence that chronic inflammation acts as a direct tumor initiator rather than a tumor promoter [41]. Furthermore, a mouse mutant defective in the repair of oxidative DNA lesion was found to be highly susceptible to induction of chronic inflammation, while exhibiting only a very small increase in oncogenic mutations and tumor load [42].

There is growing evidence, however, that chronic inflammation acts as a tumor promoter. A tumor-promoting function for the proinflammatory cytokine TNF-α has been demonstrated in two-stage chemical skin carcinogenesis and other cancer models [43, 44]. The absence of TNF-α, or its type I receptor, TNFR1, confers resistance to skin carcinogenesis [45]. TNF-α does not influence the initiation phase of carcinogenesis; DNA adducts and initiating h-*Ras* mutations occur in its absence. However, epidermal induction of TNF-α is a critical mediator of tumor promotion by phorbol esters, which acts via PKCα- and AP-1-dependent intracellular signal transduction pathway in keratinocytes [43].

In the absence of TNF-α, the epithelial induction of other cytokines and matrix-degrading proteases that

are thought to be important in skin carcinogenesis and tumor–stroma communication is delayed and/or completely absent. Similarly, in a model of chemically induced liver cancer, TNF-α production by hepatocytes has been implicated in tumor development [46]. However, in a different model of liver cancer induced by chronic inflammation, rather than a chemical carcinogen, TNF-α was found to be produced by the tumor stroma [47]. Nonetheless, this stromal TNF-α was found to serve as an important tumor promoter. In this system, as well as in inflammation-induced colon cancer [48], tumor promotion depends on activation of nuclear factor-κB (NF-κB) transcription factor. Selective inhibition of NF-κB in hepatocytes, or inhibition of TNF-α production by neighboring parenchymal cells, induced programmed cell death of transformed hepatocytes and subsequently reduced the incidence of hepatocellular carcinomas [47].

Likewise, ablation of IKKβ, a protein kinase critical for NF-κB activation in intestinal epithelial cells, was found to prevent the development of colitis-associated cancer (CAC) induced by the procarcinogen azoxymethane (AOM) and repeated cycles of dextran sulfate sodium (DSS)-induced colonic inflammation [48]. Deletion of myeloid cell IKKβ also interfered with CAC development, but in this case it mostly reduced tumor size rather than tumor multiplicity. More recent work has shown that part of this tumor-promoting function of myeloid cell IKKβ is mediated through induction of the proinflammatory cytokine IL-6 [49].

Inflammatory Infiltrates in Tumors and Their Effects on Tumor and Metastatic Progression

The outcome of primary oncogenic events in epithelial cells can be significantly modified by the nature of the surrounding nonmalignant cells, such as myeloid populations and stromal cells, underscoring the importance of the inflammatory microenvironment in tumorigenesis and metastatogenesis [3, 50]. The inflammatory microenvironment of neoplastic tissues is characterized by presence of infiltrating cells of hematopoietic origin, such as leukocytes, macrophages, dendritic cells, mast cells, and T cells [51]. However, it is still being debated whether activation of the innate immune system, whose major manifestation is inflammation, contributes to tumor promotion and progression or enhances tumor surveillance and elimination [52]. It was suggested that whereas acute inflammation may inhibit malignancy through activation of T and natural killer (NK) cells, and induction of death cytokines such as TNF-α–related apoptosis-inducing ligand (TRAIL) chronic inflammation promotes carcinogenesis through activation of macrophages and mast cells, which produce tumor-promoting cytokines [3, 50, 53].

Pollard and coworkers have shown that macrophages, major components of the inflammatory microenvironment, are important to the growth and progression of mammary carcinomas by using *macrophage colony-stimulating factor* (*MCSF)-1*-deficient mice [50]. Such tumor-associated macrophages (TAMs) can promote tumor development and metastatic progression through multiple mechanisms, including the inhibition of antitumor T-cell-dependent immunity through production of immunosuppressive indoleamine dioxygenase metabolites; inhibition of DC maturation via secretion of IL-10, transforming growth factor (TGF)-β, and M-CSF; as well as attraction of T regulatory (Treg) cells to the tumor [51]. In addition, TAMs produce numerous cytokines, such as TNF-α, IL-1β, and IL-6; chemokines, such as IL-8, macrophage inflammatory protein (MIP)1, and MIP2; and enzymes that catalyze production of inflammatory mediators, such as cyclooxygenase (COX)-2. All these act to support survival, proliferation, invasiveness, and metastasis of cancer cells. It is now evident that TNF-α, initially heralded for its anticancer activity [54], can actually serve as a tumor-promoting factor [43, 55], and a similar function has been demonstrated for IL-6 [49]. TAMs also secrete matrix metalloproteinases (MMPs) and proangiogenic factors, such as vascular endothelial growth factor (VEGF), that stimulate invasion of surrounding tissues and angiogenesis, as well as ROS and RNS, which enhance genomic instability, cell proliferation, and tumor progression [56, 57].

The capability to express distinct functional programs in response to different microenvironmental signals is a key feature of macrophages, which is typically manifested during pathological conditions such as infections and cancer [58–60]. In response to cytokines and microbial products, mononuclear phagocytes express specialized programs, manifested by different cytokine production profiles that are referred as M1 and M2 polarized macrophages. Classically activated M1 macrophages are induced by IFN-γ alone or in concert with microbial stimuli, such as LPS, or cytokines, such as TNF-α and GM-CSF. On the other hand, IL-4 and IL-13 induce alternatively activated M2 macrophages (Figure 14.2). M1 and M2 macrophages display a number of distinct features. M1 macrophages are characterized by a high capacity to present antigens, high IL-12 and IL-23 production, and consequent activation of polarized type I T cells, having cytotoxic ability toward tumor cells [61]. On the other hand, M2 macrophages have poor antigen-presenting capacity, have an IL-12^{low}, IL-10^{high} cytokine phenotype, suppress inflammatory responses as well as Th1 adaptive immunity, actively scavenge cellular debris, and promote wound healing, angiogenesis, and tissue remodeling [59] (Figure 14.2). Earlier studies with TNF-α–stimulated macrophages or TAMs indicated that under

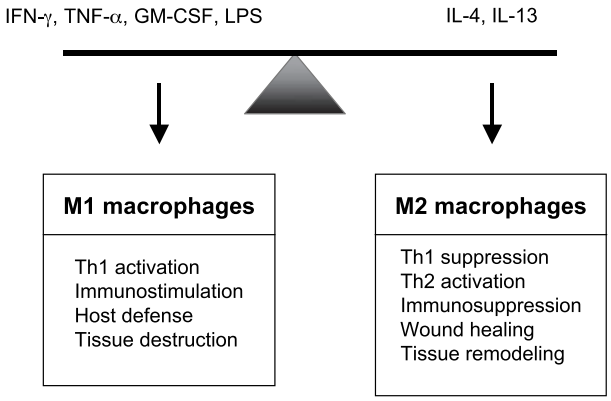

Figure 14.2. M1 vs M2 macrophages. Conventional macrophages (M1) play essential roles in host defense and immune activation. However, M2 macrophages, well observed in tumor microenvironment and injured tissues, are known to suppress Th1 immune response.

certain conditions these cells display cytotoxic functions against cancer cells [62, 63]. However, it is already clear that in the absence of M1-orienting signals, TAMs promote cancer cell growth in vitro and in vivo [60, 63, 64]. Importantly, in many human tumors, a high amount of TAMs has been associated with poor prognosis [1, 51, 64].

Lymphocyte activation and production of MMPs by inflammatory cells are important tumor promoting events in skin carcinogenesis [65]. In a model of cancer metastatic progression, the appearance of distant site metastasis was found to correlate with infiltration of the primary tumors with T cells and other inflammatory cell types that express high levels of the TNF family members RANK ligand (RANKL) and lymphotoxin (LT) α [66]. RANKL administration was found to stimulate the metastatic growth of mammary carcinoma (Tan et al. in preparation). Such findings strongly suggest that activation of nontumorigenic myeloid and lymphoid cells in the tumor microenvironment can provide a major impetus to tumor growth, survival, angiogenesis, and metastasis [3, 50, 53]. The mechanisms that lead to inflammatory cell recruitment and activation within tumors and to tumor growth, angiogenesis, and metastatic progression are not fully understood, and need to be further investigated. Our current mechanistic understanding will be discussed in the following section.

MECHANISMS

How Tumors Create Their Inflammatory Microenvironment

Cancer and inflammation can be linked by two pathways: one is dependent on an underlying inflammatory activation and the other one is not (Figure 14.3). The latter pathway is activated by the genetic events that cause neoplasia, which include activation of protooncogenes by mutations, chromosomal rearrangements, gene amplification, and genetic and epigenetic inactivation of tumor suppressor genes. Genetically transformed cancer cells can produce different inflammatory mediators, which generate an inflammatory microenvironment in tumors for which there is no underlying inflammatory condition or infection (e.g., breast cancer). For instance, it was shown that h-*Ras* activation can result in increased production of the chemokine CXCL-8/IL-8 that recruits inflammatory cells that produce factors that stimulate the growth of malignant, h-*Ras*-transformed, cells [67].

The inflammation-dependent pathway, on the other hand, is based on underlying infections or chronic inflammatory disease that generates an inflammatory microenvironment rich in cytokines and chemokines that enhance the survival and growth of genetically transformed cancer cells that arise within this environment (for example, colorectal and gastric cancers). The two pathways converge through the activation of transcription factors such as NF-κB, signal transducer and activator of transcription (STAT) 3, and hypoxia-inducible factor (HIF) 1α in malignant cells [5, 68].

Within the malignant cell these transcription factors control the expression of prosurvival genes, proangiogenic factors, and MMPs. NF-κB in the inflammatory cells controls the production of cytokines and chemokines that act on the malignant cell as well as the production of proangiogenic factors, such as VEGF. Interestingly, NF-κB is also required for full activation of HIF1α through its effect on transcription of the *Hif1α* gene [69]. In addition to its important role in activation of the proangiogenic program, HIF1α is important for the survival and activation of macrophages and other myeloid cells in the oxygen-poor environment of primary tumors [70]. The concerted action of these transcription factors and the reciprocal interactions between malignant cells and inflammatory cells are likely to play a key role in formation of the inflammatory microenvironment, typical of advanced tumors.

Chemokines, initially defined as soluble factors regulating directional migration of leukocytes during states of inflammation, can be produced by cell types including most human neoplastic cells [71], and play an important role in the formation of the inflammatory microenvironment. The importance of chemokines in malignant progression was first reported using mice that lacked T or NK cell functions but still exhibited typical inflammatory infiltrates when challenged with tumors, suggesting that neoplastic cells either produce chemotactic factors that recruit inflammatory cells or induce the expression of such factors in nearby host cells [72]. Importantly, certain tumor cells not only use chemokines to recruit inflammatory cells but can also

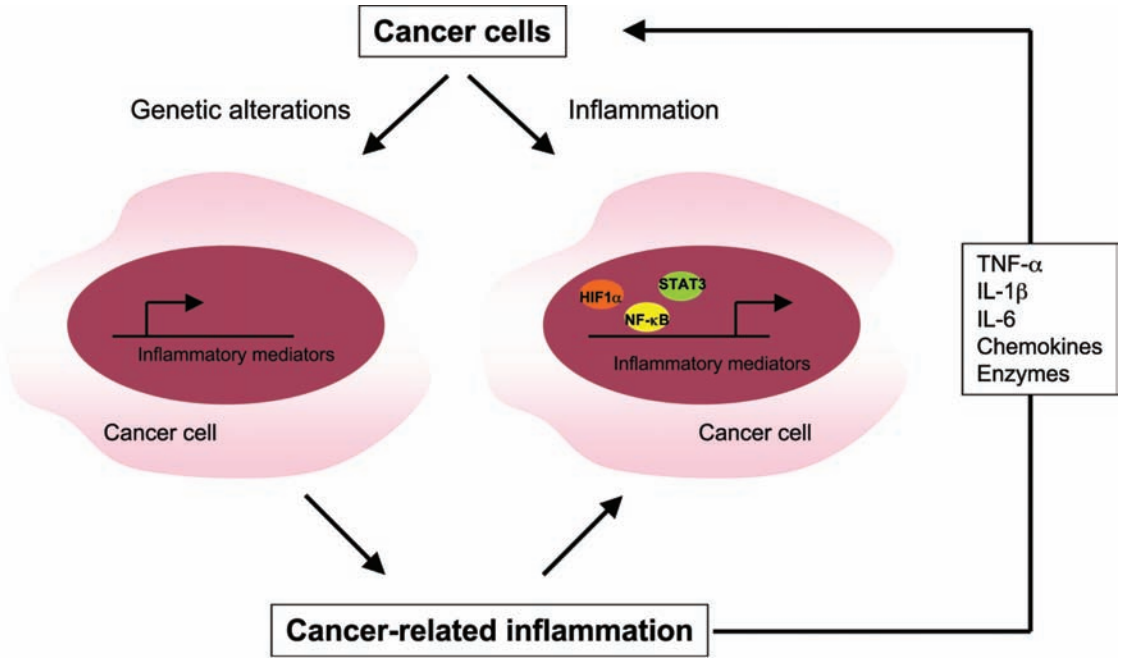

Figure 14.3. Link between cancer and inflammation. Genetically altered cancer cells may produce different inflammatory mediators, generating inflammatory microenvironments in tumors and promoting tumor progression and metastasis.

respond to these factors directly to further enhance their own growth and survival [73–75].

Inflammatory Mediators Enhance Cancer Cell Migration, Invasion, Metastasis

The microenvironment of human and murine cancers is rich in cytokines, chemokines, and enzymes that produce inflammatory mediators, which collectively modulate cancer cell migration, invasion, and metastasis [1, 2] (Figure 14.3). Of particular interest among these factors is TNF-α, a pivotal cytokine in inflammatory reactions. Induced by a wide range of pathogenic stimuli, TNF-α induces other inflammatory mediators and proteases that orchestrate inflammatory responses [44]. High doses of extrinsic TNF-α cause hemorrhagic necrosis and can stimulate antitumor immunity [76]. However, there is increasing evidence that low amounts of TNF-α are produced by malignant and stromal cells within tumors and act as endogeneous tumor promoters [44]. TNF-α is frequently detected in human cancers, being produced by epithelial tumor cells, as, for instance, in ovarian and renal cancer, or by stromal cells, as in breast cancer [44]. Tumor TNF-α production is associated with a poor prognosis, loss of hormone responsiveness, and cachexia. An interesting genetic link between TNF-α and malignancy was identified in renal cell cancer, in which the pVHL tumor suppressor gene is a translational repressor of TNF-α [77]. Despite its ability to induce necrosis at high concentrations in certain cell types, TNF-α frequently acts as a survival factor owing to its ability to promote NF-κB activation [47, 55]. TNF-α also increases vascular permeability and can stimulate the migration, as well as extravasation and intravasation, of cancer cells [78]. In certain cases, TNF-α can also act as a growth factor [55].

Another key inflammatory cytokine, IL-1β, also increases tumor invasiveness and metastasis, primarily by promoting angiogenic factor production by stromal cells in the tumor microenvironment [79–81]. IL-1β is produced mainly by myeloid cells, in which its synthesis is subject to intricate transcriptional and post-transcriptional control [82]. Curiously, whereas NF-κB stimulates IL-1β gene transcription, it inhibits the processing of pro-IL-1β to IL-1β [83]. A related cytokine is IL-1α, which, unlike IL-1β, is secreted mainly by epithelial cells undergoing necrosis [7, 84]. IL-1 receptor activation can lead to induction of IL-6. Curiously, blood levels of IL-6 are elevated with age [85, 86] owing to loss of inhibitory sex steroids [87]. The loss of hormonal regulation of IL-6 has been implicated in the pathogenesis of several chronic diseases [88], including B-cell malignancies, renal cell carcinoma, and prostate, breast, lung, colon, and ovarian cancers [89]. Many of these cancers appear with old age, when circulating IL-6 is high. In multiple myeloma, for example, IL-6 promotes the survival and proliferation of cancer cells via activation of STAT3 and ERK signaling [90]. Using a combination of in vitro experiments and mouse models, we showed that IL-1α released by necrotic hepatocytes can induce the secretion of IL-6 by resident liver macrophages (Kupffer cells) [7]. In turn, IL-6 acts to

promote chemically induced liver carcinogenesis, most likely through activation of the pro-oncogenic transcription factor STAT3 [91].

A range of inflammatory enzymes, including COX-2, that catalyzes the conversion of arachidonic acid to prostaglandins are also induced by cytokines. COX-2 is highly expressed in colorectal, gastric, esophageal, breast, and prostate cancers and non-small cell squamous lung carcinoma [92]. COX-2-produced PGE_2 increases tumor invasion and metastasis, and enhances production of IL-6, IL-8, VEGF, iNOS, MMP-2, and MMP-9 [93]. Selective and nonselective COX-2 inhibitions exhibit chemopreventive and antimetastatic activity in a variety of human cancers [94]. Most likely, this activity is the result of their ability to disrupt the inflammatory microinvironment.

Effect of TLR Agonists and Administered Cytokines on Metastatic Growth

Several publications have demonstrated that strong proinflammatory stimuli can stimulate tumor growth, and suggested that bacterial contamination during surgery or postoperative inflammation triggered by tissue damage can increase metastatic tumor growth in mice [55, 95, 96] and human patients [97]. As described previously, very high doses of TNF-α administered in close proximity to solid tumors can kill both cancer cells and the tumor neovasculature [76]. However, endogenous TNF-α at moderate amounts produced by inflammatory stimuli promotes tumor development and growth [98].

We found that administration of a sublethal dose of LPS, a TLR4 agonist, can stimulate the metastatic growth of colon carcinoma in the lung by inducing the expression of TNF-α [55]. However, LPS administration also leads to induction of the death cytokine TRAIL [55], also known as Apo2 ligand and a type II transmembrane protein of the TNF family, which, in contrast to TNF-α, is a weak inducer of inflammation [99]. Administration of recombinant TRAIL suppresses the growth of tumor xenografts with no apparent systemic toxicity [100]. Endogenously expressed TRAIL on the surface of NK cells can suppress the growth of liver and lung metastases [55, 101]. Inhibition of NF-κB activation in the metastatic cells prevents this protective effect and strongly enhances TRAIL-induced killing of the malignant cell [55]. This inhibition of NF-κB in malignant cells may be used to convert the prometastatic activity of LPS and similar proinflammatory stimuli to a potent tumorcidal effect.

In addition to TNF-α and TRAIL, other cytokines may also affect tumor progression or regression. A considerable effort has been invested in the use of cytokines in cancer therapy [102]. One of the most effective agents identified so far is IFN-α, which evokes antitumor effects in several hematological malignancies and solid tumors [103]. The infusion of high doses of IL-2 was also found to induce regression of renal cell carcinoma and melanoma in a minority of patients [104, 105]. Recently, numerous preclinical studies have established the ability of tumors that were engineered to produce cytokines to serve as cellular vaccines that augment systemic immunity against wild-type tumors (cytokine-based vaccines) [106].

LEWIS LUNG CARCINOMA, LUNG INFLAMMATION, AND METASTASIS

Lewis lung carcinoma (LLC), a commonly used mouse lung cancer cell line, has strong metastatic activity; upon tail vein injection or subcutaneous implantation, it metastasizes to lungs and, to a lesser extent, to liver, lymph nodes, adrenal glands, and bone [107, 108]. LLC cells grown as a primary subcutaneous tumor induce the expression of MMP-9 by lung endothelial cells; this was proposed to somehow "precondition" the lung to become a preferential site for LLC cells to migrate into and establish metastatic growths [107]. Induction of MMP-9 expression in the lung by subcutaneous LLC was shown to be partially dependent on type 1 VEGF receptor (VEGFR1) [107]. These results were confirmed and extended by Lyden and colleagues, who found that LLC secretes factors that stimulate the migration of bone-marrow–derived hematopoietic cells that express VEGFR1 into the lung [108]. The nature of these factors and their mode of action remained unknown until recently.

Using a biochemical approach, we have identified one of the most critical factors secreted by LLC as versican, an extracellular matrix (ECM) prometastatic protein that can induce the activation of macrophages and stimulate the secretion of TNF-α (Figure 14.4) [34]. We found that conditioned medium collected from LLC cells activated TLR2 on macrophages to induce NF-κB and MAP kinase (MAPK) signaling and thereby stimulate the expression of proinflammatory cytokines such as IL-6 and TNF-α. Importantly, subcutaneous LLC tumors led to TLR2 activation in vivo, which was important for induction of various inflammatory cytokines and chemokines in the lung, including TNF-α, IL-6, IL-1, CCL3/MIP1α, CCL4/MIP1β, CXCL1/MIP2, and CXCL2/KC. Both TLR2 on host bone-marrow–derived cells and TNF-α have turned out to be critical for optimal metastatic growth of either tail-vein injected or subcutaneously implanted LLC (Figure 14.4) [34].

We used column chromatography and mass spectrometry to identify the LLC-secreted factor that is responsible for TLR2 activation and stimulation of metastatic growth. The critical factor has turned out to be the ECM component versican [34, 35], an aggregating chondroitin sulfate proteoglycan that was

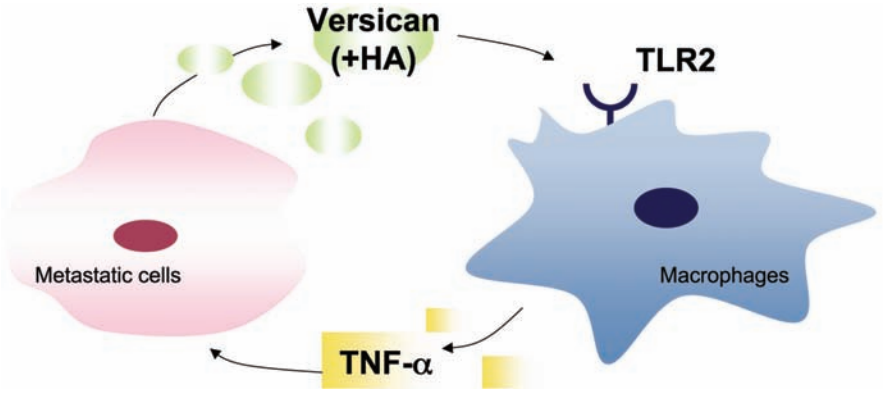

Figure 14.4. Metastatic progression via TLR2-mediated activation of macrophages. Versican, an extracellular matrix protein, can be released from cancer cells and plays as a ligand of TLR2, expressed on macrophages. Activated macrophages release inflammatory cytokines and chemokines, which are fundamental components of inflammatory microenvironment in tumors and promote metastasis progression.

previously found to accumulate both in the tumor stroma and in cancer cells, including lung cancer [109]. We found that versican can bind to TLR2 and its coreceptor CD14 and can lead to activation of TLR2:TLR6 heterodimers (Figure 14.4) [34, 35]. Versican expression, which is very high in several cancers, including lung cancer [109–111], is stimulated by signaling pathways that are known to be activated in cancer cells [112]. Furthermore, versican or its fragments can enhance tumor cell migration, growth, and angiogenesis, processes that are of direct relevance to metastasis [113].

Versican can bind HA; both versican and HA are highly expressed in non–small-cell lung cancer (NSCLC), especially in advanced disease with high recurrence rates, whereas versican expression in the normal lung is low [109]. In addition to HA, versican can also interact with several adhesion molecules expressed by inflammatory cells and is in possession of proinflammatory activity [35].

A related ECM proteoglycan, biglycan, was reported to activate both TLR2 and TLR4 on macrophages [114], but our results indicate that the proinflammatory activities of versican rely on TLR2 and TLR6 but not on TLR4, activation. Silencing of versican expression in LLC cells eliminates their metastatic behavior [34, 35].

In summary, our results suggest that LLC cells secrete versican to activate hematopoietic-derived cells and recruit them to generate an inflammatory microenvironment and produce TNF-α, stimulating the metastatic behavior of LLC. Although we still must confirm the role of versican in the metastatic progression of human NSCLC, we have observed that other metastatic cells can also lead to TLR2-dependent macrophage activation but through other secreted factors. The molecular nature of these factors remains unknown, but the principles that guide their proinflammatory and prometastatic activity are likely to be similar to those established for versican.

METASTATIC CELLS SHARE SIMILAR CHARACTERISTICS TO CELLS INVADED IN WOUND HEALING, VASCULAR REMODELING, AND INFLAMMATION

Other than the studies described previously, it has been noted that tumorigenic and metastatic progression share many features with the process of wound healing [115, 116]. For instance, solid tumors must induce new blood vessels if they are to grow beyond minimal size. As established for the neoangiogenic process that accompanies wound healing, tumors secrete vascular permeability factors, such as VEGF, that render the local microvasculature permeable to fibrinogen and to other plasma proteins. Extravasated fibrinogen is rapidly clotted and invaded by macrophages, fibroblasts, and endothelial cells. It then undergoes "organization" and is replaced by vascularized granulation tissue, which finally becomes mature connective tissue. The same sequence of events (angiogenesis) found in tumors also occurs during wound healing and in a range of chronic inflammatory diseases. However, the molecular alterations that allow tumors to behave like wounds that do not heal [116] (invading tumor cells continually render new vessels hyperpermeable to plasma, which does not occur in a normal wound healing process) are just being elucidated.

TGF-β, a key cytokine during embryonic development and tissue homeostasis [117], exerts potent inhibitory effects on epithelial cell proliferation and also can deter tumor growth [117–119]. TGF-β can be produced by myeloid cells, mesenchymal cells, and cancer cells subjected to hypoxic and inflammatory conditions during tumor progression, and is one of major

cytokines in tumor microenvironments. Interestingly, it has been found that TGF-β in breast tumors primes cancer cells for metastasis to the lungs [120]. Central to this process is TGF-β-dependent induction of *angiopoietin-like 4* (*ANGPTL4*) in cancer cells that are about to enter the circulation, which enhances their subsequent retention in the lungs [120]. Tumor-cell–derived Angptl4 disrupts vascular endothelial cell–cell junctions, increases the permeability of lung capillaries, and facilitates the transendothelial passage of tumor cells [120]. Although this work describes molecular bases for the vasculature disruptive proactivity of TGF-β, it is likely that the prometastatic activity of this cytokine depends on other processes as well.

INFLAMMATION AND TRANSCRIPTIONAL CONTROL OF METASTATIC GENES

Inflammatory cytokines, chemokines, and the proinflammatory microenvironment also have a role in shaping the gene expression profile that is required for metastatic behavior of cancer cells, and they include mediators of vascular remodeling, such as integrins, VCAM, and MMPs [51, 121].

A key transcription factor in metastasis is the helix–loop–helix protein Twist, which regulates cell movement and tissue reorganization during early embryogenesis [122]. Suppression of Twist expression in metastatic 4T1 mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung [122]. Importantly, the ability of these cells to form primary mammary tumors was not affected. Loss of Twist expression hindered the entry of metastatic cells into the circulation. Like several other controllers of tumorigenesis, Twist is likely to exert similar biological activities during metastatic progression as it does during normal development. In *Drosophila*, the *twist* gene is required for mesoderm induction [123, 124]. In vertebrates, Twist is expressed predominantly in neural crest cells; its ablation in mice causes failure in cranial neural tube closure, indicating a role in migration and differentiation of neural crest and head mesenchymal cells [125, 126]. Both mesoderm formation and neural crest development depend on a key cellular event, termed the epithelial–mesenchymal transition (EMT), which involves the conversion of a sheet of tightly attached epithelial cells into highly mobile mesenchymal or neural crest cells [127]. Indeed, ectopic expression of Twist resulted in loss of E-cadherin–mediated cell–cell adhesion, activation of mesenchymal markers, and gain of motility by malignant cells [122]. These results suggest that Twist can contribute to invasion and metastasis by promoting the EMT developmental program. Under certain circumstances, Twist expression can be induced in response to NF-κB activation [128] and therefore can be upregulated in response to inflammation. This provides a mechanism through which tumor-associated inflammation may stimulate metastatic progression through induction of Twist-dependent EMT.

We have identified another mechanism by which tumor-associated inflammation can affect the expression of key metastasis control genes. By studying the TRAMP model of metastatic prostate cancer, we found that appearance of distant site metastasis and the metastatic behavior of isolated carcinoma cells are dependent on the activation and nuclear accumulation of IKKα [66]. Analyzing which of about forty known genes that either enhance or suppress metastasis [129] is affected by inactivation of IKKα during metastatic progression revealed that IKKα exerted its prometastatic effect by repressing transcription of the *maspin* gene [66]. Maspin is a member of the serpin family with well-established antimetastatic activity in breast and prostate cancers [130, 131]. Repression of maspin expression required nuclear translocation of catalytically active IKKα; the two processes occur only in advanced prostate tumors that contain inflammatory infiltrates and cells that express RANKL and LTα [66]. Early tumors devoid of inflammatory infiltrates do not show activated IKKα and, in contrast to late tumors, express high amounts of maspin and therefore lack metastatic activity. In vitro, RANKL can lead to repression of maspin expression in an IKKα-dependent manner.

CAN WE USE ANTIINFLAMMATORY DRUGS TO BATTLE METASTATIC CANCER?

As discussed previously, there is ample evidence from experimental cancer models in rodents and investigation of human cancer that continuous/chronic inflammation stimulates tumorigenesis and metastatic progression. If so, therapies that are directed at reducing inflammation, inhibiting the function of inflammatory cytokines, or preventing the recruitment of inflammatory cells onto the tumor microenvironment could reduce cancer risk, slow tumor progression, and possibly even decrease the burden of metastasis. Indeed, the use of nonsteroidal antiinflammatory drugs (NSAIDs) and aspirin was found to reduce colon cancer risk by 40 percent to 50 percent and may also have a significant preventative effect in lung, esophageal, and stomach cancer [132, 133]. The ability of NSAIDs to inhibit COX-1 and COX-2 underlies their mechanisms of chemoprevention. Other NSAIDs – flubiprofen, for example – were found to have strong antimetastatic effects, which were attributed to their inhibition of platelet aggregation [134]. NSAIDs may act through additional mechanisms, though, as some NSAIDs lacking COX-inhibitory function are also effective in inhibiting colon carcinogenesis [135].

Unfortunately, some of the current NSAIDs, especially those that are COX-2 selective, exert side effects such as life-threatening stomach ulcers, heart attacks, and strokes in a considerable number of patients [136], which limits their utility. The continuing study of the molecular mechanisms that lead to inflammatory cell activation within tumors and to tumor growth, angiogenesis, and progression should help in the identification of new therapeutic targets and aid in the design of new drugs that are free of such side effects. This understanding may also aid the development of vaccines and other strategies to enhance antitumor immunity.

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15 Proteolytic Cascades in Invasion and Metastasis

Steven D. Mason and Johanna A. Joyce

During tumor progression, there are several key stages that require the action of proteases (Figure 15.1). First, the induction of angiogenesis, or new blood vessel growth, involves degradation of the vascular basement membrane and the release and/or activation of matrix-bound proangiogenic growth factors. Second, invasion of cancer cells into the surrounding tissue requires the dissolution of cell–cell junctions and degradation of the epithelial basement membrane/extracellular matrix (BM/ECM) for cancer cells to spread from the primary tumor. Third, at least two key steps in metastasis require proteolysis: intravasation of cancer cells into the blood or lymphatic circulation at the primary site, and extravasation at the secondary site, where proteases can again play a role in promoting the colonization and growth of cancer cells as they do in the primary tumor.

Proteases not only are essential for the degradation of BM/ECM proteins, however; they also have more specialized processing roles that are important for cell signaling, such as in the restricted cleavage of prodomains and subsequent activation of growth factors and cytokines. These functions are tightly regulated in a cascade of proteolytic interactions, allowing for control and amplification of proteolysis in metastasis.

The realization that proteolysis is necessary for multiple stages in metastasis emphasizes the importance of identifying the key tumor-promoting proteases, determining how individual proteases interact with each other, and developing therapeutic strategies to inhibit their actions. In this chapter, we discuss how proteases typically function in complex and interconnected cascades that are used by both cancer cells and stromal cells in the microenvironment to facilitate tumor invasion and metastasis.

BACKGROUND AND HISTORICAL CONTEXT

The human genome contains at least 570 proteases and 156 endogenous protease inhibitors, making this one of the largest functional groups in mammals. There are five protease classes: aspartic, cysteine, metallo, serine, and threonine proteases, which are classified based on their catalytic mechanism (e.g., cysteine proteases require a cysteine residue in the active site). With literally thousands of targets, proteases play a role in regulating virtually every cellular process, providing multiple opportunities for tumor cells to exploit their activity to promote proliferation, invasion, and metastasis. To date, much of the research on proteases and their involvement in cancer has focused on their roles in ECM remodeling and promoting invasion. Several clinical trials have examined the possibility of inhibiting matrix-remodeling proteases and/or protease families as cancer therapy.

The most notable example of protease inhibition in clinical trials is that of the broad-spectrum matrix metalloproteinase (MMP) inhibitors, which were tested in Phase I, II, and III clinical trials. Unfortunately, promising preclinical data did not translate into clinical success, as patients experienced negligible benefit from therapy [1–3]. Inhibitors targeting the proteasome have had more success, as cancer cells (for as yet unclear reasons) are more sensitive than normal cells to proteasome inhibition. To date, proteasome inhibition has shown therapeutic efficacy in multiple myeloma, in combination with traditional chemotherapeutic agents [4]. Finally, cathepsin K, a cysteine protease, which has been found to be important in bone resorption, is also upregulated by cancer cells and stromal cells in the bone metastatic microenvironment. Small-molecule inhibitors targeting cathepsin K are currently being tested in Phase II clinical trials for their ability to treat bone metastases [5].

From the converse perspective, several proteases have been implicated as suppressors of tumorigenesis through various lines of evidence [6]. Notable among this group of proteases are the caspases, which participate in a complex cascade triggering apoptosis, and

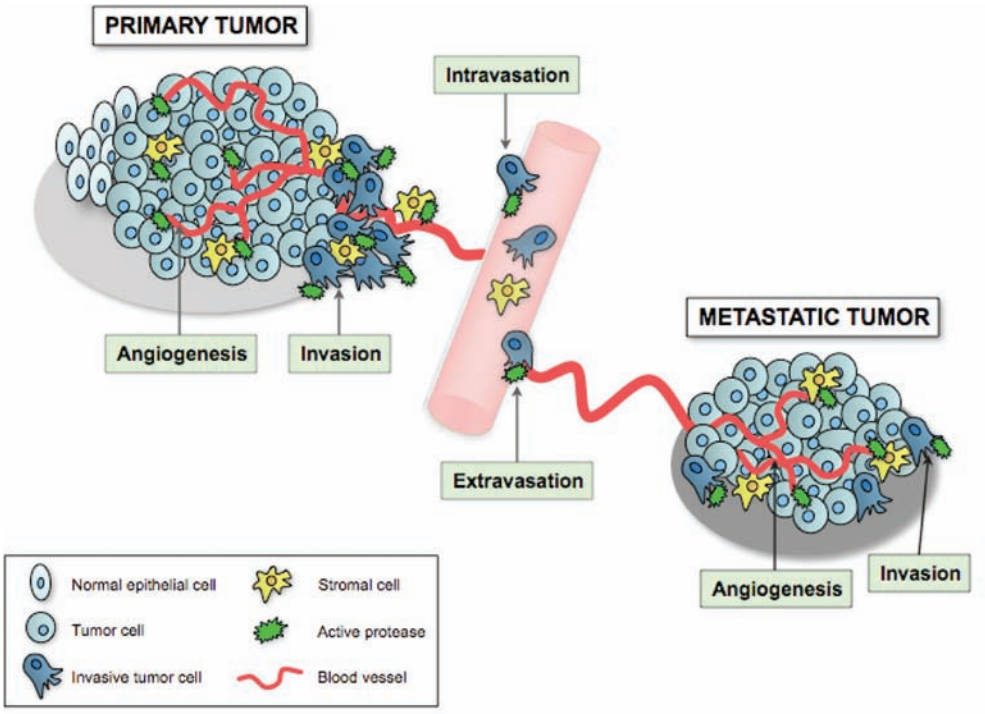

Figure 15.1. Roles of proteases in the metastatic cascade. Proteolytic activity is essential at several different steps in the metastatic cascade, including angiogenesis and invasion of the primary tumor into the surrounding stroma, intravasation and extravasation of cancer cells into and out of circulation, and angiogenesis and invasion of the metastatic tumor into the new host tissue. Proteases can be provided by both tumor cells and stromal cells, as highlighted in Figure 15.3.

are frequently mutated in tumors [7–10]. Other proteases have been found to be tumor-suppressing under some conditions, but tumor-promoting under others. Specific examples of proteases in this category are discussed throughout this chapter, but it is worth noting at this point that several MMPs also fall into this category, giving a potential explanation to the failure of MMP inhibitors in the clinical setting.

As can be inferred from the preceding information, much of the research on proteases, and targeting of their activity in cancer, to this point has focused primarily on individual proteases and their functions. However, the picture of proteases in cancer is rapidly evolving, from one of single proteases to a complex network of intricate – and sometimes overlapping – proteolytic interactions, similar to a classical view of a signaling cascade (Figure 15.2). Viewing proteases as members of a proteolytic cascade can provide the appropriate context to their activity, allowing a thorough assessment of their targets and biological impact, thus giving a clearer picture of the "degradome," a term coined by Lopez-Otin and Overall [11] to describe the entire spectrum of proteases expressed under defined conditions by a cell, tissue, or organism along with the complete array of substrates available under the same condition. One classic example of a proteolytic cascade that is essential in normal physiology is the blood coagulation pathway [12], which serves as a useful comparison to illustrate how the functions of key instigator proteases at the top of a cascade can be amplified through effector proteases at multiple steps along the way.

Within the context of a proteolytic cascade, which inherently has tremendous destructive potential, proteolytic activity must be controlled at multiple levels to safeguard the cell and organism from aberrant or excessive proteolysis. Like many genes, proteases are regulated at the transcriptional level in response to intracellular and extracellular stimuli, such as growth factors and hypoxia. Proteases are synthesized as inactive precursors that must undergo some form of conformational change or proteolytic cleavage before being able to act on their substrates. Additionally, many proteases have endogenous protein inhibitors that can directly impact their proteolytic activity. A final level of regulation lies in their subcellular localization; for example, the compartmentalization of proteases in the lysosomes or at the cell surface of normal cells limits their ability to activate a wide range of potential substrates. The network of activating proteases, their endogenous inhibitors, and targets thus forms an intricate cascade of proteolytic interactions.

Viewing protease–substrate interactions as part of a cascade reveals several important concepts for understanding proteolytic activity (Figure 15.2). First, it can

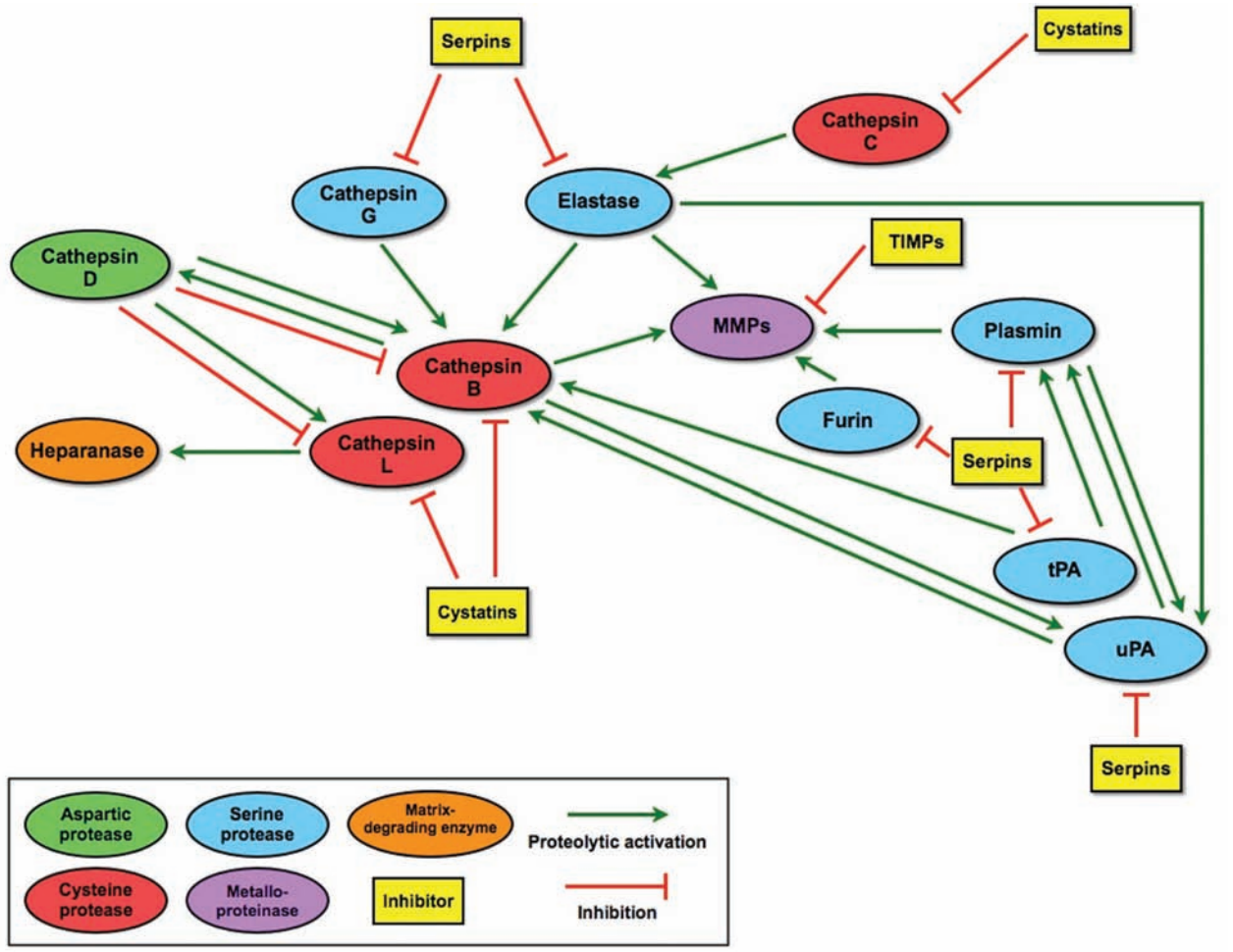

Figure 15.2. Key interactions in the proteolytic cascade. A wide range of catalytic mechanisms is involved in this example of a proteolytic cascade, which can accurately be termed a proteolytic "web," given the interconnectivity of the proteases involved. Several key "nodes" can be seen at cathepsin B, elastase, and MMPs, revealing common pathways that various cancers may exploit. Endogenous protease inhibitors can regulate many of the proteases involved, adding another layer of complexity to the cascade.

be seen that there are a few key upstream proteases through which several pathways pass, revealing points of convergence at which multiple processes can be affected through inhibition or activation of a single protease. One example of this is the aspartic protease cathepsin D, which can activate several other downstream proteases, which in turn either act on other proteases or cleave nonprotease substrates. Another key concept is the way in which signals can be amplified through the cascade. One upstream protease may activate a few direct propeptide proteases, each of which may also activate additional propeptide substrates, resulting in a pronounced increase in proteolytic activity. Finally, it can be seen that there are many feedback loops and much redundancy in the cascade; several proteases can each be activated by multiple different upstream proteases.

Although the concept of a proteolytic cascade leading to ECM degradation has been established for some time, proteolytic cascades involved in controlling other tumorigenic processes have also been elucidated. A notable example is the caspase cascade leading to apoptosis, in which different apoptotic stimuli lead to activation of initiator and executioner caspases that ultimately result in programmed cell death. Caspases and the caspase cascade are covered more extensively elsewhere and will only be given a brief overview here.

THE PROTEOLYTIC CASCADE: INTRACELLULAR AND EXTRACELLULAR PATHWAYS

The proteolytic cascade can be divided into categories based on subcellular localization of the interactions. Some reactions are intracellular, taking place in the cytosol or lysosomes, or at the intracellular surface of the plasma membrane. However, many reactions are extracellular, occurring in the ECM, on the extracellular side of the plasma membrane, or in specialized

structures such as invadopodia, podosomes, and caveolae. This mechanism of interaction and activation has been observed with cathepsin B and some of its substrates, as well as several MMPs. We will discuss proteolytic cascades operating inside and outside the cell, with several proteases acting in both locations.

Intracellular Interactions

The proteolytic cascade is initiated intracellularly through activation of upstream proteases in the cytosol or lysosomes and rapidly expands to include proteases of different types in distinct locations. The lysosome is the consensus location for the beginning of the cascade, although several lysosomal proteases are translocated to the cytosol and/or secreted by cancer cells [13], leading to open questions about where the cascade truly begins.

Cathepsin D

One family of proteases prominently involved in metastasis is the cathepsin family, which in humans is comprised of fourteen members: two proteases with an aspartic residue in the active site (cathepsins D and E), one serine protease (cathepsin G), and eleven cysteine proteases (cathepsins B, C, F, H, K, L, O, S, W, V/L2, and X/Z). As seen in Figure 15.2, cathepsin D is an upstream intracellular protease in the proteolytic cascade. Normally targeted to lysosomes [14], cathepsin D has been implicated in cancer progression and metastasis for some time [15, 16], and the proform can be secreted by cancer cells [17]. Regulation of cathepsin D occurs at many levels. Transcriptionally, it can be upregulated by hypoxia [18] and by a pathway induced by the serine protease thrombin [19]. The proteolytic activity of cathepsin D is regulated by pH (cathepsin D is active up to a pH of 6.2 in vitro [20]) and cleavage of the synthesized proform. Interestingly, there is no known endogenous protein inhibitor for cathepsin D, distinguishing it from the majority of other proteases.

Cathepsin D is initially synthesized as a 52-kDa precursor, which binds to the mannose-6-phosphate (M6P)-receptor for targeting to the lysosome and cleavage to a 48kDa intermediate that is catalytically active [21, 22] but is further cleaved into a mature enzyme consisting of two separate peptides – a 14-kDa chain and a 34-kDa chain [14]. Two cysteine proteases – cathepsins B and L – have been implicated in the processing of the 48-kDa form of cathepsin D to the 34-kDa form [14]. Cathepsin D can also be autoactivated in an acidic environment (pH <5), although the product is pseudocathepsin D and the physiological relevance is questionable [23].

Targets of cathepsin D include proteases, protease inhibitors, ECM proteins, and chemokines. Cathepsin D can affect cysteine protease activity in two different ways. First, it activates the proforms of cathepsins B and L, creating an activation feedback loop with its two activating proteases [24]. Additionally, it cleaves and inactivates several cystatins, which are endogenous cysteine cathepsin inhibitors [25], further enhancing cysteine cathepsin activity.

As indicated previously, several lines of clinical evidence implicate cathepsin D in cancer progression and metastasis. Cathepsin D (and its proform) is upregulated by up to fiftyfold in breast cancer tissue, relative to normal mammary tissue [26]. Additionally, and of relevance to the dysregulation of cathepsin D in cancer, cytosolic levels of cathepsin D function as a prognostic indicator for distant metastasis and survival [27, 28]. Finally, a large-scale analysis of patient samples from multiple data sets has found that cathepsin D levels can be used as a marker of the aggressiveness of breast cancers [29]. The importance of cathepsin D to tumor progression and metastasis has also been demonstrated in vivo, as highly metastatic breast cancer cell lines exhibit increases in cathepsin D expression [30, 31], and knockdown strategies targeting cathepsin D result in decreased breast cancer xenograft tumor growth and metastasis to the lung [19, 32], although the exact mechanisms for these effects are still unresolved.

Cathepsin B

One direct target for cathepsin D is cathepsin B, a cysteine protease that is also normally found in the lysosome. Transcriptionally, cathepsin B expression is controlled by the Sp1, Sp3, and Ets1 transcription factors [33]. The Ets1 connection is of particular relevance to cancer, as Ets1 expression often correlates with poor prognosis in various tumor types and Ets1 also upregulates several other proteases [34]. Other nonproteolytic mechanisms of cathepsin B regulation include mRNA stabilization [35], use of alternate promoters [36], and alternative splicing [37].

In addition to cathepsin D, other proteases that may activate cathepsin B include cathepsin G, elastase, tissue plasminogen activator (tPA), and urokinase plasminogen activator (uPA), giving many access points to cathepsin B in the proteolytic cascade [24] (Figure 15.2). One of the most potent inhibitors of cathepsin B is cystatin C [38], which can be inactivated by elastase, providing another means for elastase to enhance cathepsin B activity. In addition, the proteolytic targets of cathepsin B are wide and varied, ranging from proforms of other proteases, to proteolytic inhibitors, to ECM components. The variety of proteases that can regulate cathepsin B, and its wide-ranging downstream proteolytic targets, make it a central node in the proteolytic cascade (Figure 15.2). However, this view of cathepsin B must be tempered by the knowledge that it

is also one of the most extensively researched proteases; it is quite possible that other proteases have as wide a scope of regulators and targets, but the full extent of their activity has not yet been uncovered.

Some of the primary effects of cathepsin B activity have been found in ECM degradation and angiogenesis, but whether cathepsin B exerts these effects directly or through other proteases is open for debate, and may, quite possibly, involve contributions from both, or depend on the tissue context. The extracellular activities of cathepsin B will be discussed later, but several of its proteolytic interactions could occur either intracellularly or extracellularly. These include its ability to activate MMPs and uPA and to inactivate tissue inhibitor of metalloproteinase (TIMP). A role for intracellular cathepsin B activity in tumor invasion has been observed using cell-permeable inhibitors that function only in the intracellular environment, in which melanoma and prostate cancer cells exhibited reduced invasion [39], revealing that intracellular cathepsin B is important for degradation of internalized collagen IV [40]. Collagen fragments have been found inside cancer cells in several different tumor types, including breast, colon, and prostate cancer. Additionally, stromal fibroblasts and macrophages in different tumor microenvironments have also been shown to internalize collagen for degradation, highlighting the contributions of stromal cells to proteolytic degradation of the ECM [41]. The discovery that intracellular cathepsin B can degrade components of the ECM indicates the importance of considering therapeutic approaches that target intracellular as well as extracellular proteases.

Similar to cathepsin D, several clinical correlations have implicated cathepsin B in tumor progression, with distinct expression patterns in different tumor types. Expression of cathepsin B in tumors has been reported in both cancer cells and stromal cells, implicating different stromal components in tumor progression and metastasis, as this protease is detected in breast and colon carcinoma cancer cells, macrophages, and fibroblasts [42, 43]. Other tumors derive cathepsin B expression primarily from either macrophages [43] or cancer cells [44] alone. Several cell lines and tumors with high metastatic potential also exhibit elevated cathepsin B expression, making cathepsin B an important protease to target in anticancer therapy.

Two potent endogenous inhibitors of cathepsin B are cystatin C and cystatin E/M; cystatin E/M is often downregulated during tumor progression [38]. Conversely, and somewhat surprisingly, lung tumors formed by melanoma cells injected into cystatin C null mice were significantly smaller than tumors injected into wild-type mice [45]. However, this result is tempered by other studies demonstrating that overexpression of cystatin C inhibits lung metastasis in other murine models [46, 47], indicating that its role in cancer progression likely extends beyond its effects on cathepsin B and is dependent on other microenvironmental factors.

Cathepsin L

Another direct activation target of cathepsin D is cathepsin L, a protease that shares many similarities with cathepsin B in terms of regulation and substrates. The cathepsin L locus has alternative promoters and splice sites [48, 49] and is often highly upregulated in malignant cells, potentially owing to expression of a shorter transcript. One difference between cathepsin L and cathepsin B is the ability of the proform of cathepsin L to cleave fibronectin and laminin without being fully activated [50]. Fully activated cathepsin L can also cleave type I and IV collagens [50].

One important substrate of cathepsin L is the matrix-degrading enzyme heparanase, the only enzyme in the human genome with the demonstrated ability to cleave heparan sulfate proteoglycans. Heparanase has been shown to have a role in promoting tumor progression and metastasis in melanomas and pancreatic cancer, possibly through its ability to free bound growth factors from the ECM. The ability of cathepsin L to activate heparanase has been demonstrated in vitro, and deletion of cathepsin L in mice leads to increased levels of the proform of heparanase in vivo [51].

Cathepsin L is more highly expressed in cancer cells than in stromal cells in the RIP-Tag mouse model of pancreatic islet cancer [52], but it can also be secreted from activated macrophages [53]. Deletion of cathepsin L in RIP-Tag mice significantly inhibited tumor growth, proliferation, and invasion while increasing apoptosis [54], indicating that targeting the cathepsin L protease regardless of its source may be a beneficial therapeutic strategy in cancer. Additionally, targeting cathepsin L in xenograft models using a stably expressed anti-cathepsin L single chain variant [55] or a small-molecule inhibitor [56] resulted in decreased melanoma metastasis to the lung or bone, respectively. Supporting the connection between cathepsin L and tumor progression, clinical studies have revealed elevated cathepsin L activity in breast, prostate, and colon cancers [57–61].

However, some evidence indicates that cathepsin L may have tumor-suppressing functions, depending on the tissue environment. Deletion of cathepsin L in mice leads to enhanced proliferation in the skin [62] and increased tumor incidence in the Apc^{Min} colorectal adenoma model [63]. Furthermore, overexpression of human hurpin (serpin B13), a potent cathepsin L inhibitor, in mice results in increased susceptibility to chemically induced carcinogenesis [64]. Additional endogenous inhibitors of cathepsin L include cystatins C, D, E/M, SA, and SN; investigation of these inhibitors in cancer is still incomplete. A further line of evidence

that supports cathepsin L functioning as a potential tumor suppressor comes from its ability to cleave the proapoptotic protein Bid in vitro, thus potentially triggering apoptosis. Therefore, it is possible that cathepsin L has opposing roles in tumor development, in a tissue-specific manner, at least in the animal models examined to date; thus, the microenvironmental context will need to be considered before the actual scope of cathepsin L activity in cancer can be fully understood.

One important area of regulation of cathepsins with particular relevance to cancer is pH. As cathepsins are primarily lysosomal enzymes, they generally function optimally at acidic pH levels (pH < 6), with the exception of cathepsin H, which has optimal activity at pH 6.8 [65], and cathepsin S, which has optimal activity of pH 6.5 [66]. As for the question of whether cathepsins exert their functions in cancer and metastasis in an intracellular or extracellular context, the pH dependence would appear to swing the pendulum in the direction of intracellular importance. However, cathepsins B and L are stable up to pH 7, cathepsin C is stable up to pH 7.5, and cathepsins H, K, and S are stable up to pH 8 [65]. Additionally, it is widely reported that the tumor microenvironment is acidic, and in vivo pH measurements indicate extracellular pH values of ~6.2 to 6.9, depending on tumor size [67, 68]. This places the tumor microenvironment acidity at a level for which cathepsins would be expected to be stable and potentially active, which, when coupled with the upregulation of many cathepsins in cancer and metastasis, provides experimental support to their potential to have extracellular functions. Validation for this concept comes from a study in which culturing melanoma cells in slightly acidic conditions (pH 6.8) resulted in increased experimental lung metastasis. This increase could be blocked by inhibitors of MMPs and cysteine cathepsin proteases, implicating members of both protease families in this process [69].

Elastases

Elastases are serine proteases that, like cathepsins, can contribute to the metastatic process at several different steps in the proteolytic cascade (Figure 15.2). There are three main categories of elastases: porcine pancreatic elastase, neutrophil elastase (NE), and metalloelastase. NE has received the most attention with regard to cancer, and is the only one of these that, to date, has been demonstrated to break down insoluble elastin [70], thus giving it a potential role in ECM remodeling, and an obvious connection to metastasis.

Transcriptional regulation of NE occurs through the activities of core-binding factor, lymphoid enhancer-binding factor 1, Gfi-1, and C/EBP [71, 72]. Synthesized as an inactive proprotein, elastase can be activated by cathepsin C [73] or plasmin, and is a major component of neutrophil granules (along with cathepsin G, collagenase, gelatinase, and certain MMPs), in which it can activate many other proteins [74]. Proteases activated by elastase include cathepsin B, uPA [75], and several MMPs [50]. As many of the NE targets are extracellular proteins, or ECM components, further discussion of elastase targets (and inhibitors) can be found in the subsection on secreted proteases.

Several lines of evidence implicate NE in cancer progression. Inhibitors of NE (some more specific than others) can block or reduce the development of lung metastases [76, 77], and suppress the ability of cancer cells to adhere to endothelial cells in vitro [78]. Elastinolytic activity has been detected in breast cancer patients [79], and elevated levels of NE were associated with a significant reduction in disease-free survival [80]. Similarly, non–small-cell lung cancer patients with increased NE expression have decreased overall survival [81]. Interestingly, NE expression in cancer is not necessarily restricted to infiltrating neutrophils, as breast and lung cancer cell lines have also been found to express NE [82, 83].

Furin and Other Proprotein Convertases

Furin is a serine protease that belongs to the proprotein convertase (PC) family of proteases, a group of Ca^{2+}-dependent endoproteases that is also referred to as the prohormone convertase family. To date, there are eight other known human PCs in addition to furin; four of these (including furin) contain a transmembrane domain that confines their activity to the trans-Golgi network (TGN) and the cell surface [84]. PC1 and PC2 are found in secretory granules, in which they process proteins cleaved by the regulated secretory pathway [84]. Like other proteases, PCs are synthesized as inactive zymogens before undergoing autocleavage and activation [85]. For furin, this autocleavage occurs in the endoplasmic reticulum prior to its entering the TGN [86, 87]. Interestingly, the propeptide that is cleaved from PCs also functions as the endogenous inhibitor for PCs. Multiple cleavages of the propeptide result in its complete dissociation from the protease, and complete activation of furin [88]. The ability of PCs to autoactivate places them at a new entry point to the proteolytic cascade, independent of cathepsin D, the cysteine cathepsins, or elastase (Figure 15.2).

Although furin and PCs exhibit a wide range of proteolytic substrates, only a handful of those are other proteases. These include MMP-11 and MT1-MMP (also referred to as MMP-14), which are activated by furin and PACE in the TGN [50]. Other PC targets that have specific relevance to metastasis include insulin-like growth factor and its receptor, integrins, and VEGF-C [88]. Along those lines, several studies have revealed strong correlations between PC expression and

tumor progression. These include overexpression of PC1 in pheochromocytomas [89], lung carcinoids, and small-cell lung cancer [90]. Additionally, aberrant furin expression has been found in breast cancer [91], colon cancer, and head and neck cancer [92]. Upregulation of furin in hepatoma cell lines resulted in increased metastasis to the lung [93], and overexpression of the PC inhibitor alpha1-PDX (a variant of serpin A1) in colon carcinoma cells resulted in decreased liver metastasis [94]. As a result, several groups are trying to inhibit furin and PCs using inhibitors based on PC propeptides, chloromethyl ketones, and serpin A1 [84].

Matrix Metalloproteinases

Several MMPs can be activated either intracellularly or at the plasma membrane. A more detailed discussion of MMPs can be found elsewhere, and therefore only a brief discussion of MMPs in the proteolytic cascade will be mentioned here and in the section on extracellular interactions. As discussed previously, proteases that carry out activation of some MMPs include cathepsin B, elastase, and furin. Nine MMPs can be activated by furin, resulting in the removal of an electrostatic thiol interaction. As the other eighteen known MMPs lack furin-recognition domains, their mechanism of activation is still not completely clear [95], but potential mechanisms for activation include oxidizing or alkylating agents, allosteric activation, and activation by other proteases (including plasmin, cathepsin B, elastase, and other MMPs). Other nonproteolytic proteins may also be involved. For example, activation of MMP-2 by the combined interactions of MT1-MMP and TIMP2 has been demonstrated, which reveals a novel and potentially opposing role for a protein (TIMP2) generally considered to be an MMP inhibitor [96, 97]. However, many of these activation mechanisms have been demonstrated only in vitro, and in vivo evidence confirming their physiological relevance is currently lacking.

Caspases and Apoptosis

One of the best-characterized proteolytic pathways is the intrinsic apoptotic pathway consisting of a cascade of proteolytic interactions mediated by caspases. Although apoptosis in metastasis is discussed more thoroughly elsewhere, here we briefly cover the connections between the caspase cascade and some of the proteases discussed in this chapter. The caspase cascade provides an important example of the tumor-suppressing roles of proteases, as opposed to the more widely accepted view of proteases promoting cancer.

As discussed earlier, several different proteases can feed into the apoptotic cascade in various ways. Cathepsin D can have opposing effects on apoptosis in potentially proteolytic-independent pathways. Cathepsins B, H, K, L, and S, calpain, and granzyme B all cleave Bid within its bait loop in vitro, potentially leading to cytochrome C release [98–102] and activation of the intrinsic apoptotic pathway. Additionally, cytosolic release of cathepsin B was followed by caspase 2 activation, leading to activation of the intrinsic apoptotic pathway [103], providing evidence for another mechanism by which upstream proteases can promote apoptosis.

Evasion of the caspase cascade can have an important role in metastasis, as reported in neuroblastoma, in which suppression of caspase 8 in invading cancer cells prevented apoptosis and promoted metastasis [104]. The numerous pathways leading to apoptosis are continually being elucidated; in addition to an increasing number of direct proteolytic interactions, there will likely be more signaling molecules found to be activated by proteases leading to apoptosis. The centrality of escape from apoptosis in cancer progression makes this topic a continually important area of research.

Extracellular Interactions

Interactions at the Plasma Membrane

Extracellular proteolytic interactions can be divided into two main categories – interactions that occur at the plasma membrane, and interactions that occur between secreted proteases and other proteases, or ECM components (Figure 15.3). Several proteases involved in each category have already been mentioned, but they are important to cover in this context because translocation and secretion can have dramatic effects on substrate interactions, owing to the availability of different substrates in distinct cellular microenvironments. Interactions at the cell surface are facilitated by structures such as podosomes, invadopodia, and caveolae, where proteases have been shown to concentrate. Podosomes and invadopodia are actin-rich adhesion structures produced by a variety of cell types that can contribute to ECM degradation. Although podosomes tend to be evenly distributed over an area, invadopodia tend to be larger and form in small clusters. Macrophages, endothelial cells, and smooth muscle cells have all been found to form podosomes, whereas cancer cells typically form invadopodia. Their protease composition is very similar, though, and several MMPs (MMP-2, MMP-9, and MMP-14), serine proteases (seprase and dipeptidyl peptidase IV), and the uPA receptor (uPAR) have been found in podosomes and/or invadopodia [105].

Caveolae are invaginations in the lipid membrane of a cell, and contain a variety of proteases and associated proteins. One such protease is cathepsin B, which is normally a lysosomal protease but is often translocated to the cell surface in many different cancer types.

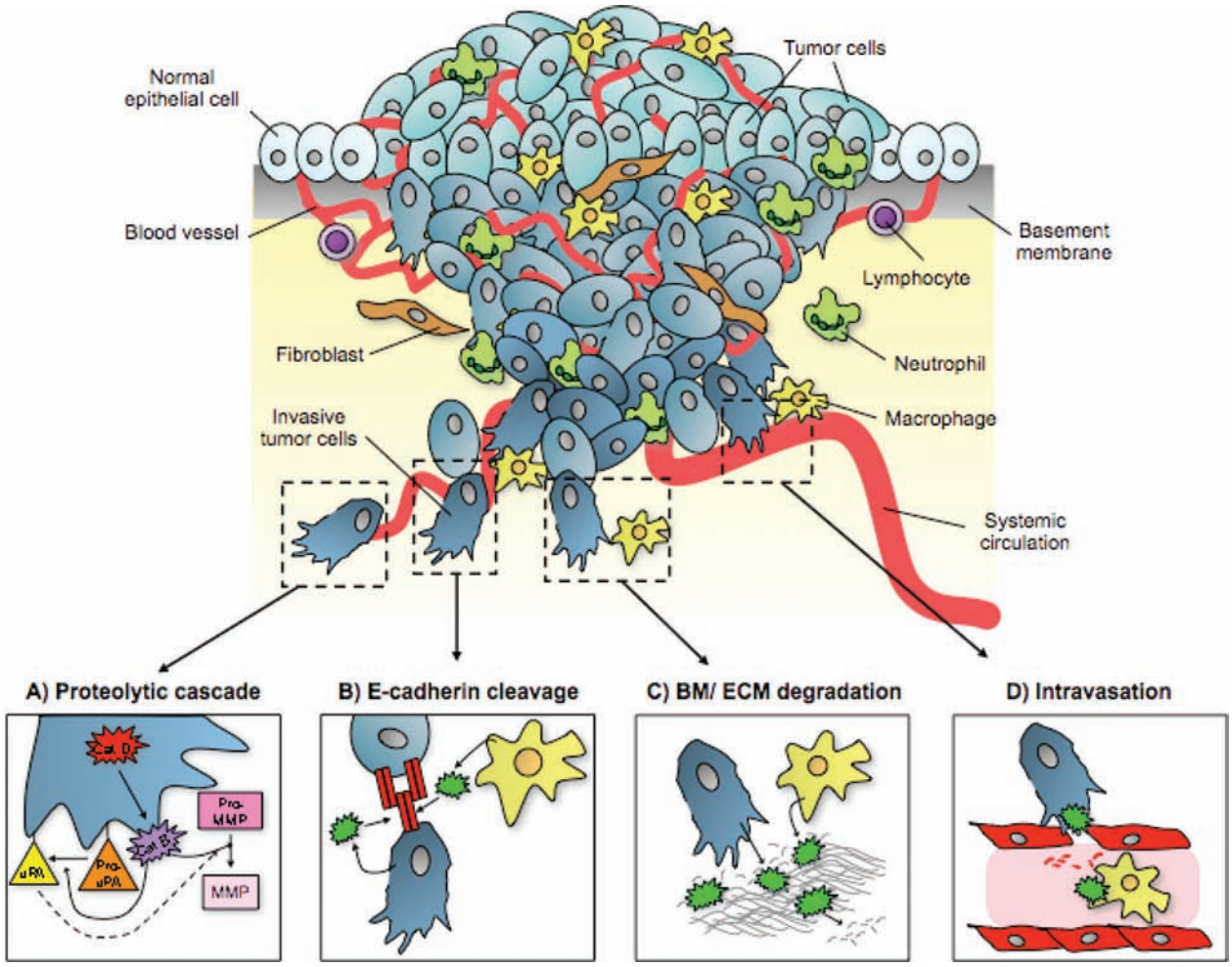

Figure 15.3. The tumor microenvironment in the proteolytic cascade. In addition to tumor cells, different stromal cells, including fibroblasts, endothelial cells, and immune cells, can contribute to the proteolytic cascade in the metastatic tumor microenvironment. These proteases participate in (A) activation of other proteases, (B) cleavage of cell-adhesion proteins, (C) ECM degradation, and (D) intravasation into circulation. Figure adapted from Joyce 2005 [159] and Gocheva and Joyce 2007 [160].

Although the exact mechanism of cathepsin B translocation is still under investigation, it is known that cathepsin B, like uPA and its receptor, localizes to caveolae in colon cancer cells with the annexin II heterotetramer (AIIt) [106]. Cathepsin B binds directly to the S100A10 light chain of AIIt [107], revealing a potential mechanism for retention of cathepsin B at the cell surface, promoting the activation of MMPs and uPA. To date, MMP-2 and MMP-3 have been shown to be activated by cathepsin B [108, 109], and the localization of cathepsin B to the cell surface provides increased potential for these interactions. Additionally, cathepsin B cleaves and inactivates TIMP1 and TIMP2, two potent negative regulators of MMP activity. The activation of pro-uPA to uPA by cathepsin B [110] would also be more likely at the cell surface, as cathepsin B and the pro-uPA/uPAR complex would be in close proximity.

In addition to protease substrates, cathepsin B also cleaves several cell surface and ECM components, including E-cadherin [54], fibronectin [111], collagen, laminin, and elastin [50, 112]. Degradation of ECM substrates can liberate sequestered growth factors, thus promoting proliferation and angiogenesis, which are vital processes for tumor development and metastasis. Support for a physiologic role for cathepsin B in this process has come from knockout studies using cathepsin B null mice in transgenic models of breast and pancreatic cancers. In the RIP-Tag pancreatic islet model, tumor growth, proliferation, invasion, and angiogenesis were impaired when cathepsin B was ablated, whereas apoptosis was increased [54]. Deletion of cathepsin B in the MMTV-PyMT breast cancer model decreased tumor growth and metastasis [113] but also revealed two interesting microenvironmental interactions. First, cancer cells compensated for loss of cathepsin B by upregulation of cathepsin X/Z (another cysteine cathepsin), highlighting the ability of redundant proteolytic interactions to potentially compensate

for each other and underscoring a possible difficulty with selectively targeting proteases. Second, deletion of cathepsin B from the stroma but not the cancer cells still resulted in a decrease in the formation and growth of lung metastases, emphasizing an important role for stromal-derived proteolytic activity in tumor progression.

Additional research has confirmed that stromal cells, particularly infiltrating immune cells and endothelial cells, contribute to protease expression, as demonstrated with cysteine cathepsins [52], NE, and several MMPs. Localization of proteases to the plasma membrane and/or secretion of proteases could enable different cell types to produce different members of the proteolytic cascade, which would facilitate cross-activation and amplification of cascade signals and proteolytic cleavage (Figure 15.3). Examples of this include the reciprocal upregulation of proteases by cancer cells and fibroblasts [114], and the increased ability of co-cultures of cancer cells with fibroblasts or macrophages to degrade collagen IV owing to enhanced pericellular and intracellular activity of MMPs, plasmin, and cysteine proteases [41]. Experiments such as these co-culture assays, or genetic experiments in vivo in which tumor and stromal contributions can be readily identified and modulated, will be essential to our increased understanding of proteolytic cascade interactions between cancer cells and stromal cells.

uPA/uPAR and tPA/tPAR

Further down the proteolytic cascade are the urokinase-type plasminogen activator and tissue-type plasminogen activator (uPA and tPA). Both serine proteases generate active plasmin from plasminogen, but uPA-generated plasmin participates in ECM degradation and therefore has a role in metastasis. A more detailed discussion of the urokinase system in invasion and metastasis can be found elsewhere, and therefore only a brief overview will be presented here, with a particular emphasis on how it integrates into the proteolytic cascade.

Activation of pro-uPA to uPA can be carried out by cathepsin B, elastase, and plasmin (Figure 15.2), and is likely dependent on binding of uPA to uPAR [115]. The primary role of uPA is to generate plasmin, leading to ECM remodeling, but it can also activate procathepsin B [24], leading to another feedback loop. uPAR is strongly induced by hypoxia in a manner that is somewhat dependent on hypoxia-inducible factor (HIF)-1α, making it part of the mechanism by which hypoxia can increase invasiveness of cancer cells [18]. Several lines of evidence implicate the uPA/uPAR system in metastasis, but one finding worth noting here highlights the complexity of the tumor microenvironment, in which direct interactions between pancreatic cancer cells and stromal cells was required for activation of the uPAR–uPA–MMP2 pathway [116]. The two main inhibitors of uPA and tPA are PAI-1 and PAI-2 (also known as serpin E1 and serpin B2, respectively). It is interesting to note that whereas hypoxia upregulates uPAR, leading to increased invasion, PAI-1 is also strongly induced by hypoxia via HIF-1α [117]. These findings indicate the lengths to which tumor cells go to control proteolytic balance during metastasis.

Secreted Proteases

As mentioned earlier, cancer cells and stromal cells secrete several cathepsins (including cathepsins B and D), enabling them to degrade ECM components such as collagen, laminin, fibrinogen, and elastin, cleave cell surface proteins, and release potent growth factors promoting angiogenesis and tumor growth. Another important cell-surface target that has been demonstrated for cathepsins B, L, and S is E-cadherin (E-Cad) (Figure 15.3). Downregulation of E-Cad has been observed in many invasive tumors from mice and humans; proteolytic turnover plays a role in this process, in addition to mutation and transcriptional silencing [118–121]. Biochemical experiments demonstrated that cathepsins B, L, and S are all able to cleave E-Cad, and genetic experiments revealed that mice lacking cathepsins B, L, or S (but not cathepsin C) were unable to downregulate E-Cad on cancer cells, resulting in less invasive tumors [54]. Loss of E-Cad leads to a loss in cell–cell adhesion, helping cancer cells to break free from the microenvironment and metastasize to distant tissues [122]. Additionally, cleavage of fibronectin by cathepsin B produces the CS-1 sequence, which can bind and activate the integrin receptor $\alpha4\beta1$, controlling cellular adhesion [111].

Neutrophil elastase is also secreted into the tumor microenvironment, where it has further opportunities to activate cathepsin B, uPA, or MMPs, and also degrade the ECM. Two other important targets for NE are cell-surface tethered epidermal growth factor (EGF) and transforming growth factor (TGF)-α [123, 124]. The importance of this interaction is in the ability of NE to release EGF and TGF-α from the cell membrane, allowing them to subsequently bind their target receptors and stimulate proliferation. Inhibiting the activity of NE could thus be of critical importance for cancers that overexpress EGFR, such as some breast and lung cancer subtypes. Endogenous inhibition of NE is achieved by several protease inhibitors that are found in the ECM, such as serpin A1 (α1-antitrypsin), α2-macroglobulin, and secretory leukoprotease inhibitors [125, 126].

Two of the major secreted protease components of the tumor microenvironment are, of course, the MMPs and plasmin. With the exception of the furin-activated MMPs, the majority of MMPs are activated

extracellularly, and MMPs target many ECM components, including proteoglycans, collagens, laminin, gelatins, fibronectin, entactin, and elastin. MMP-3 overexpression can actually drive tumor initiation and progression in the absence of an initiating oncogene, an occurrence not normally seen with proteases [127]. MMP activity in the ECM is modulated by the four TIMPs; the balance between protumor MMP activity and antitumor MMP activity depends on the tumor type and context and is discussed in more detail elsewhere. In addition to the possibility of MMPs having pro- and antitumorigenic functions, antisense inhibition of TIMP1 was surprisingly found to promote melanoma metastasis in a xenograft model [128]. However, most of the research on TIMPs has confirmed that in many different contexts and tumor types, TIMPs serve to protect against tumor growth and metastasis [127, 129, 130]. Additionally, TIMP expression has generally been associated with less aggressive tumors and a favorable prognosis [129].

Following activation of plasminogen into plasmin by uPA, plasmin degrades many different ECM targets, including fibronectin, vitronectin, and fibrin. Notably, plasmin activates the proforms of several cytokines and growth factors [131]. A more detailed description of the mechanisms of plasmin activity and its roles in cancer can be found elsewhere.

Key Unanswered Questions and Paradoxes

Although many different interactions within the proteolytic cascade have been elucidated largely through biochemical techniques, it is clear that researchers have only begun to scratch the surface of how proteolysis is regulated in cancer and metastasis. With time, more interactions and targets will be found in vivo, in addition to different mechanisms by which proteases are upregulated or inhibited. The following section will highlight several areas in which important open questions remain.

Upregulation and Translocation of Cysteine Cathepsins

As mentioned earlier, a key change observed in multiple tumor types is the increase in expression of cathepsin D and the cysteine cathepsins, and their translocation from the lysosome to the cytosol and cell surface. Although several different genes are known to potentially upregulate cathepsin expression, no specific transcription factor has been found to directly and specifically perform this role. Expression of cathepsin family members is often upregulated early in tumorigenesis [132–134] and increases throughout tumor development [52], indicating that their upregulation may be important for tumor progression and metastasis.

Although cathepsin B has been found in the caveolae of cancer cells, the route by which it is translocated and secreted is still under investigation. Bonnie Sloane's group has proposed that the caveolae pathway is responsible for this process, and that S100A10 is important for the activation of procathepsins at the cell surface [135]. Supporting this view, downregulation of caveolin 1 in vitro results in reduced association of cathepsin B, S100A10, and pro-uPA, leading to decreased collagen IV degradation [106]. However, the mechanism by which cathepsins are redirected from lysosomes to caveolae is still unclear. Translocation of cathepsins from the lysosome to the cell surface has dramatic effects on their substrate interactions, allowing them to have access to proforms of proteases and ECM proteins with which they would not interact elsewhere in the cell. Degrading ECM components or activating matrix-degrading proteins allows cathepsins to significantly enhance metastatic progression. Additionally, the findings that both cancer cells and stromal cells, especially macrophages, secrete cathepsins further complicate this picture – do they use identical mechanisms to enhance tumor development, or is the mechanism specific to the cell type?

Differences in Activity and Effects of Proteases in Different Cancers

Another important consideration in understanding the proteolytic cascade is the differences that are found in protease expression and activity in different cancers. Some proteases, such as cathepsins B and D, and some MMPs, to name a few, are associated with progression and metastasis in multiple cancers, making them potential targets for anticancer therapies with broad applications. On the other hand, several proteases exhibit very close correlations (and have been demonstrated to have causal effects) in very specific cancer types or niches. One example of this is cathepsin K, which is expressed by osteoclasts and can mediate bone resorption, and has been implicated in bone metastasis from melanoma, breast, and prostate cancers [136–138]. An example of a protease associated with a specific cancer type is prostate-specific antigen (PSA), a serine protease also known as kallikrein 3, which has been used as a prognostic factor in prostate cancer for several years [139], raising the interesting notion that some proteases may have value as prognostic indicators in addition to being potential therapeutic targets. However, the specificity of PSA for prostate cancer is currently being challenged, as new research has demonstrated that PSA levels may also serve a predictive function in breast cancer [140]. One intriguing aspect of tumor-specific proteases is that they potentially allow for specific targeting of the tumor microenvironment with minimal toxicity to normal organs.

Another challenging aspect of protease involvement in the metastatic microenvironment is proteases that appear to serve tumor-promoting functions in some cancer types yet exhibit potentially tumor-suppressing functions or correlations in other cancers. There are several notable examples of proteases in this category, including cathepsin L, which was found to be tumor-promoting in a mouse model of pancreatic cancer [54] but tumor-suppressing in a colon cancer model [63]. Other examples include hepsin [141, 142], MMP3 [127, 143], MMP9 [144, 145], and MMP12 [146, 147]. Murine models in which carefully controlled experiments can be used to address the causality of these genes in tumorigenesis and metastasis will be essential for determining the exact conditions in which proteases promote or inhibit cancer progression.

Stromal versus Tumor Cell Contributions

One rapidly growing area of research into the role of proteases in cancer is the contribution of stromal-derived proteases to tumor growth and metastasis (Figure 15.3). As already mentioned for several proteases, endothelial cells, fibroblasts, and infiltrating immune cells are often found to express high levels of certain matrix-degrading enzymes in the tumor microenvironment. These degradative enzymes include cysteine cathepsins [52, 54], heparanase [148], various MMPs [144, 149], and uPA [150]. Often, cancer cells and stromal cells signal to each other through various chemokines, causing upregulation of proteases leading to ECM remodeling, tumor cell invasion, and the release of sequestered growth factors. The list of chemokines involved in these processes is continually growing but includes factors such as CCL5 [151, 152], CCL9 [153], CSF-1 [154], CXCL12/SDF-1 [155], CXCL8, CXCL1–3, and CCL2/monocyte chemotactic protein-1 [152]. The modulation of proteolytic activity between tumor cells and stromal cells by chemokines allows for the possibility of targeting proteolytic activity indirectly by blocking chemokines or their receptors.

There are two further additions to this picture. First, several chemokines are synthesized as precursor forms and require proteolytic cleavage for full activation. Again, the list of chemokines in this category is incomplete and still growing but includes several CXCL and CCL ligands [156, 157]. The proteases that have been shown to be responsible for this are also varied and include several MMPs, uPA, plasmin, dipeptidyl peptidase IV, and some cathepsins [157]. Targeting proteases may then interfere with tumor progression not just by directly blocking processes such as ECM remodeling and invasion but also by perturbing chemokine activation, thus reducing stromal cell recruitment.

Second, a challenge to deciphering the proteolytic contributions of stromal cells is the ability of tumor cells to express proteases normally produced by stromal cells. As discussed earlier, a prominent example of this is the expression of NE by several different cancer cell lines. In these cases, inhibiting the specific proteases may be more beneficial than targeting specific cell types within the tumor microenvironment.

Extensive Redundancy in Activation or Degradation of Targets

Perhaps the greatest challenge to using proteolytic inhibition as an effective anticancer therapy is the redundancy found in proteolytic pathways. As can be appreciated from Figure 15.2, several downstream protease proforms can be activated by multiple upstream proteins, meaning that inhibition of one protease may easily lead to compensation by another. Additionally, ECM remodeling can be carried out by multiple proteases. Examples of ECM components that can be degraded by multiple proteases abound; collagens can be degraded by multiple MMPs and cathepsins; fibronectin by plasmin, cathepsins, and MMPs; elastin by elastase, cathepsins, and MMPs; and so on. This redundancy requires considering a family-centered approach to the proteolytic cascade, as was carried out by Gocheva, Joyce, and colleagues. In this research, the roles of multiple cysteine cathepsin family members were investigated in pancreatic islet cancer using cathepsin B, C, L, and S knockout mice, identifying roles for individual proteases in multiple facets of cancer development but also revealing some overlap in protease function [54]. It is likely, then, that protease profiling of tumors will be critical in determining which (multiple) proteases to target to block tumor progression, rather than attempting to halt tumor progression by targeting one individual protease at a time.

Preponderance of In Vitro versus In Vivo Data on Interactions

One of the stumbling blocks that led to difficulties targeting the proteolytic cascade in preclinical models, or in the clinic, is the lack of in vivo data to support protease–substrate interactions that have been identified only through biochemical approaches. Although in vitro assays are of considerable importance for characterizing protein–protein interactions, they are rarely able to fully recapitulate the cellular complexity of the tumor microenvironment. Therefore, important cofactors may be absent, leading to false negatives regarding interactions, or endogenous inhibitors may be absent, resulting in false positives. Additionally, in vitro interactions may not account for compartmentalization of proteases and substrates in the cell or tumor microenvironment. Therefore, it is possible that two proteins observed to interact in vitro may never actually "see"

each other in vivo. Finally, biochemical assay conditions may result in stronger or weaker binding coefficients than are found in vivo, resulting in over- or underestimation of the strength and importance of an interaction.

In addition, although several proteases have been implicated in tumor progression through in vivo studies either inhibiting or enhancing proteolytic activity, very few studies look beyond basic physiological effects to determine the actions of a protease within the proteolytic cascade. The clearly demonstrated roles of proteases in regulating angiogenesis, ECM remodeling, or cellular proliferation should not be diminished; however, elucidating the direct interacting partners that promote or inhibit these activities would add immeasurably to the data available about the proteolytic web. An example of this approach is a recent study demonstrating the ability of cathepsin L to cleave and activate heparanase, in which the authors used cathepsin L null mice to provide in vivo proof of a dramatic decrease in heparanase activation, supporting the biochemical data [51]. Examining the activation of downstream proteases in tumors in which proteases have been inhibited or upregulated could similarly provide additional information for the way in which certain physiological effects are carried out.

As a result of the difficulties translating in vitro findings into in vivo functions, several labs have taken on the challenge of characterizing in vivo proteolytic interactions. The Overall lab has used isotope labeling systems and mass spectrometry to reveal protease–substrate interactions in systems that more accurately model the tumor microenvironment, including whole-organ and animal studies [158]. It is important to note that many of these initial efforts have focused on MMPs, and therefore further research is needed to characterize the in vivo proteolytic activities of other protease families in cancer and metastasis. However, these efforts will undoubtedly lead to a clearer understanding of proteolytic interactions in the tumor microenvironment and provide essential insights into which interactions can (and need) to be blocked in cancer therapy.

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16 Role of Matrix Metalloproteinases in Tumor Invasion and Metastasis

Barbara Fingleton

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that act outside the cell to alter the environment. As implied by their name, molecules that constitute the extracellular matrix (ECM), which includes basement membranes as well as interstitial fibers, are substrates of the MMP enzyme family. Other substrates include growth, death, chemotactic, and other signaling factors, as well as proteinases and proteinase inhibitors. The ability to modulate this vast array of different types of proteins means that MMPs can be potent regulators of cellular behavior. In this chapter, we review the discovery and study of MMPs and describe the current thinking of how MMPs contribute to tumor progression, specifically the processes of invasion and metastasis. To properly understand biological functions of MMPs, in vitro and in vivo methods for their analysis have been developed. Using such analytical tools wisely requires an understanding of their limitations and possible interpretations. Despite an immense amount of research concerning this important group of proteases in cancer, as well as in other pathological and physiological conditions, there are still multiple unanswered questions; thus, we end this chapter by considering some of these.

DISCOVERY AND CHARACTERIZATION OF MMPs

In humans, the MMP family currently comprises twenty-three members, although inclusion of all mammalian as well as frog and avian MMPs bring the total known to twenty-five. These enzymes are most simply known as MMP-1 to MMP-28. Some readers may wonder at the disparity between the total number of enzymes and the numbering system; the reason is that the proteins initially labeled as MMPs-4, -5, and -6 were found to be duplicates of previously identified proteins; hence, these three designations have been retired [1].

Collagens are the most abundant protein in the body – but discovering an enzyme that could cleave them was arduous. In the end, it was the process of tadpole tail resorption that led Gross and Lapiere to discern the existence of an enzyme with collagenolytic activity at neutral pH, which was, unsurprisingly, named "collagenase" [2]. Similar collagenolytic activities were also seen in the skin, uterus, and bone. Early studies of these and similar enzymes predated the era of cloning, so enzymes were identified as "activities" rather than specific gene products. Such activities were purified based on biochemical characteristics; it was these biochemical characteristics that first resulted in the grouping of certain enzymes into a family of matrix-degrading proteinases, which we now know as the MMPs [3]. Initially, the biochemical characteristics were degradation of matrix proteins, inhibition by chelating agents such as EDTA, and activation by organomercurial compounds [1]. The response to chelating agents helps explain the "metallo" part of the name – MMPs contain zinc ions that are necessary for enzymatic activity.

In addition to identifying proteinase activities, proteins were also discovered that could interfere with proteolysis. These naturally produced enzyme inhibitors were called tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 was first purified from fibroblasts based on its ability to prevent collagenolytic activity [4]. The TIMP family has now expanded to four members with slightly different inhibition profiles [5]. In addition, TIMPs appear to have functions that are unrelated to proteinase inhibition, which is an important consideration when interpreting experiments in which TIMPs are used as tools to inhibit proteinases.

With advances in molecular biology, cloning of MMP genes became feasible. The first full-length MMP to be cloned was MMP-3, although it was called transin and not immediately identified as an MMP [6]. Transin was cloned as an oncogene and EGF-responsive gene, suggesting a possible role in tumor biology. MMP-1 was also cloned around the same time from human

fibroblasts, but in this case the investigators had already purified the protein and knew its functional capability, and thus the cDNA was immediately known to code for a metalloproteinase [7]. The advent of cloning led to changes in characterization criteria for MMPs, which became more stringent. Now, to be characterized as an MMP, the enzyme should (1) have the amino acid signature sequence HEXXHXXGXXH, which includes the histidine residues that coordinate the catalytic zinc ion, as well as a catalytic glutamic acid residue; (2) be inhibited by TIMP; and (3) have sequence similarity to collagenase [1].

As with the majority of proteinases, MMPs are produced as latent enzymes or zymogens that require proteolytic removal of a propeptide to become active. In MMPs, the propeptide includes a cysteine residue that binds to the zinc ion in the catalytic site of the enzyme. To be activated, the cysteine bond must be dissociated, in what is known as the "cysteine switch" mechanism [8]. In vitro, various chemicals, such as the organomercurial compound 4-aminophenylmercuric acetate (APMA), can be used to "open" the cysteine switch, thus activating the enzyme. In vivo, however, other proteinases are generally responsible for activation [9]. For several MMPs that possess a particular recognition sequence, the proteolytic activation event occurs intracellularly, mediated by one of a family of proprotein convertases, of which furin is the best known member [10]. Other MMPs are activated in the extracellular environment by various proteinases, including other MMPs, but also members of different classes, such as serine proteinases [11]. For this reason, it is important to remember that inhibition of any single proteinase may have far-reaching consequences on other proteinases further down an activation cascade.

ELUCIDATING MMP FUNCTION

Although early criteria for MMPs included the ability to hydrolyze extracellular matrix molecules, this criterion had to be abandoned, as it was realized that other types of proteins could be substrates for MMPs. Furthermore, some MMPs, such as MMP-11, did not appear to have any matrix substrates. Determining actual substrates for MMP enzymes is still an important area of research. At one time, a substrate was defined by its ability to be cleaved by purified enzyme when the substrate and protein were mixed in test tubes. Although these types of assays show what *can be* hydrolyzed by a particular proteinase, they do not determine what *is* hydrolyzed in the in vivo setting. Generation of mice that were null for particular members of the MMP family have greatly contributed to the finding of true *in vivo* substrates [12]. Thus far, knockout mice have been generated for MMP-2, -3, -7, -8, -9, -10, -11, -12, -13, -14, -15, -19, -20, and -28. These mice are valuable tools for assessing what the normal in vivo functions of each of the enzymes may be. Surprisingly, the majority of MMP-null mice are viable and healthy with no overt phenotype. An exception is the MMP-14-null mouse, which displays severe skeletal abnormalities and defects in angiogenesis, and dies at a young age [13, 14]. Some other MMP-null mice have subtle abnormalities, some of which are corrected as the animals age. The general lack of phenotypes associated with specific enzyme deficiency suggests that functional redundancy exists whereby, in the absence of a particular proteinase, another can perform the same role. Additionally, actual compensation has been demonstrated where, in the absence of a specific MMP, levels of other family members are increased, presumably to compensate for the loss [15]. Certainly, in test tube assays with purified proteins, many substrates appear shared among multiple family members. In vivo, however, both spatial and temporal localization, as well as activation, can control which enzymes encounter which substrates.

In addition to the MMPs, other families of metalloproteinases are present in vivo. Two closely related families are the ADAM and ADAM-TS families. The name "ADAM" derives from a description of the general structure of these proteins, which contain ***a d***isintegrin ***a***nd a ***m***etalloproteinase domain [16]. Not all ADAMs are functional proteinases; nonproteolytic ADAMs seem to be important for cell–cell interactions such as sperm–egg fusion. Proteolytic ADAM family members, including TNF-α converting enzyme, or TACE (also known as ADAM-17), are especially important for "shedding" events, in which membrane-bound factors are cleaved at the juxta-membrane region to release a soluble protein into the extracellular space. ADAM-TS proteins have a similar multidomain structure as ADAMs, with the addition of a thrombospondin (TS) domain [17].

MMPs IN INVASION AND METASTASIS

In 1980, a landmark study from Liotta and Tryggvason showed that cancer cells could degrade basement membrane and therefore invade, when they had increased levels of the enzymatic activity known as type IV collagenase [18]. This "activity" had properties corresponding to MMP-2. Critically, the levels of enzymatic activity correlated with the metastatic potential of a range of related murine cell lines. The ability to overexpress different MMPs in cell lines extended this initial observation and established MMPs as controllers of the invasive phenotype. Complementary experiments in which the endogenous inhibitor of MMPs, TIMP-1, was knocked down in fibroblasts using antisense technology further supported this concept [19].

The basic steps of metastasis can be outlined as (1) detachment of individual or small groups of cells from the tumor, (2) invasion and migration into the

surrounding parenchyma, (3) intravasation, (4) survival in the bloodstream, (5) extravasation, (6) local invasion/migration in the secondary site, and finally (7) outgrowth at the secondary site. Proteinases that are regulators of invasion then may be expected to be of great importance in at least four of these steps. The requirement for MMPs for intravasation was demonstrated in an elegant experimental design using chick chorioallantoic membrane (CAM) as a means of providing a readily accessible capillary bed [20]. In that study, Ossowski and colleagues showed that human cells placed on the upper CAM could be readily detected in the lower CAM, which is connected only by vasculature. When a broad-spectrum synthetic MMP inhibitor was added to the CAM, the number of detectable cells in the lower CAM was reduced up to 90 percent. Interestingly, these investigators also showed a necessary role for the serine protease urokinase plasminogen activator (uPA), indicating a cooperativity for two classes of proteases in the intravasation process.

Unexpectedly, another important study suggested that MMP activity was not rate-limiting for extravasation or local invasion/migration. Investigators used in vivo imaging to track the fate of mouse melanoma cells, either wild-type or transfected, to overexpress the MMP inhibitor TIMP-1 after intravenous injection [21]. The presence of TIMP-1 did not affect the ability of cells to get out of blood vessels or to invade and migrate through the lung parenchyma after extravasation. Surprisingly, it was the growth of the tumor foci in the lung that was significantly attenuated by the presence of excess MMP inhibitor. One potential explanation for the apparent noncontribution of MMPs to invasion is the methodology used to block MMP function. As previously mentioned, we now know that there are four different TIMP proteins, and the inhibitory profile is not the same for each. Importantly, membrane-type metalloproteinases (MT-MMPs 1–6, also known as MMP-14, -15, -16, -17, -21, and -25) are not all effectively inhibited by TIMP-1 but are inhibited by TIMP-2. Thus, the use of only TIMP-1 overexpression would not have blocked the activity of all MMPs. This is relevant, as elegant studies from the laboratory of Steve Weiss have shown a central role for MMP-14, but not other MMPs, in tumor cell invasion [22] (Figure 16.1). These data, suggesting the primary importance of a single MMP for tumor cell invasion, are controversial, as many researchers have identified other MMPs as important contributors to the invasive phenotype.

An important finding from the extravasation study was the demonstration that outgrowth of the metastatic focus appeared dependent on TIMP-1–sensitive MMP activity. This result highlights novel roles for MMPs in tumor growth, which have recently been expanded. It is now clear that, in vivo, a major role for MMPs is the processing and/or shedding of signaling molecules

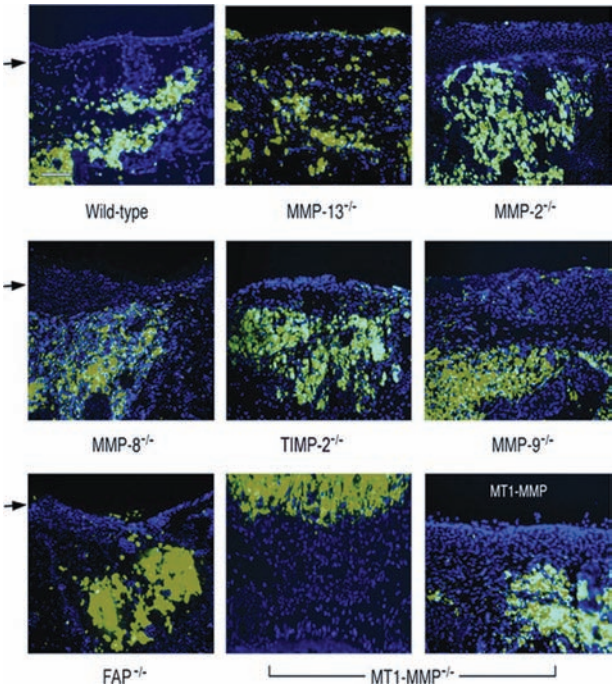

Figure 16.1. Fibroblast invasion into chicken chorioallantoic membrane (CAM) is unaffected by deletion of most MMPs, except MMP-14. Fibroblasts derived from wild-type mice or mice deficient in various MMPs ($MMP^{-/-}$), TIMP2 ($TIMP\text{-}2^{-/-}$), or the serine protease fibroblast activation protein ($FAP^{-/-}$) were labeled with green fluorescent microbeads and placed on top of a CAM. The arrows indicate the CAM surface. After three days, all fibroblasts, except those deficient in MMP14 ($MT1\text{-}MMP^{-/-}$) had invaded into the CAM. Reexpression of MMP14 into the null fibroblasts rescued the invasive phenotype. © Sabeh et al. 2004. Originally published in *Journal of Cell Biology*. Doi:10.1083/jcb.200408028.

such as growth, death, and angiogenic factors [23]. Changing the size of these molecules, or releasing them from membranes, can alter their biological function and greatly affect tumor cell behavior. An example of this is the activation of transforming growth factor β (TGF-β) by MMP-9 or MMP-2 [24].

The development of knockout animals greatly expanded our knowledge of the different roles MMPs could play. Perhaps the most surprising revelation has been that MMPs have both deleterious and beneficial functions in cancer biology [25]. One of the first demonstrations of this double-edged sword came from the identification of angiostatin as a proteinase cleavage product of plasminogen. MMP-12, -7, and -9 have all been shown to be important for the production of angiostatin, an inhibitor of angiogenesis [26]. In various cancer models, the absence of any one of these enzymes can result in larger, more aggressive tumors, as angiogenesis can proceed unchecked. In a complex scenario, some of the same MMPs can also promote angiogenesis through release of angiogenic signaling molecules, such as VEGF [27, 28]. Other angiogenic inhibitory peptides such as tumstatin have since

been identified as MMP-cleavage products of matrix proteins [29].

METHODS FOR ANALYZING MMP ACTIVITY

Although there are now known to be more than twenty MMP enzymes, the vast majority of the literature still focuses on the so-called gelatinases MMP-2 and MMP-9. Part of the reason for this is the ease with which these enzymes can be detected using simple zymography. Zymography is a gel electrophoresis technique in which an enzyme substrate, such as gelatin, is incorporated into the polymerized gel. Nonreduced or boiled samples – whether tissue lysates, body fluids, or cell culture supernatants – are then electrophoresed through the gel as usual, so separation occurs based on molecular weight. Once electrophoresis is complete, the gel is washed to remove the detergent sodium dodecyl sulfate (SDS), thus allowing unfolded proteins to refold. The gel is then incubated with a substrate buffer to provide optimal conditions for enzymatic activity. Following incubation, staining of the gel with Coomassie blue allows visualization of degraded substrate as clear bands on an otherwise blue gel (Figure 16.2). These clear patches correspond to particular molecular weights, and thus the proteinases responsible for the lytic activity can be identified based on their size.

One noteworthy, and often confusing, feature of zymography is that it allows visualization of both latent and active proteinases. As a result of the unfolding that occurs while the gel is being run, the "cysteine switch" is released and pro-MMPs are activated in the absence of proteolytic cleavage of the prodomain. Already active enzymes that have been proteolytically cleaved run at the size corresponding to an active enzyme, approximately 10 kDa smaller than the corresponding proform of the enzyme. In Figure 16.2 (vi), for example, one band corresponding to MMP-9 is shown, whereas there are two bands for MMP-2. The MMP-9 band is at approximately 92 kDa and thus corresponds to latent or pro–MMP-9, which has not been activated in the biological sample being examined. The two bands for MMP-2 correspond to latent or pro–MMP-2 at approximately 72 kDa, and active MMP-2 at approximately 62 kDa. In the biological sample that was used for the zymographic analysis, only active MMP-2 would be expected to have enzymatic activity. It should be remembered, however, that if the active MMP-2 was in the presence of a TIMP or of a synthetic MMP inhibitor, then the enzymatic activity would have been inhibited. Because the interaction between enzyme and inhibitor is noncovalent, the complex comes apart during electrophoresis, and the enzyme appears active.

Even though gel zymography has been a valuable tool for identifying the presence of MMPs without the need for specific antibodies, it does have some drawbacks. One is that identification is based on a combination of incubation conditions (neutral pH, presence of metal ions) and molecular size. This is not an infallible system, and verification that putative MMP bands are indeed so should come from other methods. Another problem is that any localization information is lost from tissue samples that must be homogenized before running on a gel. The technique of in situ zymography was thus developed in an attempt to provide information as to what areas of a tissue demonstrated enzymatic activity. In its simplest form, a substrate is overlaid on a frozen section of tissue and turnover

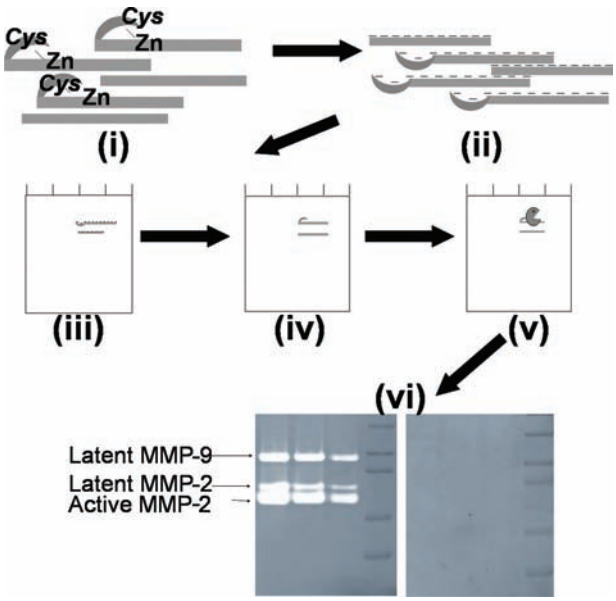

Figure 16.2. Schematic explanation of the process of gelatin zymography. **(i)** A biological sample contains a mixture of latent and active MMPs. The proteinases are in their properly folded configurations, which, in the case of latent MMPs, includes the presence of the cysteine–zinc bond, the "cysteine switch." **(ii)** On mixing with sample buffer containing SDS, the proteinases unfold as a result of the excess negative charge donated by the SDS. **(iii)** Samples are loaded onto a polyacrylamide gel that has been copolymerized with gelatin; proteins run toward the anode, and separation is based on size. **(iv)** Once the gel is run, the SDS is removed by washing, which allows refolding of proteins in the location on the gel to which they had migrated. The cysteine–zinc bond does not re-form, so now even latent size MMPs are in an active conformation. **(v)** Gels are then incubated in a reaction buffer containing Ca^{2+} ions at 37°C to allow proteolytic activity to occur. Inclusion of an inhibitor such as EDTA in the reaction buffer will prevent activity of metal-dependent proteinases. **(vi)** When gels are stained with Coomassie blue, the incorporated gelatin stains darkly except in regions in which it has been hydrolyzed by the actions of MMPs. The size of the bands, as determined by molecular weight markers, suggests the identity of the MMP. Confirmation that these bands are indeed MMPs is provided by the lack of bands in the EDTA-incubated gel on the right. Samples shown are increasing volumes of conditioned medium from the HT1080 fibrosarcoma cell line, commonly used as a positive control for zymography.

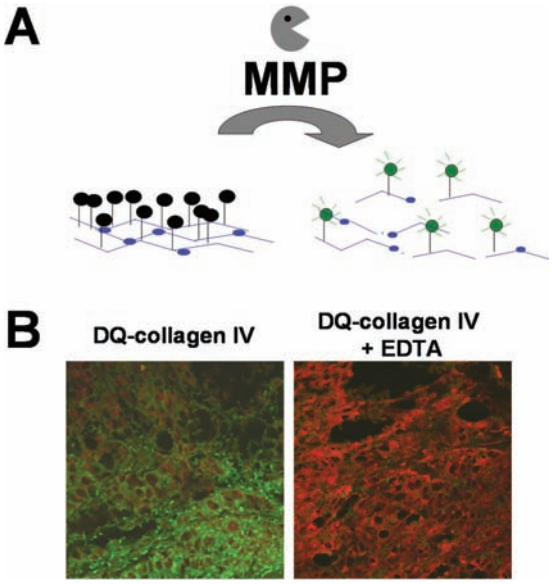

Figure 16.3. In situ zymography. (A) Dye-quenched matrix proteins are labeled with fluorophores at a high density such that proximity interactions prevent any light energy being released and they appear optically silent. After proteolysis, the proximity interactions are disrupted, allowing light energy to escape, which can be detected optically. (B) An example of in situ protease activity in a tissue section of a human skin cancer. The frozen tissue was placed on a slide that had been coated with a layer of DQ-collagen IV (Molecular Probes/Invitrogen) and incubated overnight. A second section was similarly incubated but with inclusion of EDTA to inhibit MMP activity. Both sections were then briefly stained with a red nucleic acid dye, Syto-17 (Molecular Probes/Invitrogen) and then viewed on a fluorescent microscope. Green signal indicates proteolyzed collagen IV, and red shows the presence of cells.

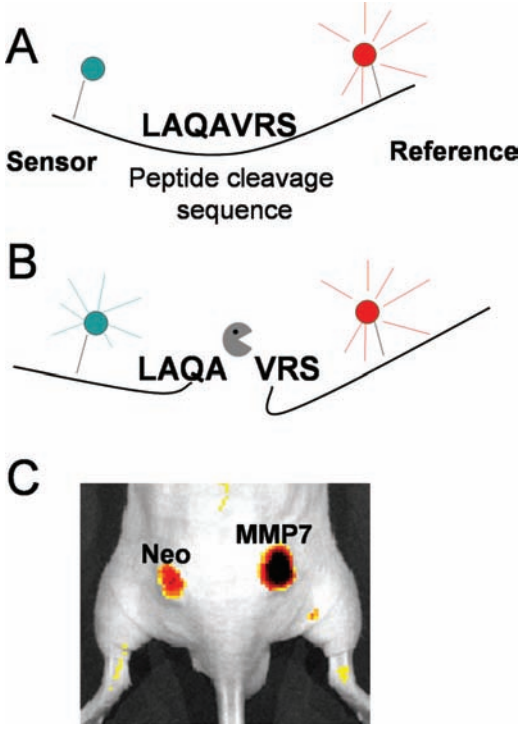

Figure 16.4. In vivo imaging using proteolytic beacons. (A) The proteolytic beacons are designed with three important moieties: (1) a reference fluorophore that will emit a signal independently of proteolysis, which is used to demonstrate the presence of the beacon in a given tissue; (2) a sensor fluorophore that emits a signal only after proximity interactions are disrupted; and (3) a peptide sequence that is optimal for cleavage by particular MMPs. (B) Although the reference always gives a signal, the sensor is detectable only after MMP activity. The amount of signal detectable is reported as a ratio of signal to reference. (C) An example of a proteolytic beacon for MMP-7 being used in a xenograft tumor model. The mouse had been injected four weeks prior to imaging with SW480 control cells ("neo") on the left flank and SW480 cells stably transfected with MMP-7 on the right flank. Twenty-four hours prior to imaging, the mouse was administered the beacon into the bloodstream. The fluorescent signals are overlaid on a white-light image of the mouse. The darker the signal, the higher the ratio of sensor to reference. Figure courtesy of RL Scherer, JO McIntyre, and LM Matrisian.

of that substrate is visualized in situ as a change in some visible marker. Early substrates included photographic emulsion, which contains gelatin. Fluorescent substrates in which proteins, such as gelatin or collagen, are labeled with fluorophores are often used. Dye-quenched versions, in which the concentration of adjacent fluorophores on the protein molecule is so heavy as to interfere with fluorescence release, are a popular method. They offer the advantage of looking for a signal – release of fluorescence – to identify areas in which proteolytic activity is present (Figure 16.3). A major drawback of these in situ techniques is the lack of specificity, and hence uncertainty as to the absolute identity of the protease responsible for any activity seen.

The most recent iteration of fluorescent substrates for MMPs can be used for in vivo imaging in real time. These "molecular beacons" contain peptides that are equivalent to MMP-preferred cleavage sites, linked to fluorophores that are optically silent owing to the proximity on the same molecule of a quencher [30]. Proteolytic cleavage of the peptide then releases the quencher from the fluorophore and a fluorescent signal can be detected (Figure 16.4). Use of long-wavelength near-infrared fluorophores permits detection from within tissues with suitable instrumentation.

The methods described here are useful precisely because they can detect whether an enzyme is active – that is, capable of turning over a substrate – and not just whether it is present. Assuming that the functions of MMPs are related to their ability to process specific substrates, it is more important to know how much actual activity is present in any given situation than how much MMP is expressed. Latent MMPs may not be activated, and active MMPs may be bound by endogenous inhibitors, such as TIMPs; either situation would not result in substrate processing.

INHIBITING MMPs AS A THERAPEUTIC APPROACH

After it was established that MMPs contributed to the invasive activity and hence to the metastatic potential of tumor cells, inhibition of MMPs appeared to be a logical goal. Initially, the endogenous MMP inhibitors – the TIMPs – were proposed as a therapeutic option. However, reliable production and delivery of these polypeptides were not considered commercially feasible. Because the MMPs were initially purified as collagen-degrading enzymes, designing small molecules that mimicked the cleavage site of collagen was a reasonable approach to designing an inhibitor that would specifically target to MMPs. Incorporation of a metal chelating moiety, such as hydroxamic acid, provided the means of actually blocking enzyme activity [31]. When these small-molecule peptide mimetic inhibitors were first developed, the number of different MMPs known was quite small; thus it was a relatively simple task to test whether the inhibitor would block enzymatic ability. This first generation of MMP inhibitors did have problems with solubility; nevertheless, impressive results were reported in preclinical animal models of different cancer types [32].

One of the first MMP inhibitors, called batimastat or BB-94, was tested in patients with metastatic ovarian cancer who developed massive ascites in the peritoneum that had to be drained on a regular basis. This disease scenario allowed administration of a suspension of the insoluble drug directly into the peritoneum [33]. Although the initial results from this trial appeared somewhat promising, the method of drug delivery was considered unreasonable and a second generation of soluble MMP inhibitors was developed. Second- and third-generation MMP inhibitors moved away from the peptide mimetic approach and instead used information from studies of three-dimensional structure of the enzymes to design drugs that would fit into the catalytic "pockets" of MMPs [31]. This was considered a more specific method of targeting. However, the family of MMP enzymes has quite similar structures in the catalytic site; drugs designed using this approach may have higher selectivity for certain members of the family, but in general will still inhibit multiple family members if concentrations are high enough. When these drugs went to clinical trials, it became apparent that blocking MMPs was not a completely benign scenario, and patients showed signs of debilitating side effects [34].

One of the most common types of side effects that showed up with multiple MMP inhibitor drugs was a musculoskeletal syndrome that left patients with extremely painful muscles and joints. The only way to relieve the pain was to give patients a "drug holiday" or to reduce the dose of MMP inhibitor. Despite a massive investment by multiple biotechnology and pharmaceutical companies and the participation of large numbers of cancer patients, no MMP inhibitor successfully made it through Phase III clinical trials, in which the requirement is to show efficacy or quality of life better than can be achieved with already-approved drugs. As might be imagined, this was a huge failure and led to a significant loss of enthusiasm for the idea of MMP inhibitors as a therapeutic approach in cancer and other diseases. The challenge now is to understand why these initial drugs failed and whether there are better ways of considering MMP inhibition.

There have been many analyses of the MMP inhibitor failure, but some issues seem particularly important. A key study from the laboratory of Doug Hanahan made use of the Rip-Tag model of progressive tumor development to illustrate the time frames at which MMP inhibition might be expected to be useful [35]. This model, in which the rat insulin promoter is used to drive expression of the SV40 T antigen oncogene specifically in islet cells of the pancreas, results in hyperplastic tumors that then convert in a well-defined time frame to angiogenic tumors, and then to invasive carcinoma. In their study, the investigators treated Rip-Tag mice with MMP inhibitors either before the tumors underwent angiogenic switch, after the angiogenic switch but before malignant conversion, or after invasive tumors were already present. They found that the MMP inhibitor had a benefit when given at either of the early stages, but had absolutely no impact in the scenario in which invasive carcinoma was already present. Unfortunately, this last group is equivalent to the majority of the cancer patients who were on clinical trials.

The other big problem with the MMP inhibitors is that they were generally broad-spectrum in nature, meaning that they inhibited most, if not all, of the MMP enzymes, and often also related to ADAM and ADAM-TS proteinases. As the cause of the musculoskeletal side effects is not known, it is possible that inhibiting certain MMPs/ADAMs/ADAM-TSs is a direct cause of the joint problems, and designing inhibitors that would avoid these particular enzymes would be beneficial. Even more noteworthy is the fact that our understanding of MMP biology now means that we recognize that some MMPs are antitumor, and inhibiting these might actually promote tumor development, definitely an unwanted outcome. Together, these considerations have prompted rethinking of how to make truly specific MMP inhibitors that focus on one enzyme at a time. One of the best ways of achieving this type of specificity is to use antibodies rather than small molecules; several companies are now pursuing this approach. It remains to be seen, however, whether this will translate into a successful clinical treatment for cancer patients.

KEY UNANSWERED QUESTIONS

Although MMPs have been associated with tumor invasion and metastasis for almost thirty years, and have been identified as pharmaceutical targets, there are still surprising gaps in our understanding of these enzymes. Identifying the true in vivo substrates is very important for determining the contribution of MMPs to health and disease. The most useful way of identifying substrates is to assess what proteins are cleaved in a physiological setting when active enzyme is present [36]. This is not a trivial undertaking, as it requires that all the proteins present in a setting in which active protease is present be compared with all the proteins present in the same setting, but in which the enzyme is inhibited or removed. Various gel and mass spectroscopic methodologies have been developed to attempt such a comparison, but none is truly complete, as there are issues with sensitivity as well as ensuring that all types of proteins are detectable. There are currently major research efforts to identify the true in vivo "proteome" in different disease states [37]. Understanding which substrates are processed by which enzymes should contribute greatly to our ability to pick the right targets for therapeutic inhibition.

Currently there are multiple suggestions as to what roles MMPs play in metastatic events. An example of an enzyme with complex, multifunctional roles is MMP9. Recent data from the laboratory of David Lyden showed that MMP-9 contributed to formation of a premetastatic niche through processing of the matrix molecule fibronectin [38]. Other researchers had also suggested that MMP-9 was critical for future development of metastasis, although the mechanism was not clearly defined [39]. MMP-9 activity also appears important for initial survival of metastasizing cells at secondary sites, at least for some tumor types [40]. Again, the mechanism for this effect is not clear. Finally, MMP-9 can contribute to outgrowth of a metastatic focus at its secondary site [41]. This appears to be related to the ability of MMP-9 to release the angiogenic factor VEGF from matrix [27], thus generating an angiogenic event. It is unknown what conditions lead to these disparate functions of MMP-9 in metastasis, why only some of these functions are manifested in different tumor types, and which of them would represent the most useful step to target.

As mentioned earlier, identifying the true MMPs responsible for invasion has also been difficult. Although the evidence that MMP-14 is the rate-limiting enzyme for invasion is especially convincing, there have been indications that tumor cell movement can proceed in the absence of any proteolytic activity. Data from the laboratory of Peter Friedl showed tumor cells moving through areas of matrix proteins despite the presence of a cocktail of proteinase inhibitors [42]. The investigators suggested that tumor cells gained "amoeboid movement" capability that resembled the way leukocytes move in the absence of proteolysis. Whether this is a regularly occurring event remains unclear and the subject of much debate.

In conclusion, although MMPs have been regarded as the quintessential regulators of tumor invasion, accumulating evidence suggests that they contribute in many more ways to tumor biology. Furthermore, it is likely that many MMPs are not critical for invasion itself but may control other aspects of metastasis. Understanding the myriad functions of the different MMPs, and under what conditions each is manifested, remains a major research goal with huge translational potential.

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17 Cell-Derived Microvesicles and Metastasis

Hector Peinado, Bethan Psaila, and David Lyden

OVERVIEW

Cell-membrane–derived vesicles are spherical membrane fragments shed by several cell types during their normal functioning. In the past, this "cellular dust" was overlooked and dismissed as insignificant debris. However, a role for these particles in physiological processes such as coagulation, immune regulation, intercellular crosstalk, and molecule delivery has now been established.[1,2] In addition, increasing attention has been focused on the role of membrane vesicles in pathological processes, particularly as a cell–cell communication system that promotes tumorigenesis and malignant progression. This chapter outlines the complex interplay between membrane vesicles derived from tumor cells and host cells. Although molecular pathways involved in membrane vesicle biology and function have not been delineated, therapeutic manipulation of membrane-derived vesicles in patients with cancer has already entered the clinical arena. In the future, targeting membrane-derived vesicles may prove to be an effective approach in reducing morbidity and mortality of advanced malignancy, particularly in metastatic disease.

INTRODUCTION

Membrane-derived vesicles have been broadly classified into two types based on their size and mechanism of release. Microvesicles are small, heterogeneous membrane particles between 100 nm and 1 μm in size that are released from the intracellular endosome by membrane blebbing.[2] In contrast, exosomes are even smaller membrane particles (30 nm–100 nm) thought to originate from multivesicular bodies during endocytosis.[3,4] Although membrane vesicles derived from cells of hematopoietic origin were the first to be identified, including those released from B and T lymphocytes, platelets, dendritic cells, mast cells, and reticulocytes,[5] recent evidence indicates that non-hematopoietic cell types, such as neurons, epithelial cells, and tumor cells, can also shed microvesicles.[6–9] Membrane-derived vesicles may be further classified according to the cell type from which they originate. For example, platelet-derived microvesicles are known as *microparticles*, whereas those originating from polymorphonuclear leukocytes are described as *ectosomes*.[2] Although more studies are necessary to elucidate the functional similarities and differences between microvesicles and exosomes, it is likely that the two types of membrane vesicles share many biological functions.[10,11]

Because of their extremely small size, microvesicles may be mistakenly dismissed as insignificant cellular debris. However, accumulating evidence suggests that microvesicles play an active role in cell regulation and cell–cell communication in both physiological and pathological processes, and may act either locally or systemically.[2,12] Thus, studies have shown that morphogen-enriched microvesicles known as *argosomes* play an important role in tissue patterning by creating a gradient of morphogenic proteins over large distances in *Drosophila* development.[13,14] In addition, membrane-derived vesicles have been shown to directly activate cells via ligand–receptor interactions, to mediate the transference of membrane receptors, and to shuttle mRNA, proteins, and lipids among different cell types (Figure 17.1).[3,15–17] In this regard, microvesicles constitute a novel system for horizontal cell–cell cross-regulation and as messenger vehicles for intercellular communication.

Given these data and the observation that the rate of vesicle shedding is markedly increased in most neoplastic cells, it is not surprising that microvesicles have been implicated in carcinogenesis and metastasis. During carcinogenesis, tumor cells interact with their

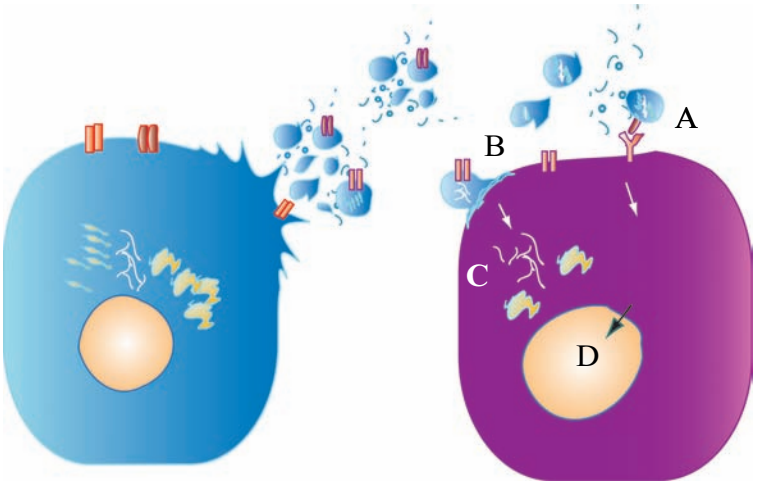

Figure 17.1. Mechanisms by which microvesicles may act as a cell–cell communication system. (A) Direct cellular activation via receptor–ligand interactions; (B) transference of surface receptors from one cell type to another; (C) deliverance of proteins, mRNA, or other bioactive molecules from once cell to another; (D) genetic and/or epigenetic reprogramming of target cells.

microenvironment directly via cell–cell communication and indirectly via secreted factors. Since the advent of microvesicle research, membrane vesicles derived from both tumor and host cells have been recognized as prime candidates for playing important roles in the promotion of tumor growth and malignant transformation. Furthermore, the tumor microenvironment may also modulate the presence and function of membrane-shed vesicles.[18]

Tumor Cell-Derived Microvesicles

Microvesicles secreted by tumor cells have been implicated in all stages of the metastatic cascade: tumor cell invasion and matrix degradation,[19,20] angiogenesis,[21–23] immune evasion,[12,24–31] and arrest at the secondary site.[6] Of note, microvesicle shedding by tumor cells has been also implicated as a drug efflux mechanism, potentially explaining certain cases of drug resistance.[32]

Proteomic analyses of the contents of microvesicles and exosomes secreted by cancer cells have revealed the presence of many proteins implicated in the migration, invasion, proliferation, angiogenesis, metastasis, chemotaxis of host stromal cells, and immune escape.[2,6,12,33–35] One implication of these findings is that the process of packaging proteins into microvesicles is an organized and controlled process, as microvesicle contents appear to be specifically enriched in enhancers of carcinogenesis and metastasis. The details of the processes underlying membrane vesicle formation remain largely unknown.

In addition to vesicle contents, the membrane itself appears to be biologically active, and the process of membrane vesicle shedding by tumor cells is thought to be spatially regulated. Vesicles are more likely to be derived from selected areas of the plasma membrane and are enriched in certain surface molecules and proteases present on tumor cells.[36] Many of these molecules, including CD44, CD63, CD147, and CD95L, have been correlated with malignant behavior.[7,19,37,38] In particular, CD147, also known as extracellular matrix metalloproteinase (MMP) inducer, has been shown to interact with host stromal fibroblasts to stimulate their production of MMP-1, MMP-2, and MMP-3,[39] as well as to enhance the angiogenic capacity of endothelial cells.[31] Moreover, microvesicles have been found to be a new agent for protease delivery and activity within the primary tumor, or even at distant sites. For example, the metalloproteinase ADAM10 has been found to be released in membrane microvesicles actively participating in cleavage and secretion of the adhesion molecule L1, known to promote tumor cell migration.[40] Similarly, the membrane tetraspanin protein CO-029/D6.1A can be delivered in exosomes playing a role in angiogenesis in distant organ. This protein is delivered in tumor exosomes reaching distant organs, where they seem to collaborate in angiogenesis, promoting increased MMP and urokinase-type plasminogen activator secretion, and pronounced vascular endothelial growth factor expression by fibroblasts and endothelium.[41] Altogether these data suggest that microvesicles could play an active role both within the tumor microenviroment and future metastatic sites, generating a microenvironment for the tumor cell growth.

Some glycoproteins, such as EMMPRIN, have also been found to be released from the surface of tumor cells via microvesicle shedding. During this process, the vesicles rapidly break down, releasing bioactive EMMPRIN and stimulating MMP expression in

fibroblasts to promote tumor invasion and metastasis.[42] Indeed, recent reports have demonstrated that glioblastoma-derived microvesicles contain a specific marker, the tumor-specific protein EGFRvIII, that can be detected in serum microvesicles from glioblastoma patients as well as expressed in human glioblastoma or glioma-derived cell lines, indicating its potential use as a new diagnostic marker in cancer patients.[43,44]

In addition to the membrane proteins, nonprotein components of the membrane have been also shown to function in oncogenesis and metastatic progression. Notably, the membrane of microvesicles is composed of large amounts of sphingomyelins, major membrane phospholipids that are particularly enriched on the surface of highly metastatic cancer cells relative to the parent cell plasma membrane.[45] In a study of human fibrosarcoma and prostate cancer cell lines, microvesicle sphingomyelin was identified as the primary factor responsible for microvesicle-induced angiogenesis, as it stimulated endothelial cell migration and invasion, formation of capillary-like structures in Matrigel, and in vivo angiogenesis in the embryonic chick chorioallantoic membrane assay.[21]

Microvesicles shed by tumor cells also have a procoagulatory effect similar to platelet-derived microparticles, additionally contributing to metastatic progression. In particular, tumor release of microvesicles containing tissue factor (TF) is thought to contribute to the systemic prothrombotic state that frequently characterizes malignancy.[46] Furthermore, TF-containing microvesicles support tumorigenesis by creating niches for tumor cell proliferation and expansion of cancer stem cells within the local tumor microenvironment. TF leads to fibrin deposition, which in turn promotes angiogenesis, and $CD133^+$ tumor stem cells are thought to express elevated levels of TF to deposit fibrin and to concentrate growth factors in these niches, in part by enhancing the expression of fibroblast growth factor (FGF)-2.[46,47]

In addition to autologous promotion of growth and survival at the tumor site, tumor-derived membrane vesicles have also been implicated in tumor evasion of immune attack, thereby enabling systemic dissemination of metastatic cells and seeding of secondary tumors at distant sites.[12,24,27,33] A variety of human cancer cells, including melanoma, colorectal, and ovarian cancer cells, has been shown to produce microvesicles that induce apoptosis of activated tumor-specific T cells via the expression of FasL and other proapoptotic molecules.[26–30] Furthermore, tumor-derived microvesicles impair the differentiation of $CD14^+$ monocytes to dendritic cells (DCs), thereby interfering with antigen presentation and T cell priming.[31] Finally, tumor-released microvesicles also promoted the generation of a subset of myeloid cells that suppressed T cell function via release of TGF-β.[27,31]

Host Cell–Derived Microparticles in Metastasis

Platelet-Derived Microparticles

Currently, platelet-derived microparticles are the most extensively studied microvesicles. Originally described as procoagulatory particles, they are now known to play an additional role in inflammation, immunomodulation, hematopoietic stem cell engraftment, and angiogenesis, and have been shown to strongly enhance tumor metastasis via several mechanisms.[5,48,49] Incubation of human breast cancer cells with microparticles derived from platelet concentrates enhanced their invasive potential by inducing transfer of platelet surface integrin CD41 to the breast cancer cell surface and promoting enhanced adhesion to endothelial cells.[48] Expression of the chemokine receptor CXCR4 by the tumor cells was also upregulated, enhancing chemotaxis toward stromal-derived factor 1 (SDF1) gradient.[48] Platelet microparticles also altered intracellular signaling in lung cancer cells by upregulating activity of STAT kinase pathways and enhancing expression of proangiogenic factors such as vascular endothelial growth factor (VEGF), interleukin-8, and hepatocyte growth factor. Secretion of MMPs by stromal fibroblasts and lung or breast tumor cells was also increased in the presence of platelet-derived microparticles, thereby accelerating tumor growth, extracellular matrix proteolysis, and invasion.[48,49]

Interestingly, the diagnostic and prognostic utility of microparticles in patients with malignancy has also been investigated. Studies in gastric and lung cancer patients demonstrated that the platelet or monocyte microvesicle number correlates with the presence of distant metastasis, although further studies are required to confirm a causal relationship.[22,50]

A pivotal role for bone-marrow–derived cells of hematopoietic and endothelial lineage in primary tumor angiogenesis has been well established.[51–53] Bone-marrow–derived cells have been implicated as pivotal in both initiation of metastasis[51] (initiating premetastatic niches – see recent reviews[52]) and mediation of the micrometastatic to macrometastatic switch.[53] To date, the mechanisms suggested to underlie the site-specific recruitment of bone-marrow–derived cells to sites of tumor angiogenesis and metastasis have described only soluble factors and chemokines; the potential role of microvesicles in this process has not yet been evaluated. However, it is possible that membrane-derived vesicles may play a role in the crosstalk between the primary tumor and host cells that leads to homing of both cell types to metastatic niches.

Several reports indicate that platelets can transfer several of their surface antigens to the surface of

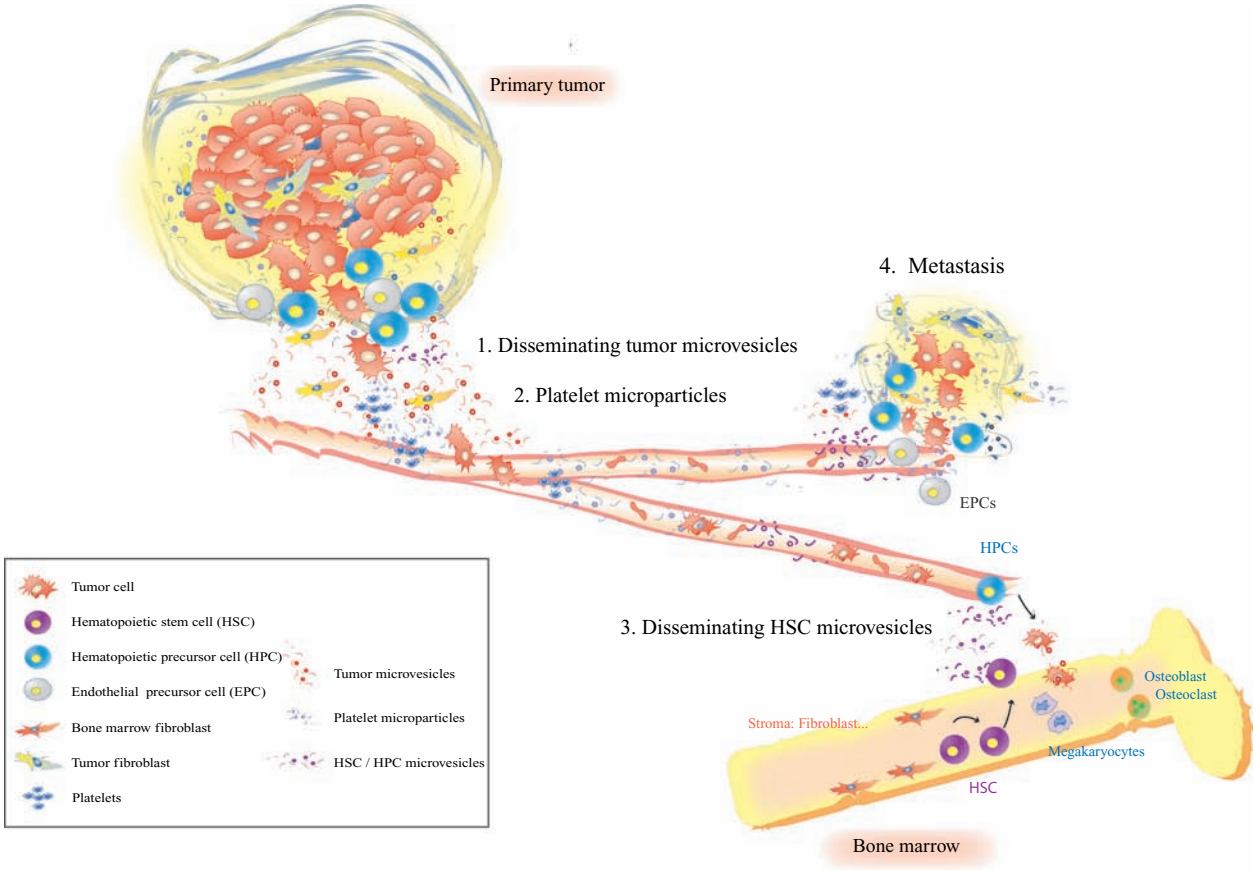

Figure 17.2. Suggested model of the interactions among tumor cells, host cells, and bone-marrow–derived cells. In addition to the actual theories, microvesicles derived from tumor cells and hematopoietic cells could potentially mediate the formation of metastatic niches. In this model, (1) microvesicles secreted by the primary tumor could promote hematopoietic cell attraction to the primary tumor. (2) In the primary tumor, microparticles secreted by platelets can contribute to generate the tumor microenvironment, promoting malignant tumor cell behavior. (3) Hematopoietic stem cell microvesicles could attract tumor cells to specific niches in the bone and in future metastatic sites (4) where the secondary metastatic foci will develop.

hematopoietic stem/progenitor cells via release of microparticles, promoting engraftment of those cells in the bone marrow.[16] Similarly, transfer of receptors such as CXCR4 from platelet microparticles to bone-marrow–derived cells may promote their chemotaxis toward SDF1 gradients, which are known to guide the homing of bone-marrow–derived cells and tumor cells in the bone marrow and in the periphery. Given the evidence for horizontal transfer of material between hematopoietic progenitors and other cell types,[15–17] it is appealing to speculate that microvesicles derived from tumor cells and hematopoietic cells could potentially mediate the formation of metastatic niches. A hypothetical working model is suggested in Figure 17.2. This model illustrates the potential pathologic collaboration between tumor and hematopoietic cell-derived membrane vesicles to promote tumor progression and metastasis. Detailed analysis of the contribution of microvesicles to metastasis may shed new light on the molecular mechanisms involved in metastatic niche formation.

Host Immune Cell–Derived Membrane Vesicles

Microvesicles and exosomes may mediate both positive and negative immune regulatory functions in the context of malignancy, depending on their cells of origin.[27] Whereas tumor-derived membrane vesicles promote immune suppression, host microvesicles/exosomes derived from DCs and T cells are immunostimulatory and may promote antitumor immunity.[54] DCs release large quantities of exosomes, known as *dexosomes*. Dexosomes are enriched in major histocompatibility complex (MHC) class II molecules; class I, costimulatory molecules (CD80, CD86); and tetraspanins.[55] Generally, the function of dexosomes is to transfer antigen-loaded MHC molecules from mature DCs to naive DCs, potentially leading to amplification of the cellular immune response.[56] However, the mechanisms by which exosomes induce antigen-specific immune responses have not yet been elucidated.

It has also been demonstrated that dexosomes can trigger a potent T cell-dependent response.[57,58] It

appears that mature bone-marrow–derived DCs activate T cells more efficiently than immature DCs do.[59,60] Activation of nuclear factor kappa B (NF-κB) induced by dexosomes may be one of the main pathways that contribute to increased T-cell survival and activation;[61] however, the molecular mechanisms involved in this response are still being investigated. Interestingly, functional analysis using intercellular adhesion molecule (ICAM)-1 knockout mice showed that MHC class II and ICAM-1 molecules are required for dexosome priming of naive T cells. In this study, the authors proposed a model in which dexosomes are secreted by mature DCs into the lymph nodes, acting as MHC and adhesion molecule microdomains that, once bound to other antigen-presenting cells (APCs), induce T-cell activation.[62]

Because of the immunostimulatory properties of DC microvesicles, attempts have been made to isolate dexosomes from cultured DCs that have been loaded with specific tumor-associated antigens.[63] Pioneering studies by Zitvogel et al. first demonstrated that vaccination with dexosomes resulted in eradication of tumors in animal models via immunogenic T-cell–based response.[64] These studies showed that dexosomes were indeed active vesicles with an immunoregulatory capacity and potent antitumor effects. Moreover, it has been suggested that a combination of dexosome therapy and immunosuppressive agents, such as cyclophosphamide, could be a promising approach for immunotherapy against certain types of cancers.[65] To date, the possibility of therapeutic utilization of the ability of dexosomes to deliver antigens has been tested in several Phase I clinical trials in non–small-cell lung cancer (NSCLC), adenocarcinoma, prostate cancer, and melanoma patients.[66–70] These early preclinical and clinical data indicate that dexosomes loaded with tumor antigen-derived peptides may be used in some cancers as a treatment modality. It has been suggested that dexosomes would constitute a more effective exosome-based vaccine for the induction of antitumor immunity than exosomes derived directly from tumor cells.[71] However, exosomes derived from patient tumors have been suggested as the "natural" source of antigens because they contain the same unmodified antigens as tumor cells; in consequence tumor microvesicles or exosomes could be potentially used as tumor-rejection agents by using them as immunization material against cancer.[72]

MEMBRANE-DERIVED VESICLES AS FUTURE ANTIMETASTATIC THERAPY?

A wealth of data accumulated over the past decade suggests that membrane-derived vesicles play an important role in oncogenesis and metastatic progression. Microvesicle biology and function have emerged as highly complex fields, and much remains unknown regarding their release mechanisms and the regulation of their contents and surface components. However, the studies to date have suggested that therapeutic manipulation of membrane-derived vesicles may be highly fruitful in the management of malignancy.

For example, tumor-derived microvesicles could serve as a new diagnostic/prognostic indicator of tumor stage, based on analysis of their number and/or composition. This hypothesis is already under investigation in the context of ovarian carcinoma, in which studies have indicated that microvesicle number is correlated with tumor aggressiveness and metastatic phenotype.[73,74] Furthermore, exosomes derived from host dendritic cells and tumor cells are under clinical investigation for cancer immunotherapy following preclinical studies that demonstrated that they may serve as a cell-free vaccine to induce strong antitumor immunity,[64,75] A Phase I clinical trial of therapeutic administration of exosomes derived from malignant ascites in combination with granulocyte macrophage–colony stimulating factor (GM-CSF) in patients with colorectal carcinoma[76] has been performed. This study reported successful induction of specific antitumor immunity against the carcinoembryonic antigen (CEA).[76] Alternatively, it has been suggested that extracorporeal removal of tumor-derived immunosuppressive exosomes may be an effective approach to combat immune suppression in cancer.[77] However, therapies that specifically target the metastatic phase of oncogenesis are lacking. Targeting the microvesicle communication system may prove to be a novel and effective means for tackling metastatic disease, for which there is an urgent need for new therapeutic modalities.

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18 Exploring the Earliest Steps in Metastasis: The Pre-metastatic Niche

Marianna Papaspyridonos, David Lyden, and Rosandra N. Kaplan

Even though significant advances have been made in recent years in the treatment of localized malignancies, metastatic disease remains the primary cause of morbidity and mortality in cancer. Steven Paget's "seed and soil" hypothesis for metastasis first set forth the concept that a nutritive microenvironment is required to enable the engraftment of disseminating malignant cells in distant tissues. Since his observation more than one hundred years ago, our understanding of the microenvironment at the primary tumor site has expanded. However, the pathophysiology of the local cellular context, or "niche," at distant metastatic sites that addresses Paget's original hypothesis has not been a focus of intensive research until very recently.

Of the millions of cancer cells that enter the circulatory system, very few will successfully engraft, survive, and proliferate at secondary sites.[1–4] The well-documented inefficiency of the metastatic process is thought to be the result of the inability of the vast majority of disseminating cells to successfully initiate tumor growth at distant sites.[5] The efficiency of survival and proliferation of tumor cells after arrival at these sites is likely to be a major factor in determining whether metastatic growth is successful[5] and is thought to require a receptive microenvironment at the destination site.

In stem cell biology, the "niche" describes the specialized microenvironment that supports stem cell maintenance and controls the balance between proliferation and quiescence of the stem cells.[6] Studies of the earlier stages of metastatic progression have uncovered a distinct step prior to the arrival of the tumor cells that involves the creation of niche-like hotspots in organs that are future sites of metastasis. These aberrant niches form by the accumulation of clusters of bone-marrow–derived cells (BMDCs) that interact with the host stromal cells and may create a microenvironment that is permissive for the subsequent engraftment and growth of tumor cells.[7–10] (Figure 18.1). Pre-metastatic niches in organs such as the lung are composed of immature myeloid cells that are characterized by their expression of VEGFR1, CD11b, and c-kit[8,11] and, as shown more recently, the chemokines S100A9 and S100A8.[9,12]

In response to factors secreted by the primary tumor, such as VEGF, placental growth factor (PlGF) and fibroblast growth factor (FGF), BMDCs preferentially adhere to areas of increased fibronectin, which is thought to be upregulated by putative fibroblasts, also in response to tumor-secreted factors.[8] VEGFR1$^+$ BMDCs express the integrin VLA-4 ($\alpha_4\beta_1$), which allows them to adhere to fibronectin and initiate cellular cluster formation. Furthermore, matrix metalloproteinase (MMP)-9 expression by bone-marrow–derived myeloid cells allows extracellular matrix remodeling, thus accelerating the extravasation of additional myeloid cells and, later on, tumor cells into the niche.[9]

Recently, the enzyme lysyl oxidase (LOX) has also been implicated in the remodeling of the extracellular matrix. LOX is an amine oxidase, secreted by tumor cells, and is thought to induce crosslinking of collagen IV in the basement membrane.[13] As a result, LOX facilitates CD11b$^+$ myeloid cell recruitment by allowing CD11b$^+$ cells to adhere to cross-linked collagen IV and produce MMP-2. Cleavage of collagen by MMP-2 enhances the invasion and recruitment of BMDCs and metastasizing tumor cells to the engraftment site.[11]

Together with fibronectin, stromal cells, and proteolytic enzymes, VEGFR1$^+$ BMDCs alter the local microenvironment by upregulating the expression of a variety of integrins and chemokines, such as SDF-1, that promote attachment, survival, and growth of tumor cells. Moreover, VEGFR2$^+$ endothelial progenitor cells (EPCs) are recruited to promote vasculogenesis soon after the implantation of tumor cells, enabling maturation to a fully developed metastatic lesion at the site of the pre-metastatic niche.[8] The influx of bone-marrow–derived VEGFR2$^+$ cells is a hallmark of the

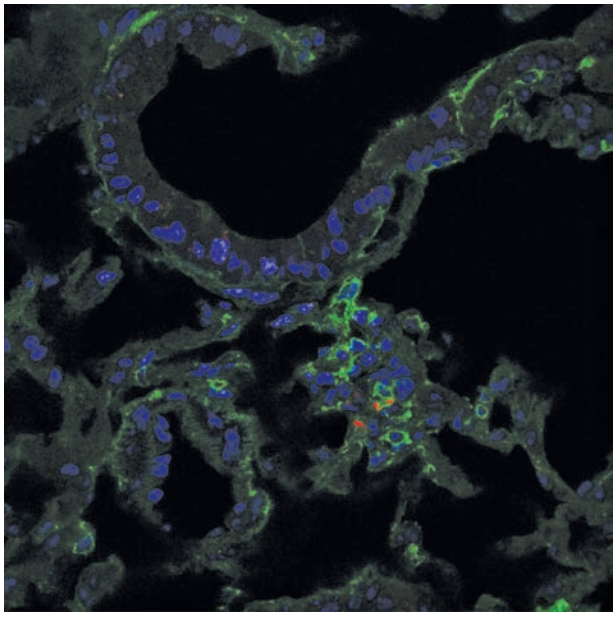

Figure 18.1. Lung tissue 18 days after injection of mCherry-labeled B16 tumor cells in the flank in a mouse that had previously received a GFP+ bone marrow transplant. mCherry B16 tumor cells (red) can be seen adhering to clusters of GFP+ bone marrow-derived cells (green). Blue = nuclei. Magnification = 200×.

angiogenic switch essential to metastatic progression and formation of a vascularized tumor at the metastatic site.[14]

The dependence of the evolving metastatic process on the changes at the engraftment site is illustrated by experiments using monoclonal antibodies against VEGF receptors. Neutralizing antibodies against VEGFR1 eliminated the pre-metastatic niche, whereas use of VEGFR^2-specific antibodies allowed the formation of small micrometastases without vascularization, preventing formation of full metastatic lesions.[8] These experiments highlight the role of immature myeloid cells expressing VEGFR1$^+$ in initiating metastasis and recruiting EPCs for neoangiogenesis and metastatic tumor growth. Similar to carcinoma-associated fibroblasts and tumor-associated macrophages, which promote tumor progression via the creation of a supportive microenvironment, the VEGFR1$^+$ BMDCs may also foster inflammation and sustain tumor cell growth at distant organ sites,[15–17] as discussed later. In fact, these BMDCs have the potential to mature into tumor-associated macrophages (TAMs) at the metastatic site and recruit both bone-marrow–derived endothelial cells and fibroblasts.

TUMOR-INDUCED IMPAIRED MYELOID CELL DIFFERENTIATION

Tumor-secreted factors are known to affect myelopoiesis by inhibiting the differentiation and maturation of antigen-presenting cells from their bone-marrow–derived myeloid precursors, leading to the accumulation of immature myeloid cells in cancer patients and tumor-bearing mice.[18] These immature myeloid cells are thought to be instrumental in cancer progression in multiple ways, by inhibiting of adaptive immune responses against tumors in lymphoid organs[19–26] and by their differentiation into immune-suppressive TAMs.[27–30]

The BM-derived VEGFR1$^+$ cells that form the pre-metastatic niche may represent subsets of circulating immature myeloid cells termed *myeloid-derived suppressor cells* (MDSCs).[31] 'MDSCs, also characterized by the expression of the markers Gr-1 and CD11b in mice, may underlie the limited effectiveness of cancer vaccines and other therapies, such as anti-vascular endothelial growth factor (VEGF) treatment.[32,33] Despite the wealth of information regarding the functional importance of MDSCs, the mechanism governing their accumulation in the tumor setting in association with inhibition of dendritic cell (DC) and macrophage differentiation remains unknown. It is thought that in normal conditions, BMDCs migrate to peripheral organs, where they differentiate into macrophages and DCs. However, in the tumor microenvironment, various tumor-derived factors (VEGF, IL-6, IL-10, M-CSF, and GM-CSF) are thought to prevent their differentiation into fully mature immune cells and induce the expansion of MDSCs that remain in an immature state.[34]

The block in myeloid differentiation during cancer progression has three main consequences. First, the number of functionally competent DCs and macrophages is reduced. Second, the expansion of an immature myeloid population negatively regulates the immune response by suppressing T-cell responses.[35] Third, via their high reactive oxygen species (ROS), the immature myeloid cells may induce local changes that resemble an inflammatory state and that facilitate adherence of incoming tumor cells into these distant pre-metastatic sites.[8]

Recent studies have identified the S100A9 and S100A8 as key factors in promoting abnormal myeloid cell differentiation in cancer.[36] Specifically, S100A9, expressed together with its dimerization partner, S100A8, was found to be consistently upregulated in MDSCs. Importantly, these two proteins appear to have a functional role in myeloid cell differentiation.[37] Tumor-induced expression of STAT3 has been shown to upregulate S100A9 levels, which, in turn, were found to be critically important for accumulation of MDSCs. This may represent a universal molecular mechanism of tumor-induced abnormalities in myeloid cells in cancer, directly linking STAT3, a key inflammatory pathway and the role of MDSCs in immune suppression. In addition, the upregulation of S100A8 and S100A9

in the pre-metastatic sites[9,12] could support the notion that the BM-derived myeloid cells that contribute to the pre-metastatic niche may constitute subsets of MDSCs, or vice versa.

ROLE OF S100A8 AND S100A9 AT THE PRE-METASTATIC NICHE

The recent evidence, in studies by Hiratsuka et al.,[9] for the presence and functional role of S100A8 and S100A9 in the formation of the pre-metastatic niche in the lungs in tumor-bearing animals has demonstrated that upregulation of these chemokines by immature myeloid and endothelial cells may serve to further enhance the influx of $CD11b^+$ myeloid cells to the pre-metastatic sites. The expression of S100A8 and S100A9 was induced in $CD11b^+$ myeloid cells and endothelial cells exposed to tumor-secreted factors unlike those in the absence of tumor-secreted factors, suggesting the incomplete differentiation status of these cells. Moreover, neutralizing anti-S100A8 and anti-S100A9 antibodies blocked the migration of both tumor cells and $CD11b^+$ myeloid cells, indicating that the S100A8/S100A9 pathway may function in myeloid cell differentiation, recruitment, and tumor cell invasion.

A follow-up study on the role of S100A8 and S100A9 identified a novel pathway by which these proteins induce cell accumulation.[12] It was proposed that these chemoattractants serve to upregulate serum amyloid A 3 (SAA3), which then acts as a positive-feedback regulator for chemoattractant secretion via toll-like receptor 4 (TLR4) and NF-κB. Activation of these additional inflammatory pathways creates an inflammatory-like state that accelerates the migration of primary tumor cells to lung tissues. Thus, blocking SAA3-TLR4 function in the premetastatic phase could prove to be an effective strategy for the prevention of pulmonary metastasis.

NOVEL TUMOR-SECRETED FACTORS IN BMDC RECRUITMENT

The mobilization and recruitment of BM-derived myeloid cells is thought to result via the action of chemoattractant and angiogenic cytokines such as VEGF and PlGF (a VEGF family member that binds specifically to the VEGF receptor, VEGFR-1) secreted by the primary tumor.[8] A new study shed further light on the tumor-secreted proteins that may initiate the migration of BMDCs in peripheral sites and the formation of the pre-metastatic niche by conducting a screen to identify macrophage activating factors that are secreted by metastatic carcinomas.[38] A biochemical analysis of Lewis lung carcinoma (LLC)-conditioned medium (LCM) led to identification of versican, an extracellular matrix proteoglycan upregulated in many human tumors, including lung cancer.[39,40] Versican was shown to be a potent macrophage activator via TLR2 and its coreceptors TLR6 and CD14. By activating TLR2:TLR6 complexes and inducing TNF-α secretion by myeloid cells, a potent inducer of vascular permeability, versican strongly enhanced LLC metastatic growth. These findings further support the idea that advanced cancer cells usurp components of the host innate immune system, including bone-marrow–derived myeloid progenitors,[8] to generate an inflammatory microenvironment hospitable for metastatic growth.

PRE-METASTATIC NICHE FORMATION: NOVEL EMERGING THERAPEUTIC TARGETS

As discussed earlier, targeting metastatic growth is crucial in reducing cancer-associated morbidity and mortality. Inhibition of $VEGFR2^+$ EPCs has already proven moderately efficacious in controlling metastatic disease.[41] However, tumors containing $CD11b^+$ $Gr\text{-}1^+$ cells show a decreased response to anti-VEGF therapy.[33] In patients with gastric, prostate, and colorectal cancer, the presence of VEGFR1 has been shown to be associated with an increased risk of metastasis.[42–45] VEGFR1 expression has been shown to be mediated by oxidative stress, as seen in pro-inflammatory states, and may help to explain the links among inflammation, immune suppression, and metastatic progression.[46] Targeting this process can have a dual role in decreasing tumor-induced immune suppression and reducing metastatic progression. The close relationship between the pathways that control vascularization and myeloid cell recruitment suggest that combined blockade of VEGFR1 and VEGFR2 may be more effective than monotherapy targeting each individual pathway. Use of both neutralizing antibodies in conjunction with small-molecule inhibitors of these receptors may circumvent resistance seen with internalization of the receptor and activation through autocrine mechanisms. In addition to VEGF receptor targeting therapies, agents inhibiting these BMDCs through inhibition of integrin VLA-4, MMPs, and Id proteins may prove a useful adjunct to alter the milieu established by these BMDCs.[8] Such approaches to target immune-modulating and angiogenic functions can serve to be more effective in preventing metastatic spread.

As mentioned previously, LOX was recently implicated in the formation of the pre-metastatic niche.[11] $CD11b^+$ cells and LOX were found to co-localize in biopsies of human metastases, and increased levels of LOX correlated with poor survival in patients with breast or head and neck cancer.[11] More importantly, inhibition of LOX activity prevented $CD11b^+$ cell recruitment as well as metastatic growth, indicating a critical role for LOX in pre-metastatic niche formation and suggesting that LOX may be a therapeutic

target for the treatment and prevention of metastatic disease.

TSU68, an inhibitor of VEGFR2, platelet-derived growth factor receptor beta (PDGFR-β), and FGFR1, has been shown to prevent orthotopic colon tumors from metastasizing to the liver by targeting the pre-metastatic niche.[47] CXCR2 and IL-12, chemokines produced by infiltrating myeloid cells that contribute to the creation of a pro-inflammatory environment, were shown to be inhibited with the use of the therapeutic TSU68, underscoring the importance of myeloid cell recruitment in metastatic growth initiation and progression. Therapeutic use of anti-inflammatory agents such as TSU68, antibodies targeting versican or S100A8/S100A9, or inhibitors of LOX may inhibit alternative pathways to angiogenesis and act synergistically with anti-angiogenic agents to prevent the metastatic cascade.

In addition, therapeutic strategies to promote myeloid cell differentiation[48,49] or prevent the accumulation of the immature myeloid cell population[46] in combination with chemotherapeutic agents that reduce their numbers[50,51] may assist in mounting an effective anti-tumor response and preventing tumor progression.[35]

Current and future work on tumor-derived factors that are involved in the initiation of metastatic growth, impaired differentiation of host myeloid cells, and migration of those immature myeloid cells and tumor cells to distant sites should aid our understanding of the metastatic process and accelerate the development of novel anti-metastatic therapies.

CONCLUSIONS

Current findings provide evidence that cellular/molecular events at distant metastatic sites, including immature myeloid VLA4$^+$ VEGFR1$^+$ cellular infiltration and activation of inflammatory pathways, accelerate the development of metastatic lesions. Many details regarding the interactions among tumor cells, tumor-associated cells, and the resident stroma at premetastatic sites remain to be elucidated. The molecular and functional phenotype of the myeloid cells and other bone-marrow–derived cells that are recruited to premetastatic sites has yet to be fully characterized; the variation in surface markers used to characterize the cells compounds this challenge. It is likely that both immature progenitors and fully differentiated cells are involved. Both CD11b and VEGFR1 are expressed on a wide variety of myeloid cells, including progenitor cells and MDSCs, and these studies may have examined overlapping cell subsets. Furthermore, in addition to targeting the myeloid cell compartment, therapies aimed at the putative fibronectin-expressing fibroblasts could be another approach to prevent metastatic progression by preventing myeloid cell adherence to these pockets of fibronectin.

Dissecting the precise mechanisms underlying the dysfunctional myeloid cell differentiation and the interactions between BMDCs and their niches during the development of metastatic disease may lead to the development of specific therapies that prevent aberrant niche formation. Identifying and inhibiting the cytokines and growth factors that promote cellular migration may provide an additional arsenal for abrogation of the early and late processes of tumor growth and metastasis. Perhaps it is through this approach that therapeutic targeting of metastatic progression will prove most beneficial.

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19 Growth Regulatory Pathways Contributing to Organ Selectivity of Metastasis

Suzanne A. Eccles

HISTORICAL CONTEXT

There has long been an appreciation that when cancer spreads, the secondary sites colonized are more or less predictable; however, each cancer type displays different predilections. The presence of cancer in both a primary site (e.g., breast) and elsewhere (e.g., nodes or lungs) and the appreciation that these were pathologically linked was accurately recorded in the seventeenth century. In 1829, Joseph Recamier, a gynecologist in Paris, recognized the discontiguous dissemination of cancer. He described the invasion of veins and distant metastases in the brain of a breast cancer patient but did not realize that it was cancer *cells* that were spreading the malignant disease. The surgeon James Paget wrote that there was no need to invoke corpuscles or germs to explain the spread of cancer, and that "an unformed cancerous blastema" must be assumed when dissemination occurred in organs beyond the lungs in the course of the circulation [1].

Most famous of all are the observations of James Paget's son Stephen, who took up the challenge to answer the question, "What is it that decides what organ shall suffer in a case of disseminated cancer?" in an article published in the *Lancet* in 1889 [2]. Based on his own observations on the nonrandom distribution of secondary growths in breast cancer patients, and those of Fuchs and Cohnheim, who both proposed that different organs exhibited either "diminished resistance" or "predisposition" to the growth of disseminated cancer cells, Paget formalized the "seed and soil" hypothesis. This hypothesis, stating that seeds (cancer cells) may spread widely but grow only in certain congenial soils (organ sites), has been amply demonstrated in both clinical and experimental studies. Not least, extensive postmortem examinations by Leonard Weiss attest to the nonrandom nature of metastasis [3].

Even though hematogenous and lymphatic circulatory pathways can account for about 50 percent to 60 percent of metastases, there is undoubtedly preferential homing, survival, and/or growth of certain tumor cells at certain sites. However, more than one hundred years later, we are only just beginning to unravel the molecular mechanisms responsible for this phenomenon. Key experiments that underpinned this revolution were the isolation of tumor cell populations differing in metastatic potential [4, 5], identification of genes that suppressed metastasis quite independently of tumor growth, [6–9], and, most recently, the selection of clonal populations from within primary tumors that not only had different metastatic capacities, but also showed site-selectivity [10, 11].

ROLE OF THE MICROENVIRONMENT

When exploring the site-selectivity of metastasis, we can consider the mechanisms that obtain at the cellular level: how tumor cells interact with each other, their environment (host cells and their products, the extracellular matrix), and the molecular machinery that drives the phenotypic effects observed. It is now clear that cells may disseminate very early in the evolution of some cancers [12]. They may grow out, remain dormant (see Chapter 13), and/or seed other sites. Bone marrow and lymph nodes, in particular, are well recognized as "staging posts" for further dissemination [2, 13]. Later waves of pioneer cells may be genetically distinct from the trailblazers, and additional genetic and epigenetic changes may be induced (or selected for) by the new environment. Clearly, tumor cells must have exceptional properties if they are to survive the rigors of detachment (usually a trigger for anoikis), extravasation, and, most important, survival and proliferation in an alien (ectopic) site. Normal cells are designed to thrive only in their correct tissue

environment, responding to microenvironmental cues that allow them to proliferate appropriately, differentiate, perform specialized functions, and ultimately die. Multicellular organisms, even very simple ones, have powerful mechanisms to avoid the taboo of cells surviving and growing in the wrong place. How do cancer cells overcome this? Although many processes and signaling pathways are generic and contribute to tumor growth, invasion, and metastasis per se, here we will focus on the particular ways in which site-selective metastasis may be controlled.

Most cellular behavior is dependent on cues from the environment including systemic hormones, locally acting cytokines, or growth factors. They may be autocrine (cells responding to their own ligands), juxtacrine (receptors and ligands on neighboring cells), or paracrine (cells responding to factors produced by different cells, often at a distance). Organ selectivity may be regulated at several levels: tumor cells may attach preferentially to the microvasculature in different organs or more readily extravasate. This may be caused by simple mechanical factors (the lower resistance of fenestrated capillaries or lymphatic vessels that lack a contiguous basement membrane) or selective transendothelial migration. Of more significance in this chapter is the notion that tumor cells may show apparent tropism to – or selective proliferation within – specific microenvironments, depending on the cell membrane receptors they express and the ligands they encounter (reviewed in [14, 15]). For therapeutic considerations, the so-called metastasis virulence genes, which enable tumor cells to grow and thrive in ectopic secondary sites, are the most important [16], as many patients will present with established micro- or macrometastases. Factors involved in metastasis initiation (which may provide an advantage to tumor cells at primary sites, e.g., via effects on angiogenesis) and progression (which may influence choice of destination) are also of interest mechanistically, although therapeutic intervention at these steps is problematic and possibly of limited clinical value.

Tissues colonized by tumor cells themselves become modified. Initially the cells may react as if to damage or infection; indeed, malignancy has been likened to a wound that does not heal [17], and many of the molecular processes used are similar. Sites of metastasis may become hypoxic, enhancing local invasion and angiogenesis via the upregulation of key genes such as vascular endothelial growth factor (VEGF), lysyl oxidase (LOX), cell membrane receptors, and particularly proteases, which release local growth factors from sequestration in the matrix [18]. Interactions with existing stromal fibroblasts – and those newly recruited from the bone marrow – can enhance tumor cell survival at both primary and secondary sites, although the degree to which this dictates organ selectivity is not entirely clear.

Inflammatory cells are often involved in a cascade of events that can further cancer progression. For example, macrophages stimulated by colony-stimulating factor (CSF)-1 produce growth factors such as PDGF, FGF, EGF, and VEGF, which stimulate proliferation of fibroblasts, tumor cells, and endothelial cells, respectively. Responding fibroblasts and myofibroblasts then secrete chemokines, such as CXCL12 (a specific CXCR4 ligand) and CCL5 (a pleiotropic cytokine), to stimulate tumor growth, invasion, and metastasis. Tumor cell motility and extravasation have been linked with reciprocal activity of tumor-derived CSF-1 and macrophage-derived EGF in experimental models, although it remains to be proven that perivascular macrophages secrete EGF. Lung metastasis was shown to be reduced in the PyVMT transgenic mammary carcinoma model when the CSF-1 gene was inactivated. Because metastasis in these animals is generally restricted to the lung, any organ-specific effects cannot be addressed. However, evidence suggests that tumor cell growth in the lungs is dependent on VEGF-induced expression of matrix metalloproteinase (MMP)-9 by macrophages, and mice whose livers were depleted of macrophages supported colon carcinoma growth less well (reviewed in [19]).

Although tumor cells may show a degree of autonomy from exogenous growth factors, they often take advantage of local ligands that activate their particular repertoire of receptors. Most so-called growth factors actually induce pleiotropic effects on cells, being capable of protecting against apoptosis, stimulating proliferation and motility, and promoting release of proteases and angiogenesis factors. Tumor dormancy may be a result of a lack of appropriate growth factors or the presence of suppressors (including inducers and inhibitors of angiogenesis). In addition, context-specific inactivation of specific signaling pathways may prevent colonization by tumor cells that are fully competent for growth at other sites: for example, activation of the metastasis suppressors MKK4 and MKK7 occurs in prostate carcinoma cells in the lung, but not at the primary site [20].

Initial stages of metastatic organ colonization may be stochastic or promoted by specific adhesive interactions between tumor cells and host endothelial cells or matrix proteins (e.g., mediated by integrins) or response to gradients of chemokines or cytokines. In addition, the importance of the complex two-way interactions in premetastatic niches has recently been recognized. However, for a metastasis to become life-threatening, its growth must be sustained by both intrinsic and extrinsic factors promoting survival, proliferation, and a vascular supply.

KEY SIGNALING PATHWAYS

Receptor Tyrosine Kinases/Ligands

EGFR (ERB-B/HER2, 3, and 4)

The ERB-B/HER family of receptor tyrosine kinases (RTK) – EGFR and HER2, -3 and -4 – are frequently overexpressed (and in some cases mutated) in human cancers. Ligand binding induces dimerization and activation of the MAP kinase, PI3 kinase, and other signaling pathways. Interestingly, EGF and TGFα have been shown to mediate cell motility by distinct matrix-dependent mechanisms involving, respectively, either p70S6K or PLCγ. Both ligands require functional EGFR, but EGF activity is mediated via CD44, whereas TGFα uses integrin $\alpha V\beta 3$. Once triggered, motility is independent of the EGFR ligand but requires continued activity of the matrix receptors and their ligands (CD44-hyaluronan or vitronectin-$\alpha V\beta 3$). Thus, local invasion or metastatic organ colonization may be influenced by matrix composition and availability of multiple ligands [21].

Bone marrow micrometastases express HER2/ERB-B2 more frequently than do primary tumors [22], suggesting that this phenotype is selected for during dissemination, perhaps owing to activation of catenins, enhanced motility, or preferential survival at this site [23]. The predilection of breast cancer cells expressing ERB-B oncogenes to generate CNS metastasis [24, 25] may be explained by the fact that their cognate ligands (heregulins/neuregulins) are brain-derived growth factors. More recently, it has been shown that HER2 overexpression in mammary carcinoma cell lines increases the proportion of stem/progenitor cells and their tumorigenicity and invasiveness [26].

MET/RON

The c-MET proto-oncogene, encoding the tyrosine kinase receptor for HGF, drives cell invasion and metastasis [27]. Like other RTKs, it can interact with a wide variety of SH2-domain-containing proteins to signal via multiple downstream pathways (including PI3K and ERK) leading to many of the key hallmarks of cancer. MET overexpression, often induced by hypoxia, leads to constitutive activation of the receptor and correlates with poor prognosis. Encouragingly, recent experimental studies showed that silencing the endogenous MET gene by shRNA not only inhibited primary tumor growth and invasion but also induced regression of established metastases. This suggests that sustained MET expression is required for maintenance of metastases [28]. RON is a related receptor that can form a complex with MET, and both can be transactivated by EGFR, certain plexins, or integrins. MET overexpression has been linked to lymphatic, bone, lung, and liver metastases. Colon or pancreatic carcinoma cells overexpressing EGFR or MET may respond, respectively, to high levels of TGFα or HGF in the liver [29, 30]. In the case of lung metastases, it appears that MET expression is induced at the metastatic site, rather than being due to preferential survival of MET-expressing cells [31]. MET may also be activated by mutation, especially in metastases, when it may become ligand-independent. RON overexpression in the mammary epithelium of transgenic mice induced invasive tumors that metastasized to the lungs and liver in association with phosphorylation of β-catenin and upregulation of cyclin D1 and c-myc [32]. RON is also upregulated and/or mutated in a variety of human cancers, where it too may be associated with metastasis, although there are as yet no clear examples of associations with site-selectivity [33].

IGF-1R

IGF-1R has been linked to progression and metastasis in many tumor types. It can be transcriptionally upregulated by hypoxia and cooperates with other RTK and GPCR (such as EGFR and CXCR4) to enhance oncogenic signaling. There is evidence that IGF-1R-expressing tumor cells respond chemotactically to ligand gradients, and preferentially adhere to and transmigrate through endothelium in tissues in which IGFs are highly expressed (e.g., bone and liver stroma). IGF-1R expression has been shown to promote the targeting of neuroblastoma cells to bone following systemic injection, their sustained growth at this site, and subsequent metastasis to the liver [34]. IGF-1 and IGF-2 can also induce lymphangiogenesis, as IGF-1R is expressed on lymphatic endothelial cells. In addition, IGF-1R positively regulates expression of the lymphangiogenic cytokine VEGF-C, and by either or both of these mechanisms may potentiate lymphatic metastasis, as shown in the RIP-Tag2 transgenic pancreatic cancer model and Lewis lung carcinoma [35, 36].

PDGFR and FGFR

Platelet-derived growth factors act as autocrine ligands in certain nonepithelial cancers, and as paracrine inducers of stromal recruitment in many epithelial cancers. PDGFRα correlates with lymphatic metastasis in colon cancer [37]; this may be linked to the angiogenic (and, in particular, lymphangiogenic) potential of the ligand PDGF-BB [38]. PDGFRα has also been implicated in prostate cancer metastasis to bone, as its ligands are synthesized by both osteoclasts and osteoblasts. [39].

Fibroblast growth factors (FGFs) often collaborate with PDGF, especially in neoangiogenesis. FGF-2 also synergizes with N-cadherin, which stabilizes FGFR and

leads to sustained activation of the MAPK pathway, MMP-9 induction, and invasion. FGF2 is also implicated in EMT, causally linked to early steps in metastasis in several malignancies. With regard to site-specific metastasis, overexpression of FGFR1 has recently been linked to colorectal cancer liver metastasis [40].

TRKB

TRKB is an RTK whose primary ligand is brain-derived neurotrophic factor (BDNF). It has been recognized as a poor prognostic factor in neuroblastoma (where its expression is increased by hypoxia) and is associated with drug resistance. When stably expressed in neuroblastoma cells, TRKB increased invasive potency both in vitro and in vivo via upregulation of HGF and its receptor c-MET, resulting in an autocrine loop [41]. TRKB – and, also commonly, BDNF – are also overexpressed in metastatic myeloma, pancreatic, hepatic, gastric, prostate, and head and neck cancers. Overexpression of TRKB and BDNF in ovarian cancers is also associated with drug resistance, ascites formation, and omental metastasis [42]. More recently, TRKB was identified as a potent suppressor of anoikis (detachment-induced apoptosis), putatively enhancing tumor cell survival during dissemination in a PI3K-dependent manner [43]. TRKB overexpression and activation increases production of MMPs, uPA, and VEGF, thus promoting invasion and angiogenesis in addition to anoikis resistance.

Eph Receptors and Ephrins

Eph receptors are the largest RTK family; their membrane-bound ephrin ligands are involved in axon guidance but also have been implicated in tumor angiogenesis and invasion. As with semaphorins (which are discussed later), again, a "yin and yang" function for these families is evident, with both increases and decreases of specific molecules associated with cancer progression. Of note to the present discussion is the finding that high levels of EphA2 in NSCLC is associated with metastasis to the brain [44], where one of its ligands (ephrin A3) is preferentially expressed. Overexpression of EphA4 and reduced EphB2 levels correlates with colon cancer liver metastasis [45]. Ephrin A1 is increased in metastatic breast cancer cells and acts as an autocrine growth factor; it also stimulates angiogenesis by inducing release of VEGF and/or attracting EphA2-expressing endothelial cells. Ephrin B2 is upregulated in the most aggressive melanoma cells and is also proangiogenic. EphA2 levels also increase as prostate cancers progress (with metastatic cells expressing ten- to 100-fold more than noninvasive cells) and levels are high in metastatic colon, pancreatic, and esophageal cancers [46]. EphA2 promotes experimental breast cancer progression and lung metastasis by inducing a permissive (angiogenesis-rich) microenvironment. Additional prometastatic effects are oncogene-dependent, as EphA2 was shown to form a complex with ERB-B2, thus amplifying signaling via Ras-MAPK and RhoA GTPase and enhancing metastasis; no such effects were seen in breast cancers induced by transgenic PyVMT [47]. The diversity of effects of EphA2 in different cancer types may therefore again depend on context and oncogene expression. Under normal circumstances, ligand availability may downregulate EphA2, whereas in the absence of juxtacrine stimulation (low ligand levels or dissociated tumor cells), EphA2 may be free to associate with other receptors, such as ERB-B2, and potentiate invasion. It will therefore be important to take these considerations into account when planning therapeutic interventions.

G-Protein–Coupled Receptors/Ligands

Chemokines are small chemoattractant cytokines that bind with varying degrees of selectivity to 7-transmembrane G-protein–coupled receptors (GPCRs). One of their main roles is in the regulation of leukocyte trafficking and the control of inflammatory responses. However, it is now recognized that tumor cells may hijack this signaling pathway to achieve site-selectivity of metastasis [48, 49]. CXCR4 was one of the first receptors to be implicated in the "homing" of breast cancer cells to their favored sites of metastasis (e.g., nodes, lung, bone marrow), in which high levels of its specific ligand (CXCL12) were found. Also, CCR7-expressing breast cells responded to high levels of one of its ligands (CCL21) in nodes, whereas melanoma cells showed high levels of CCR10, which was associated with skin metastasis [50]. CXCR5, responding to CXCL13, has been described as crucial for the growth of colon carcinoma cells in the liver [51]. Since then, many other examples of associations (and, in some cases, mechanistic evidence) of specific chemokine-GPCR interactions and site-selective metastasis have been described (see Chapter 14). They may function as chemoattractants and promote adhesion to vasculature (mimicking lymphocyte trafficking) and survival/proliferation in chemokine-rich secondary sites. CXCR4 is of particular interest, as it is thought to be a master regulator of trafficking of both normal and cancer stem cells and has been implicated in establishment of premetastatic niches (especially in the bone marrow) via mobilization of VEGFR-1–expressing hematopoietic progenitor cells [52, 53] (see Chapter 18).

Semaphorins and Plexins

Although initially described as axon guidance factors, cell-bound or secreted semaphorins and their receptors

(plexins and neuropilins) are now also implicated in angiogenesis, invasion, and metastasis, primarily by acting as chemotactic sensors [54]. Semaphorins can provide both "stop" and "go" signals and, indeed, can signal forward as ligands and backward as receptors. Their roles are complex and sometimes seemingly contradictory, indicating cell-type selective activities. Because the neuropilins, in particular, also bind a number of other molecules (e.g., receptors such as MET, ligands including HGF, PDGF-BB, VEGF, and TGFβ), they act as key regulators, having positive or negative effects depending on context and availability of partners. The secreted class 3 semaphorins bind to NRP1 and 2. SEMA3B and SEMA3F were originally described as a tumor suppressors, which experimentally inhibited migration and invasion of prostate and breast cancer cells and melanoma metastasis, possibly in this case partly by acting as an inhibitor of angiogenesis [55]. However, SEMA3A has been implicated in the progression of pancreatic and colon cancer. and SEMA3C is reportedly proangiogenic. Recently, SEMA3B, although inhibiting primary tumor growth, was found to increase lung metastasis via NRP1 and p38-dependent upregulation of IL-8 and macrophage recruitment [56].

SEMA4D, which binds plexin B1, induces tumor cell invasiveness via MET and RON activation. Plexin B1 and B2 also form complexes with ErbB2; thus, SEMA4D is pro- or antimigratory, depending on which RTKs are expressed [57]. In addition, the cleavage product of SEMA3E is prometastatic, whereas the full-length protein is antiangiogenic [58]. Recently, somatic missense mutations in the cytoplasmic domain of the plexin B1 gene have been identified in 89 percent of prostate cancer bone metastases, 41 percent of lymph node metastases, and in 46 percent of primary cancers. The mutations hinder Rac and R-Ras binding and R-RasGAP activity, resulting in increased cell motility and invasion [59]. These results identify a key role for plexin B1 and the semaphorin signaling pathway in prostate cancer, and given the enrichment of mutants in bone metastases, potentially provide some organ-selective advantage. Because neuropilins and plexins are expressed on endothelial cells, these signaling pathways also play important roles in angiogenesis.

ORGAN SELECTIVITY (SEE CHAPTER 20)

Bone and Bone Marrow

The bone is clearly one of the most alien sites for cancers derived from soft tissues; this is reflected in the clear gene expression signatures that have been described for cancer cells that preferentially colonize it [10]. Bone metastasis is particularly common for breast cancers and prostate cancers, although the former preferentially induce mainly osteolytic lesions, and the latter osteoblastic lesions. This can partly be explained by the production of growth factors that differentially activate osteoclasts (e.g., PTHrP; TNFα; IL-1, 6, 8) or osteoblasts (e.g., BMPs, PDGF, and Wnt ligands). In the former case, activated osteoclasts release from the bone matrix factors such as FGF, TGFβ, and IGF-1, which support the survival and proliferation of tumor cells expressing the cognate receptors. Tumor cells may also release GM-CSF, which stimulates the bone marrow to produce more osteoclasts. Finally, breast cancer cells were shown to induce bone-derived mesenchymal stem cells within their stroma to secrete CCL5 in co-cultures. This enhanced the invasion and metastatic potential of the tumor cells [60]. Metastatic cells may show "osteomimicry" – responding to bone-derived chemotactic and mitogenic factors and entering a vicious cycle of bone degradation and remodeling [61]. The vicious cycle is potentiated by growth factors, such as PTHrP, secreted by or expressed on tumor cells, which activate osteoblasts and osteoclasts to produce cytokines such as RANKL; in contrast, osteoprotegerin (OPG) is downregulated. Remodeling and osteolysis releases growth factors such as TGFβ and IGF1, which then stimulate tumor cell growth and motility and further release of PTHrP (Figure 19.1).

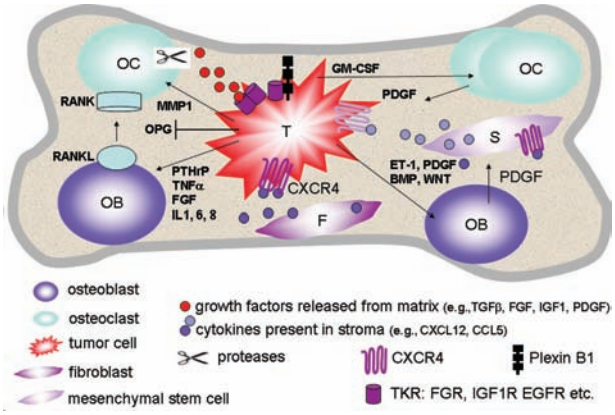

Figure 19.1. Key signaling pathways implicated in bone metastases. Factors released by tumor cells activate osteoclasts and/or osteoblasts. A vicious cycle between these three cell types and growth factors released from the stroma potentiates tumor cell invasion and bone destruction/remodeling. Cytokines and their receptors may also contribute.

Cancers expressing the chemokine receptor CXCR4 (such as breast, ovarian, prostate, rhabdomyosarcoma, and neuroblastoma) have been shown to metastasize to the bone/bone marrow in a CXCL12-dependent manner (reviewed in [62]). The bone marrow can be considered a distinct microenvironment and a primary "staging post" for breast cancer, although one issue is that tumor cells may remain dormant at this site for

many years. It has recently been suggested that the uPA/u-PAR signaling pathway may be responsible for maintaining survival of tumor cells during dormancy and for later reactivating proliferation. The molecular mechanism was proposed to involve uPAR interactions with fibronectin, EGFR, and integrins and a switch between ERK and p38 MAPK signaling pathways [63]. Others have suggested that FGFR–MAPK signaling is important for breast cancer metastasis to bone. Five genes from this pathway were identified in bone metastasis signatures, and the ligands FGF1 and 2 are stored in mineralized bone matrices, from which they can be released by proteases [64].

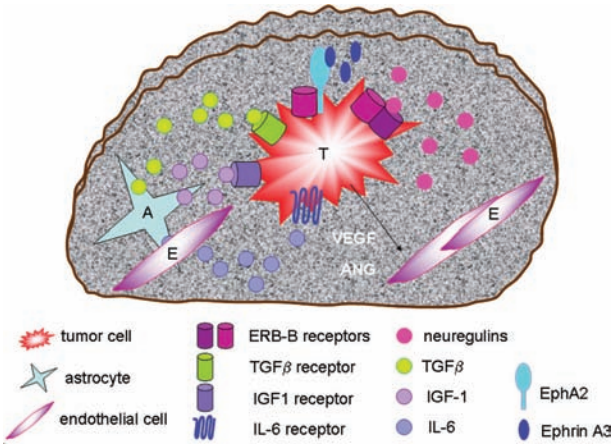

Figure 19.2. Key signaling pathways implicated in brain metastases. Growth factors are produced, notably by astrocytes, which can stimulate the proliferation and invasion of tumor cells expressing the cognate receptors. Angiogenic factors released by tumor cells are also implicated.

Brain

The CNS is increasingly recognized as a sanctuary site for women with breast cancer who have received herceptin treatment [65]. This may be compounded by both HER2-expressing cells having a higher probability of metastasizing to the brain and the failure of antibodies to cross the blood–brain barrier. It is intriguing to speculate that the major ligands for the ERB-B receptors (neuregulins), which are naturally present in the brain, may promote the survival and proliferation of ERB-B receptor-expressing cells. In a murine model, breast cancer brain metastatic cells expressed receptors for ligands commonly produced by astrocytes (IL-6, IGF, TGFβ), suggesting key local paracrine tumor–host interactions [66]. This is consistent with the recruitment of reactive glial cells observed in the MDA MB 231 xenograft model and the fact that co-cultures between these cell types enhanced breast cancer cell proliferation, putatively owing to secreted growth factors [67, 68]. Others have implicated Notch signaling (plus upregulation of angiogenic growth factors, such as angiopoietins and VEGF) in MDA MB 231 brain metastases [69] (Figure 19.2).

Lung

The lung represents a common site of metastasis of several common cancers, especially sarcomas, as it is the first capillary bed encountered by tumor cells released into the venous circulation. However, it is clear that not all tumor cells are capable of colonizing the lung and that there is a significant contribution from selective processes. Several genes in the Massagué "lung metastasis signature" (LMS), such as those encoding such ligands as EREG (epiregulin) and the TGFβ-regulated angiopoietin-like 4 (ANGPTL4) adaptor protein, have been implicated in the ability of cells to extravasate through the tight endothelial junctions in this organ. They are not required for access to sites with fenestrated capillaries, such as bone marrow and liver, and have been associated with human breast cancer metastasis to the lung but not other sites [70]. However, the LMS also provides a growth advantage for primary tumor cells [71].

In elegant studies from Weinberg's lab, a crucial role for paracrine CCL5-CCR5 interactions in lung metastasis was recently discovered. Bone-marrow–derived mesenchymal stem cells localized in experimental breast carcinomas were stimulated to secrete CCL5. This, in turn, bound to CCR5 receptors on the tumor cells and specifically promoted extravasation and invasion from the microvasculature into the lung parenchyma in a PI3 kinase-dependent fashion. The enhanced metastasis was independent of effects on tumor cell survival and proliferation and was reversible [60]. These findings have significant implications, as the data suggest that cellular functions associated with metastasis may be not constitutive but rather expressed transiently and in response to a specific microenvironmental context, and specifically via signals from the host. CXCL12 (CXCR4 ligand) is also expressed at high levels in lung tissue and may be associated with metastasis to the lung, at least in experimental models of breast cancer and melanoma. CXCL1 also appears in the LMS, as described by Minn et al. [71]. In these studies, however, it was not possible to discriminate contributions to initial colonization/extravasation from sustained survival and growth (Figure 19.3).

It has been suggested that tumor cells may also preferentially secrete factors that are required for survival in the early stages of colonization – for example, dynamic activation of the adhesion molecule ezrin by PKC has been implicated in osteosarcoma lung metastasis [72, 73]. Other studies have shown that release of

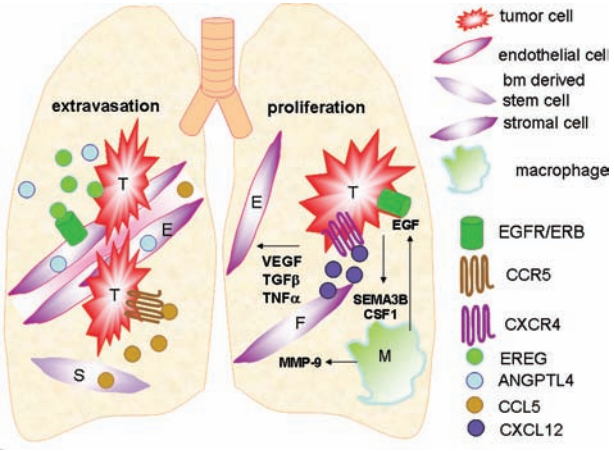

Figure 19.3. Key signaling pathways implicated in lung metastases. An important determinant of lung metastasis is the specific ability of tumor cells to effect transmigration of the lung endothelium. Factors implicated include those released by tumor cells, which act on endothelial cells (e.g., EREG and ANGPTL4), and paracrine stimulation of tumor cells mediated by bone marrow stem cell-derived CCL5. Within the lung, growth may be stimulated by CXCR4-CCL12 interactions.

VEGF, TGFβ, and TNFα by tumor cells leads to upregulation of S100A8 and A9 in lung myeloid and endothelial cells, which enhances tumor cell colonization of the lung [74].

Liver

Gene expression signatures have also been described for liver metastases, although these share several features in common with lung metastases and are not always distinguishable. Two signaling pathways commonly associated with liver metastasis are TGFα-EGFR and HGF-MET. In an orthotopic experimental model, Sasaki et al. showed that KM12 colon carcinoma cells expressing high levels of TGFα, when implanted into the cecum, invoked autocrine and paracrine signaling networks that potentiated lymph node and liver metastasis. Compared with nonmetastatic KM12 cells with low TGFα, the primary tumor sites were enriched for VEGF, IL-8, and MMP-2 and -9 and had a high density of vascular and lymphatic channels. They also contained high numbers of macrophages that secreted VEGF-C. Thus TGFα activation of EGFR can simultaneously potentiate both lymphatic and vascular dissemination via complementary mechanisms involving host inflammatory cells [75].

Most human colon cancers express PDGF-A and -B subunits but not their corresponding receptors. PDGF-Rβ was predominantly expressed by tumor-associated stromal cells and vascular pericytes and was associated with advanced-stage disease. The expression of PDGF-Rβ in the stroma was higher in highly metastatic orthotopic KM12 tumors than in low metastatic KM12 tumors (or in either tumor grown sc), suggesting that in both experimental models and human cancers, paracrine interactions between PDGF and its receptors may be associated with metastasis and are influenced by the organ-specific microenvironment [76] (Figure 19.4).

Lymph Nodes

Some studies have suggested that lymphatic metastasis is primarily stochastic/nonselective and caused simply by the passive transport of tumor cells in draining lymph and their ready extravasation through the leaky vessels characterized by discontinuous basement membranes. However, others have identified gene expression signatures that correlate with the probability of lymphatic spread [77]. There are clearly some significant mechanistic determinants, as epithelial malignancies have a high propensity for lymphatic dissemination, whereas sarcomas, even when developing in the same anatomical location, do not. One key factor is the lymphangiogenic cytokine VEGF-C. High levels in primary tumors correlate with nodal metastasis in several types of cancer, transfection of the gene into tumor cells increases lymphangiogenesis and/or lymphatic spread, and inhibition of its major receptor VEGFR-3 inhibits the process (see chapter by Alitalo).

In addition, it has been proposed that tumors may use the chemokines and receptors designed to enable lymphocyte homing to nodes and their extravasation through high endothelial venules, notably CCR7 and its ligands CCL19 or CCL21. VEGF-C and CCR7 may act synergistically to promote tumor cell invasion toward lymphatics. VEGF-C increases lymphatic secretion of CCL21, which induces CCR7-dependent tumor

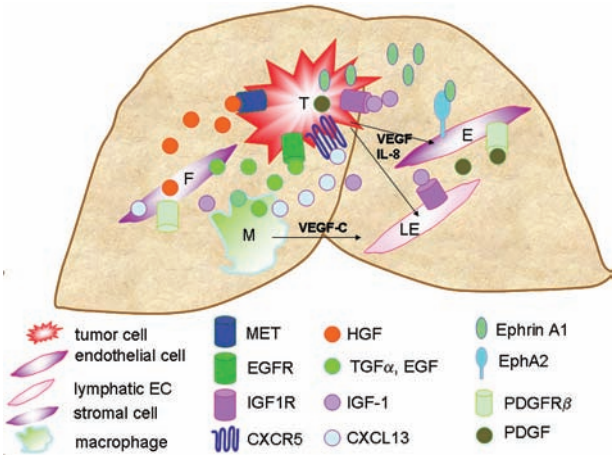

Figure 19.4. Key signaling pathways implicated in liver metastases. Receptors on tumor cells such as EGFR and MET may respond to high levels of their ligands in the liver. Paracrine interactions among tumor cells, host stromal cells, and endothelial cells involving ephrins and chemokines are also evident.

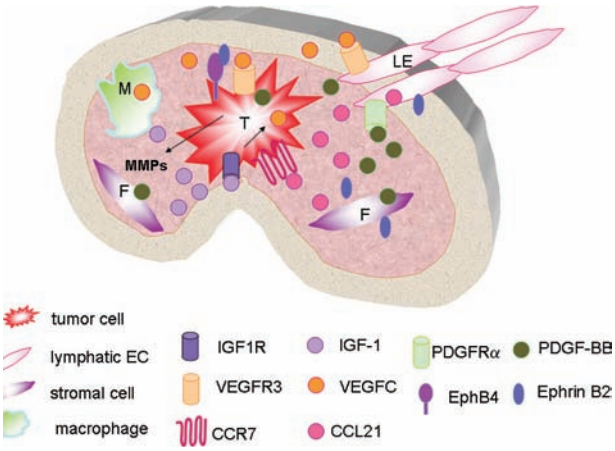

Figure 19.5. Key signaling pathways implicated in lymph node metastases. The major signaling pathways implicated in lymphatic metastasis are the VEGF-C–VEGFR3 and CCL21–CCR7 systems. PDGF-BB acting through RTK receptors may also play a role.

movement toward lymphatics. VEGF-C also acts in an autocrine loop via VEGFR-3 receptors on tumor cells to promote invasion by increasing proteolytic activity and motility in three-dimensional matrices [78, 79] (Figure 19.5).

Examples of growth factor–ligand interactions implicated in site-selective metastases are shown in Table 19.1.

Therapeutic Implications

Although factors controlling the initial dissemination and spread of cancer cells (including conditions at the primary site, distant preconditioning of niches by tumor-derived factors, circulation in blood or lymph, extravasation, and lodgment at secondary sites) are of great scientific interest, unless growth of *established* micrometastases is controlled, cancer cures will remain limited. Although cytotoxic therapy is designed to attack systemic disease, too often it fails, perhaps because of metastatic heterogeneity [80], innate or acquired resistance, or the failure stringently to evaluate such agents in appropriate metastatic tumor models. Too often, control of metastasis in preclinical studies is contingent on a reduction in primary tumor growth, or therapy is commenced before or at the same time as systemic injection of tumor cells.

Although studies of metastasis *prevention* are of interest mechanistically, it is essential that more attention be given to the mechanisms controlling ectopic tumor survival and sustained growth at secondary sites. For example, in spite of the key role of CXCR4 in promoting breast cancer xenograft metastasis, the potent inhibitor AMD3100 failed to prolong survival of mice bearing established lung metastases, showing that its role (perhaps as predicted) is primarily in early phases of metastasis initiation [81]. Also, if indeed stemlike

TABLE 19.1. Examples of growth factor/ligand–receptor interactions potentially enabling site-selective tropism to (or growth within) different organ sites.

Receptor on tumor cell	Ligand	Primary tumor type	Metastases
EGFR	TGFα/EGF	Colon, pancreas	Liver
c-MET	HGF	Colon, pancreas	Liver
HER2/3/4	NRGs	Breast	Brain
FGFR FGFR1	FGF FGF	Prostate Colon	Bone Liver
PDGFRα	PDGF	Colon Prostate	Lymph nodes Bone
IGF1R	IGFI and II	Neuroblastoma	Bone, liver
EphA2	Ephrins A1, A3, A4 A5	NSCLC	Brain
EphA2	Ephrins A1, A3, A4 A5	Colon, gastric	Liver
EphB4	Ephrin B2	Esophageal	Lymph nodes
CXCR2	CXCL1	Breast	Lung
CXCR5	CXCL13	Colon	Liver
CXCR4	CXCL12	Breast, prostate, neuroblastoma	Lung, bone, lymph nodes
CCR7	CCL19/21	SCCHN, melanoma, gastric	Lymph nodes
CCR10	CCL27	Melanoma	Skin
uPAR	uPA	Breast?	Bone marrow

cells are primarily responsible for treatment failures, we need to address their key signaling pathways too, to effect complete control [82]. Indeed, we need to target the bulk population using cytoreductive therapies *and* the putative stemlike cells that may be responsible for later relapse. The Massagué group has shown that combinations of genes are required for successful metastasis, and many of these differ between target organs. Of particular significance is the fact that genetic knockdown of more than one gene (or pharmacological inhibition of the encoded proteins) was required to inhibit metastasis, and some combinations were more effective than others [83]. Clearly there are challenges ahead to discover key pivotal (or complementary) points for intervention – especially to identify molecular targets that override site selectivity – but with our rapidly increasing knowledge of molecular mechanisms, that goal may one day be within reach [15, 82, 84, 85].

ABBREVIATIONS

ANG	angiopoietin
BMP	bone morphogenic protein
BDNF	bone-derived neurotrophic factor
BTC	betacellulin
CNS	central nervous system
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
EREG	epiregulin
FGF	fibroblast growth factor
HRG/NRG	heregulin (neuregulin)
IFN	interferon
IL	interleukin
IGF	insulin-like growth factor
MMP	matrix metalloproteinase
NSCLC	non–small-cell lung cancer
PDGF	platelet-derived growth factor
PTHrP	parathyroid hormone releasing peptide
RANKL	receptor activator of NFκB ligand
SCCHN	squamous cell carcinoma of the head and neck
TGF	transforming growth factor
TRKB	neurotrophin tyrosine kinase receptor
uPA	urokinase plasminogen activator

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20 Determinants of Organ-Specific Metastasis

Yibin Kang

For cancer patients, the risk of developing metastasis in vital organs brings a great deal of anxiety and uncertainty. After tumor cells are released into systemic blood circulation, metastasis can potentially develop in any organ that becomes the recipient of tumor emboli. However, extensive analysis of autopsy records in cancer patients revealed that the relative distribution of metastases in different organs is far from random. Certain organs, such as bone, lung, and liver, are frequently victimized by metastatic cancers, whereas other organs and tissues, such as the spleen and muscles, are rarely affected [1].

Metastasis organotropism is a term to reflect the well-documented fact that each type of cancer manifests a distinct pattern of metastatic involvement in secondary organs (Figure 20.1) [1, 2]. For example, almost 85 percent of advanced-stage prostate cancer patients suffer from bone metastasis. In contrast, liver metastasis is predominant among late-stage colorectal cancer patients, who rarely develop bone metastasis (Figure 20.1). Breast cancer often metastasizes to the bone, liver, and lungs; metastases to each of these organs were found in at least 25 percent of advanced-stage patients at the time of diagnosis (Figure 20.1) and in more than 60 percent at the time of autopsy [3]. Metastasis from the primary breast tumor to other distant organs, such as the kidney, spleen, or uterus, is relatively rare.

HEMODYNAMIC AND "SEED-AND-SOIL" HYPOTHESES OF ORGAN-SPECIFIC METASTASIS

Two major theories have been proposed to explain the organotropism of cancer metastasis. As hematogenous dissemination is the major route of metastatic spread, the anatomy of vascular connection between the primary tumor and the secondary organs may have a significant influence on the relative metastasis risk. The importance of vascular anatomy and blood flow mechanics in metastasis organotropism – the hemodynamic hypothesis – was proposed by James Ewing (1866–1943), a prominent American pathologist and founder of the Memorial Sloan-Kettering Cancer Center in New York. In his influential textbook, *Neoplastic Diseases*, Ewing observes that "the particular susceptibility of a tissue to develop secondary tumors, is an interesting phase of study of metastases. . . . The mechanisms of the circulation will doubtless explain most of these peculiarities, for there is as yet no evidence that any one parenchymatous organ is more adapted than others to the growth of embolic tumor cells. The spleen seems to escape with peculiar frequency." Indeed, the blood flow pattern can at least partly explain the high frequency of liver metastasis in patients with cancer in the gut, such as colorectal cancer, and the prevalence of spinal bone metastasis in prostate cancer patients (Figure 20.2).

Whereas hematogenous metastases are seeded mostly through arterial circulation, venous circulation allows the trapping of tumor emboli from most solid tumors, such as breast cancer, in the lung microvasculature before the blood returns to systemic arterial circulation. This largely explains the high prevalence of metastasis to the lungs. In contrast, tumor cells disseminated from colorectal cancer are first delivered to the liver through the hepatic–portal venous system before they reach the lungs. Thus, disseminated tumor emboli from colon cancer are more likely to be entrapped in the liver microvasculature before they have the chance to seed metastases in other visceral organs. Similarly, prostatic carcinoma cells are often distributed to the spine via the paravertebral venous plexus of Baston, likely accounting for the high incidence of skeletal metastasis in advanced-stage prostate cancer patients (Figure 20.2).

Although the blood flow pattern certainly contributes to metastasis, a thorough analysis of metastasis pattern of sixteen primary tumor types and eight

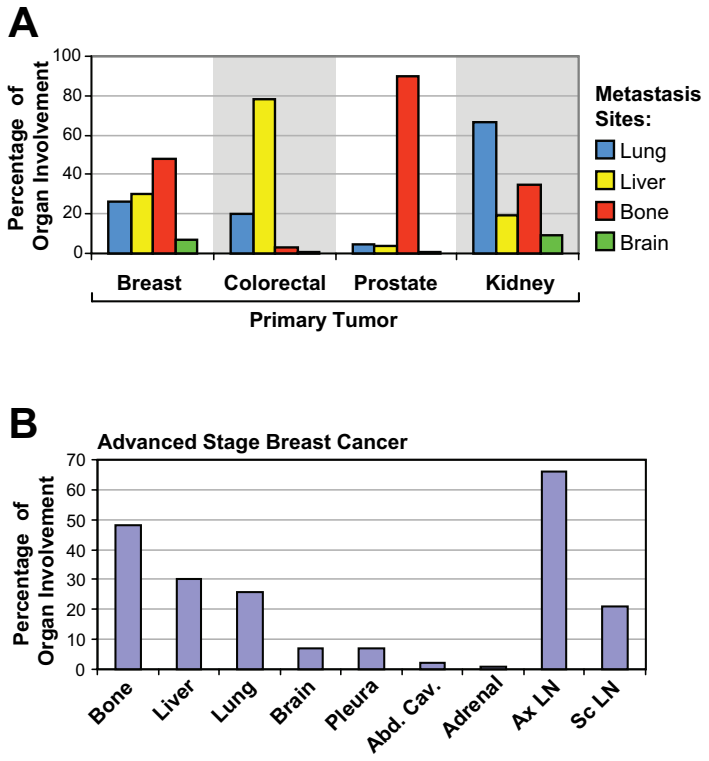

Figure 20.1. Organ distribution patterns of metastases from breast, colorectal, prostate, and kidney cancers. (A) Percentage of organ involvement in four common metastasis sites (lung, liver, bone, and brain) among patients with distant-stage cancers at the time of initial diagnosis [1]. (B) Pattern of organ distribution in advanced-stage breast cancer patients at the time of diagnosis [1]. Percentage of organ involvement is often significantly higher at autopsy. Data presented in this figure are from a study by Hess et al [1]. Figure adapted from [2].

metastasis target organs by Leonard Weiss revealed that only 66 percent of the primary-secondary organ pairs of metastasis can be adequately explained by the hemodynamic hypothesis alone [4]. For the remaining cases, it appeared that certain organs are more favorable for the growth of metastatic lesions than predicted by the amount of vascular input alone, whereas others are more "hostile" for the growth of metastasis. The concept of positive or negative interaction between the tumor cells and the secondary organ was best encapsulated in the timeless "seed-and-soil" hypothesis proposed by British surgeon Stephen Paget in 1889 [5]. He speculated that malignant tumor cells are shed from the primary tumor and disseminated throughout the body, but metastases form only when the seed (disseminated tumor cells) and soil (secondary organ) are compatible [5, 6].

The determining factors for the seed-and-soil interaction may include selective retention, or homing, of tumor cells to certain organs; the unique properties of certain tumor cells to survive and thrive in a foreign microenvironment; and the particular combination of stimulating factors provided by the secondary organs to foster the formation of metastatic lesions. Identifying the molecular properties of these specific "seed" or "soil" factors, however, is a daunting task because of the difficulty of modeling organ-specific metastasis and the genetic complexity of metastatic tumors.

A particularly productive experimental approach for the study of metastasis organotropism is based on the observation by Josh Fidler and his colleagues in the early 1970s that metastatic cancer cells derived from a particular metastasis site often display enhanced metastasis ability to that specific organ [7]. Such organ-specific metastatic cells were found to be preexisting in the parental cell line and were simply enriched in vivo in their target organs through Darwinian selection in experimental metastasis assays [7]. Application of DNA microarray profiling technology in animal models of metastasis (Figure 20.3) by Joan Massagué, Richard Hynes, Robert Weinberg, and others allowed the identification of distinct sets of genes that are differentially expressed in organ-specific metastatic variants selected in vivo [8–13]. Many genes identified in these organ-specific metastasis gene signatures were later proven to be functionally important and clinically relevant in metastasis. These types of functional genomic studies have greatly accelerated the study of organotropism in recent years and have provided unprecedented insights into the mechanism of this mysterious phenomenon. Here, we will use bone and lung metastasis to illustrate our current understanding of the intricate tumor–stroma interactions that promote organ-specific metastasis.

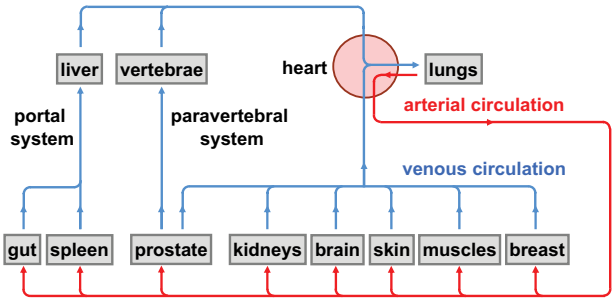

Figure 20.2. Vascular flow patterns influence organ distribution patterns of metastases from different cancers. The venous blood of most tissues drains to the right side of the heart and thereafter into the lungs, whereas the veins draining the spleen and gut empty directly into the liver via the hepatic–portal vein. Consequently, lung metastasis is a frequent occurrence in most cancer types, whereas colorectal cancer often leads to liver metastasis. Prostate cancer cells travel to the spine via the paravertebral blood vessels and generate skeletal metastases in prostate cancer patients. (Adapted from [51])

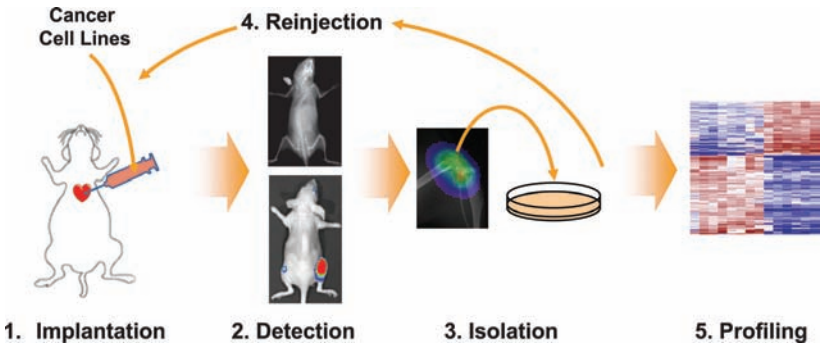

Figure 20.3. Schematic representation of the in vivo selection approach for the identification of tissue-specific metastasis genes. Experiments are usually carried in five steps: (1) Cancer cell lines are injected into either syngeneic or immunocompromised animals through one of two injection routes: orthotopic injection into an anatomically relevant site, or direct injection into blood circulation. (2) The development of metastasis is monitored by appropriate noninvasive imaging technologies, such as X-ray imaging (top) or bioluminescent imaging (bottom). (3) Sublines of cancer cells are established from tumor cells isolated from metastases. (4) The enhanced metastasis potential of sublines is tested through a second round of injection into a new cohort of animals. Successive rounds of selection may be carried out to enrich the most metastatic subpopulation. (5) After a sufficient number of variant cell lines are established and tested, microarray profiling and statistical analysis are carried out to identify candidate metastasis genes, which could be further tested in animal models or correlated with immunohistological analysis of human tumor samples. (Adapted from [52])

BONE METASTASIS: THE VICIOUS CYCLE AND THE RANK–RANKL–OPG AXIS

Bone metastasis has become one of the best-studied metastasis tropisms for two main reasons. First, bone metastasis is very common in many late-stage solid tumors, including breast, prostate, and lung cancers. In patients with metastatic diseases, a significant proportion of tumor burden is often in the bone [14], and patients with bone metastasis suffer from debilitating complications such as bone fracture, severe bone pain, nerve compression, and hypercalcemia. Current available treatments for bone metastasis are mainly palliative; more effective therapies are urgently needed. Second, normal bone homeostasis is maintained by two distinctive cell types, with opposing and yet tightly coupled functions: the bone-building osteoblasts and the bone-degrading osteoclasts. Lessons learned from the study of bone homeostasis have greatly facilitated bone metastasis research, as pathological manifestations of skeletal metastasis are often caused by the disruption of the delicate balance of osteoclast and osteoblast activities, leading to either osteoblastic or osteolytic bone lesions.

Osteoblasts differentiate from mesenchymal progenitor cells that also give rise to myocytes, adipocytes, and chondrocytes [15]. Their differentiation is largely controlled by Runx2, a master transcription factor that is active specifically in the osteoblast lineage. In contrast, osteoclasts are derived from the monocyte/macrophage lineage of hematopoietic cells; the signaling molecules essential for their differentiation include RANKL and M-CSF. Binding of RANKL to its receptor RANK on osteoclast precursor cells results in the recruitment of TRAF family proteins, such as TRAF6, which signals through the NFκB and JNK pathways to activate the gene program for osteoclast differentiation and maturation. In addition to secreting RANKL to activate osteoclast differentiation, osteoblasts also express osteoprotegerin (OPG), a decoy receptor that binds and sequesters RANKL, preventing its activation of RANK. RANKL and OPG can also be secreted by many stromal cells in the bone microenvironment, including fibroblasts, activated T cells or dendritic cells, and endothelial cells [16, 17]. The relative abundance of RANKL and OPG in the bone microenvironment determines the relative activity of osteoclasts. Under normal physiological conditions, the coupling of osteoblast and osteoclast functions allows active remodeling of bone (resorption followed by new bone buildup) to maintain the strength of the skeletal system. Loss of this delicate balance leads to pathological conditions such as osteoporosis (bone loss) and osteopetrosis (excessive thickening of bone).

In bone metastasis, the balance of osteoclast and osteoblast activities is altered, and, as a result, the integrity of bone is compromised to favor the growth of disseminated tumor cells in the bone marrow [14]. Most bone lesions in prostate cancer are osteoblastic, with new bone fragments frequently found adjacent to tumor cells. Breast cancer can generate both osteolytic and osteoblastic bone metastases, although most bone lesions in breast cancer patients are osteolytic because of the tendency of breast cancer cells to tip the balance toward enhanced osteoclast activation [14, 18]. It is believed that breast cancer cells are not capable of carrying out the highly specialized function

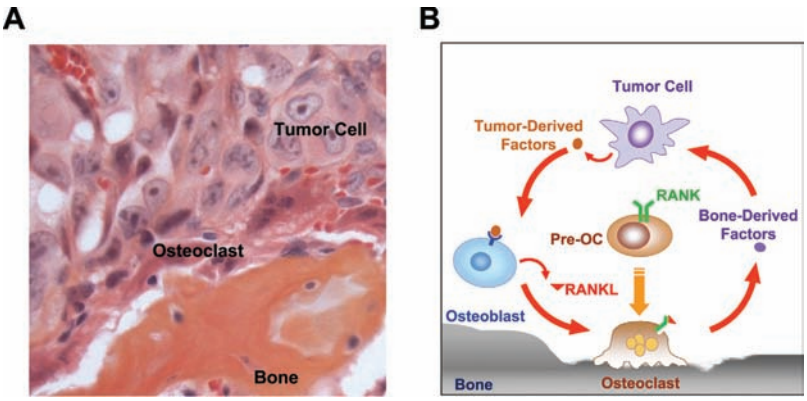

Figure 20.4 Tumor–stroma interactions in osteolytic bone metastasis. (A) A typical osteolytic bone metastasis generated by breast cancer cell line MDA-MB-231 in the hind limb of an athymic mouse. Histological image demonstrates tumor cells (upper left) destroying the mineralized bone matrix (lower right) by activating and recruiting multinucleated osteoclasts, which are located between tumor cells and the bone surface. (B) The vicious cycle in osteolytic bone metastasis. Bone homeostasis is maintained by the balance of bone-building osteoblasts and bone-degrading osteoclasts. Differentiation and activation of osteoclasts depends on the binding of chemokine RANKL to its receptor RANK at the surface of osteoclast presursors. RANKL is produced in osteoblasts as well as other bone stromal cells, such as fibroblasts, activated T cells and dendritic cells, and endothelial cells. Cancer cells produce a series of factors to influence bone and stromal cells and modify the bone matrix, usually tilting the balance of bone homeostasis toward osteolytic bone destruction. These tumor-derived factors include angiogenic factors such as FGF and VEGF, immune cell regulators such as TNFα, TGFβ, and GM-CSF, and fibroblast activators such as FGF and TGFβ. Several cancer-cell-derived proteases can participate in bone degradation either directly (e.g., MMP-1/collagenase 1) or indirectly by enhancing the solubilization of RANKL from the cell surface. Other tumor-derived cytokines and cell surface/ECM proteins promote osteoclast or osteoblast differentiation, activation, and recruitment (e.g., BMP, IL-11, OPN, and endothelin-1) or homing of cancer cells to bone (e.g., CXCR4). Growth factors released by osteolysis from bone matrix promote the growth and malignant phenotypes of cancer cells. (Adapted from [2])

of bone resorption. Instead, cancer cells secrete factors to promote the production of RANKL and reduce the expression of OPG in osteoblasts and bone stromal cells, including tumor-associated fibroblasts, immune cells (e.g., activated T cells and macrophages), platelets, and endothelial cells (Figure 20.4[B]). One such tumor-derived factor is parathyroid hormone-related protein (PTHrP), which stimulates osteoblasts to secrete RANKL. In addition to PTHrP, tumor cells can also secrete, or induce stromal cells to produce, other factors to increase osteoclast formation [14, 17, 19], including IL-1 [20], IL-8 [21], GM-CSF [22], and prostaglandin E2 [23] (Figure 20.3).

Genomic profiling of the bone metastasis-derived subpopulations of the MDA-MB-231 breast cancer cell line revealed a "bone metastasis gene signature" that includes *osteopontin* (*OPN*), *CTGF*, *FGF5*, *IL-11*, *CXCR4*, *MMP1*, *ADAMTS1*, and many other genes [9, 24]. These genes constitute an organ-specific "toolbox" that promotes the homing of tumor cells to bone (mediated by chemokine receptor CXCR4), degradation of collagen in bone matrix (by MMP-1), osteoclast activation (via the induction of prostaglandin E2 in osteoblasts by IL-11, suppression of OPG expression in osteoblasts through an EGFR-dependent paracrine signaling cascade initiated by MMP1 and ADAMTS1) [25], and angiogenesis (by CTGF and FGF5). Experimental validation of these candidate bone metastasis genes showed that simultaneous overexpression of multiple metastasis genes is often required to significantly enhance the bone metastasis capacity in weakly metastatic cells [9]. This observation underscores the multigenic nature of organ-specific metastasis.

Bone matrix represents a fertile soil for fueling the pathological interactions among tumor cells, bone cells, and other stromal components. Bone resorption by osteoclasts releases a number of growth factors embedded in the bone matrix, including IGFs, TGFβ, PDGF, and BMP (Figure 20.4(B)). These growth factors can stimulate the growth of cancer cells as well as enhance the production of bone metastasis factors, such as PTHrP, CTGF, IL-11, and VEGF, thus forming the so-called vicious cycle in bone metastasis [14, 26, 27].

Interestingly, mounting evidence suggests that breast cancer cells may be endowed with intrinsic properties to engage in profitable interactions with the bone tissue. The ability of breast carcinoma cells to activate osteoclasts is very similar to what their counterparts in the normal mammary tissue, mammary epithelial cells (MECs), do during lactation. To deposit enough calcium into milk, MECs can overexpress PTHrP, which causes bone resorption and calcium release by stimulating the activity of osteoclasts [28]. When the PTHrP gene is specifically deleted during lactation in mice, bone loss is significantly reduced [29]. Furthermore, many of the factors critical for bone cell development also play important roles in mammary gland function and in breast cancer malignancy. For example, the osteoclast differentiation factor RANKL was found to be essential for mammary gland development by signaling through the NFκB pathway to activate cyclin D1 expression in mammary epithelial cells [30, 31] and has also been shown to play a key role in progestin-induced breast cancer [32]. Mice lacking RANKL or its receptor, RANK, fail to form lobuloaveolar mammary structures during pregnancy, resulting in the death of newborns. RANK was also found to be expressed in breast cancer cells and to promote

invasion, migration, and tissue-specific metastasis to bone in response to stimulation by RANKL, which is abundant in bone [33].

The master regulator of osteoblast differentiation, Runx2, is expressed in nascent mammary epithelial cells and contributes to the expression of β-casein and OPN during lactation [34]. Strikingly, Runx2 is found to be overexpressed in bone-metastatic breast cancer cells and activates the expression of several genes that are associated with bone metastasis, such as *MMP9*, *OPN*, *BSP,* and *RANKL* [35, 36]. Expression of a dominant negative Runx2 in breast cancer cells significantly suppressed bone metastasis [37]. Furthermore, gene expression profiling of bone-metastatic breast cancer cells revealed significant levels of bone-related gene expression [38], consistent with the osteomimicry phenotype that has been previously described in bone-tropic breast and prostate cancer cells.

Overall, these studies highlight the capacity of malignant breast cancer to make use of the preexisting functionality of mammary tissue or take on a phenotypic transition to coopt or mimic the resident cells in the bone microenvironment. Thus, breast cancer bone metastasis serves as a compelling example for Stephen Paget's "seed-and-soil" theory. Our insights about the underlying molecular mechanisms of bone metastasis have also lead to better targeted therapeutics. For example, bisphophonates are extensively used to alleviate bone loss and bone pain in patients with osteolytic breast cancer, as bisphophonates can block the aforementioned vicious cycle by coating the bone matrix surface and inducing osteoclast apoptosis [14]. Other bone metastasis inhibitory agents are currently in clinical trials, including RANKL and PTHrP blocking antibodies, endothelin-1 inhibitors, TGFβ inhibitors, and vitamin-D analogs [14].

LUNG METASTASIS: VASCULAR ENDOTHELIUM TARGETING AND LUNG METASTASIS VIRULENCE GENES

Much effort in the study of lung-tropic metastasis has focused on the identification of cell–cell interaction molecules that mediate the adhesion of cancer cells to the lung vascular endothelium. As breast tumor cells arrive at the pulmonary capillaries, they can be physically trapped by the narrowing blood vessels. Recent evidence suggests that the adhesion and extravasation of cancer cells in the lung are also mediated by specific surface adhesion molecules on tumor cells and receptors on the endothelial cells. Chemokine receptors CXCR4 and CCR7 are highly expressed in human breast cancer cells and mediate the homing of these cells to metastasis sites in the lung, bone, and regional lymph nodes that have abundant expression of their respective ligands CXCL12/SDF-1 and CCL21 [39]. Brown et al. used a phage expression library of cDNAs from a metastatic mouse mammary tumor to identify protein domains that bind to the vasculature of the lung [40]. A transmembrane domain of the protein metadherin was found to mediate the targeting of tumor cells specifically to the lung, but not to other organs, through binding to an unknown receptor expressed in lung endothelium [40]. In a separate study, adhesion of breast cancer cells to the lung endothelium was shown to be mediated by cell surface expression of $\alpha 6\beta 4$ integrin and adhesion to the human CLCA2 protein, which is a calcium-sensitive chloride channel protein that is expressed on the endothelial cell luminal surface of pulmonary arteries, arterioles, and venules [41]. Additionally, fibronectin assembled on the cell surface of breast cancer cells was shown to interact with dipeptidyl peptidase IV (DPP IV) on lung endothelial cells to mediate adhesion in a rat breast cancer model [42]. If one or several of these adhesion proteins prove to be significantly involved in clinical lung metastasis, neutralizing peptides against the ligands and/or the receptors can be developed to block lung metastasis.

Genomic profiling of lung-tropic variant cells from the MDA-MB-231 breast cancer cell line uncovered a lung metastasis gene signature that includes the EGF family member epiregulin, cell adhesion molecules SPARC and VCAM1, MMP-1, IL13 decoy receptor IL13Rα2, and others [10]. Similar to what was observed in a functional genomics study of bone metastasis [9], overexpression of individual genes in this signature led to only a marginal increase of lung metastasis, whereas coexpression of multiple genes dramatically increased the virulent growth of lung metastases [10]. Among the lung metastasis genes, the epidermal growth factor receptor ligand epiregulin, the cyclooxygenase COX2, and the matrix metalloproteinases 1 and 2 were found to collectively facilitate neoangiogenesis in primary tumors as well as intravasation and extravasation of tumor cells to seed pulmonary metastasis [43]. Importantly, when the lung metastasis gene signature was used to analyze the gene expression profile of primary breast tumors, it successfully distinguished patients with a high risk of lung metastasis (but not bone metastasis) from those with a low risk [10].

There is only a limited overlap (e.g., *CXCR4* and *MMP1*) between genes in the bone and lung metastasis signatures, suggesting the distinct functional requirements for different types of organ-specific metastasis. Nevertheless, several signaling pathways have been found to be important mediators of both bone and lung metastases. For example, NFκB was found to be critical for lung metastasis; accordingly, inhibitors of the NFκB pathway reduce lung metastasis [44]. NFκB also promotes osteolytic bone metastasis of breast cancer by stimulating osteoclastogenesis through the secretion

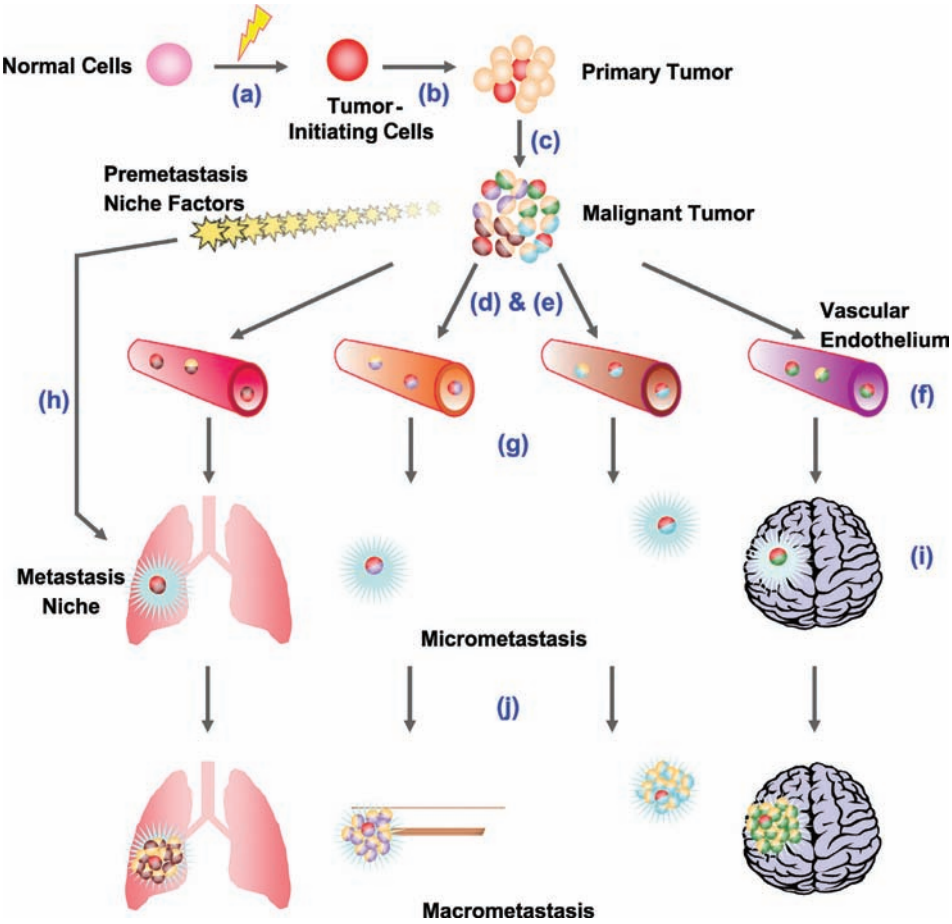

Figure 20.5 An integrative model for organotropic metastasis. Oncogenic events transform normal cells into tumor-initiating cells (shown in red) (A), which give rise to a heterogeneous primary tumor (B). After acquiring a combination of general and organ-specific metastasis capabilities (represented by different colors) (C), the primary tumor becomes malignant and starts distributing tumor cells through the blood circulation to different organs in the body (D). The number of tumor cells that reach and stay in a particular target organ depends on both the blood flow pattern and molecular interactions between tumor cells and the target organ. Chemokines such as SDF1 may mediate organ-specific homing of tumor cells that overexpress their cognate receptors (E). Capillary beds in different organs (shown as blood vessels with different colors) arrest different subpopulations of tumor cells through specific adhesive interactions between tumor cells and the endothelium (F). Attached tumor cells invade through the endothelial layer and basement membrane (G) and reach the tissue parenchyma. A premetastasis niche formed by the stimulatory factors released by the primary tumor (H) facilitates the initial establishment of micrometastases (I). Only metastatic CSCs are shown in the niches (I), as they are presumed to be the only cells capable of seeding secondary tumors. Active tumor–stroma interactions (J) support the outgrowth of micrometastases into life-threatening macrometastases (K). (Adapted from [2])

of the osteoclast differentiation factor GM-CSF [22]. TGFβ is an important bone-matrix–derived "soil factor" in the vicious cycle of osteolytic bone metastasis; it stimulates the expression of bone metastasis genes such as *PTHrP*, *IL-11*, and *CTGF* [9, 24] but has also been shown to promote lung metastasis [45, 46, 47]. The TGFβ pathway has opposing roles during the progression of breast cancer [48]. It inhibits the proliferation of normal mammary epithelial cells and represses early stage cancer but promotes malignancy of late-stage tumors. Therefore, prevention or treatment of metastasis by targeting the TGFβ pathway may be suitable for only a subset of cancer patients during a specific late-stage treatment window [49, 50].

CURRENT MODEL OF METASTASIS ORGANOTROPISM AND FUTURE DIRECTIONS

In addition to the effect of blood flow dynamics and "seed-and-soil" interactions in the metastasis site, metastasis organotropism may also be influenced by many factors that are not covered here but are discussed in other chapters – for example, the cellular and molecular components of premetastasis niches and

organ-specific survival factors that allow tumor cells to avoid apoptosis in a foreign microenvironment distinct from the primary tumor. Although metastasis organotropism still remains an underexplored area of cancer research, an increasingly sophisticated understanding of this phenomenon is emerging (Figure 20.5). Oncogenic transformation gives rise to tumor-initiating cells, which form a heterogeneous primary tumor. Primary tumor cells may further develop genetic programs for organotropic metastasis or remain naïve in tissue specificity and evolve only the ability to thrive in secondary organs under the intense selection pressure in a foreign microenvironment. Disseminated tumor cells are transported through systemic blood circulation to various distant target organs. Blood flow patterns undoubtedly contribute the relative risk of different organs to receive tumor emboli, and subsequently the risk to develop metastasis from a particular type of primary tumors. Tumor cells may use elevated expression of chemokine receptors to sniff out a favorable target organ that produces a particular set of chemokines. Target organ specificity is further defined by the binding of tumor cells to endothelial cells via specific sets of adhesion molecules. Afterward, tumor cells extravasate, migrate, and eventually arrive at the premetastasis niches that the primary tumor has prepared in advance by long-distance mobilization of premetastasis niche cells. To colonize target organs, tumor cells must rely on their ability to adapt to the host organ microenvironment and interact profitably with a variety of cell types within that organ.

FUTURE DIRECTIONS

Metastasis organotropism is one of the oldest problems in cancer research and has received intense interest in the modern era because of its important implication in the prevention and treatment of metastatic diseases. Although rapid progress in recent years has generated enormous excitement and interest in the study of cancer metastasis organotropism, several questions remain to be addressed by future studies.

- What are the particular sets of functions that are required for each organ-specific metastasis? Do they differ among different types of cancer? What are the combinations of metastasis genes that fulfill these functions for metastatic cells?
- Can we identify master regulators of metastasis organotropism? Candidate master regulators such as Runx2 have been proposed based on our understanding of their function in normal mammary gland development and bone physiology. However, a sophisticated computational or systems biology approach may pave the way for more rapid and comprehensive identification of other such master regulators.
- Do prometastasis signaling pathways, such as those of TGFβ and NFκB, use different downstream mediators to promote organ-specific metastasis? What are the molecular effectors of prometastasis pathways?
- Can we improve our experimental animal models for organ-specific metastasis through the integration of advanced in vivo imaging technology and conditional gene expression techniques? Such experimental platforms will provide much needed information about the temporal–spatial requirement of metastasis genes and allow us to assess the tumor–host interactions more accurately. It will also facilitate the development and improvement of antimetastasis agents and increase their chance of succeeding in clinical trials.
- Is there a link between the tumor-initiating cancer stem cell and metastasis organotropism? Do different cellular origins of cancer stem cells determine their metastasis propensity, including metastasis organotropisms? Do different host tissue microenvironments select different variants of cancer stem cells? Do cancer cells use normal tissue stem cell niches to colonize a distant organ?
- How can we translate our growing knowledge of organ-specific metastasis into better preventive and curative measures for metastatic breast cancer? How do we improve clinical trial design for testing the efficacy of novel antimetastasis therapeutics?

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21 Function and Expression of the uPA/uPAR System in Cancer Metastasis

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Metastatic disease is responsible for most cancer lethality; therefore, understanding the intricate interplay among tumor cells, soluble factors, extracellular matrix (ECM), and host cells during cancer progression to metastasis is crucial to designing successful therapies [1]. Metastasis is formed when a cell, or a group of cells, leaves the original site of the primary tumor and establishes a new colony of tumor cells in a distant, anatomically separate, site in the body [1]. To form an overt metastasis, the cells must overcome the regulatory and physical constraints imposed by the tissue milieu and initiate proliferation and invasive growth. Proteases and their inhibitors and receptors, such as those that comprise the urokinase-type plasminogen activator (uPA) system, play a crucial role in determining the ability of tumor cells to metastasize (Figure 21.1). Interestingly, dissemination of tumor cells and metastatic growth promoted by uPA and its receptor (uPAR) may be caused not only by proteolysis but also by novel functions related to cell signaling necessary for tumor cells to migrate, survive, and proliferate in target organs [1, 2]. The role of uPAR in regulating cell motility, which appears to operate similarly in normal and tumor cells, has been covered recently in extensive reviews [3]. This chapter focuses on recent advances that focus on how the uPA system coordinates proteolysis and signal transduction for invasion, dissemination, survival, and mitogenesis during tumor progression. We also review the strategies designed to target both the proteolytic and signaling properties of this complex.

PLASMINOGEN ACTIVATOR SYSTEM

Discovery and Early Pioneering Studies in Cancer

The first studies leading to the discovery of the plasminogen activator system focused on the normal fibrin matrix degradation (fibrinolysis) and in thrombosis in the late 1940s [4]. The ability to produce firbrin matrices and measure their degradation launched the studies on fibrinolysis, a process in which an inactive proenzyme-like plasminogen is activated to plasmin by zymogens such as the tissue-type (tPA) and/or urokinase-type (uPA) plasminogen activators. In those early studies, these fibrinolytic factors were defined as fibrinokinases. In the early 1970s, with the development of more sophisticated techniques to obtain highly purified proteins, the molecular interactions between the components involved in fibrinolysis process were elucidated [5, 6]. During that time, studies in the Reich lab and other labs delineated to a great extent the enzymology and biological function of plasminogen activators in normal tissue remodeling processes [7–9], as well as in pathological ones such as cancer invasion and metastasis. In consequence, uPA, which was isolated in the supernatant fluid of transformed chicken embryo fibroblasts, was the first protease ever identified to be involved in tumor-associated fibrinolysis [10]. These pioneering studies were then confirmed as a common feature in malignant cells and have since then been relentlessly studied as targets of cancer therapy. In opposition to uPA, tPA was, but for some exceptions, not strongly linked to malignancy; in fact, it may be more associated with a differentiated state in several tissues [11, 12]. The impact of the discovery of the PAs went beyond cancer. A good example is that both tPA and uPA have been used since their discovery as profibrinolytic agents in the clinic to treat thrombotic diseases [13].

The degradation of ECMs is a key process during normal development, wound healing, and tumor dissemination and metastasis. ECMs differ in their molecular composition in different tissues. Thus, it is expected that a similar diversity exists in the molecules responsible for their processing. Both normal and tumor cells produce a wide variety of proteolytic enzymes. In some cases, tumor cells can also use those produced by stromal cells [14]. Regardless of the source

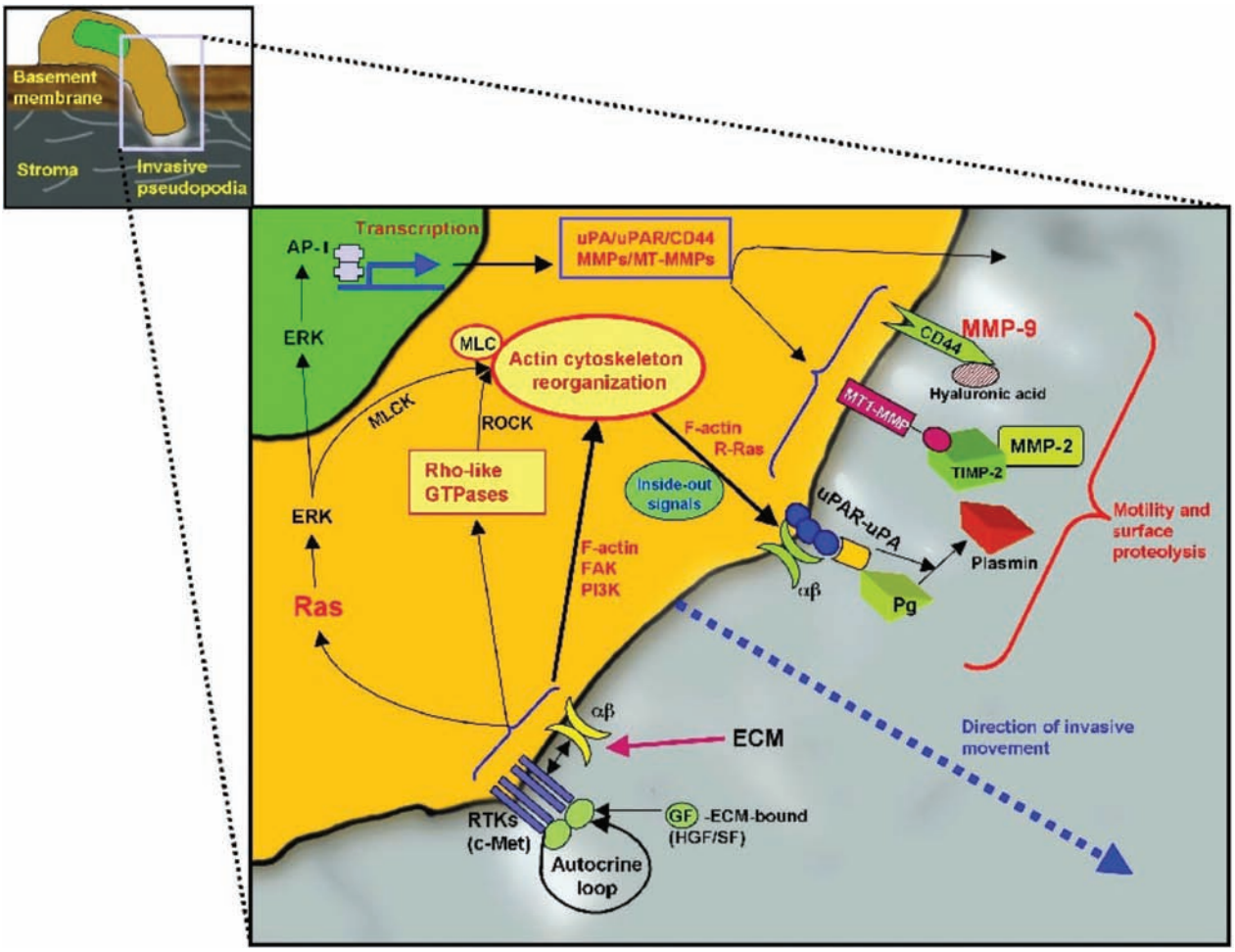

Figure 21.1. Coordination of motility and proteolysis in invasion. The scheme depicts a protrusion of an invading cell and molecules, and the flow of signals outside the cell and in the cytoplasm and the nucleus. Growth factor (GF) receptors (RTKs) activated through autocrine loops, or through activating mutations in their receptors, or by GFs such as hepatocyte growth factor/scatter factor (HGF/SF) released form matrix, cooperate with integrins ($\alpha\beta$) to activate the Ras-ERK pathway, leading to sustained expression of proteases, their receptors, or "docking" molecules, which then interact with their matrix ligands. Integrins and GF receptors activate pathways dependent on the Rho family of GTPases, causing, through activation of Rho kinase (ROCK) and phosphorylation of myosin light chain (MLC) actin cytoskeleton reorganization, contractility, and motility. The actin cytoskeleton, through inside-out signaling, can activate integrins and engage uPAR, CD44, and proteases in migration and invasion. Controlled surface proteolysis by uPAR-uPA-plasmin and MMPs clears a path for movement. Matrix molecules, or their proteolytic fragments, may serve as chemokines and as inducers of protease production. MT-MMPs can degrade matrix but can also regulate MMP-2 activity by binding it to the cell surface through a TIMP-2 bridge. Thus, the cells can respond in a coordinate fashion to migratory cues by localizing surface proteolysis, adhesion molecules, and GF receptors at the invading front.

of the protease, it ultimately allows tumor cells to remodel the ECM, affording migration and invasion into other tissues (Figure 21.1 and Table 21.1).

A comprehensive analysis of human and mouse tissue samples showed a significant increase of uPA in malignant tumors as compared with normal tissues or benign lesions [14, 15]. In fact, uPA plays a central role as one of the master regulators of tumor-cell–associated proteolysis during dissemination [16, 17]. Production of uPA by tumor cells activates a cascade of events that start with the activation of plasminogen to plasmin, leading to the activation of other proteinases and degradation of several components of the ECM (Table 21.1 and Figures 21.1 and 21.2). These events are highly regulated in normal processes, such as ovulation, trophoblast invasive growth, mammary gland remodeling after lactation, inflammation, angiogenesis, and nervous system development, to mention just a few [18–23]. In tumors, uPA production and the enzymatic cascade activated are greatly enhanced (see the section "uPA Receptor"), leading to the degradation of the ECM and creating an invasion front that will allow tumor cell migration and invasion of other tissues and the vasculature [24] (Figures 21.1 and 21.2). This particular capacity to degrade the ECM and promote invasion has been actively studied by academic and industry

TABLE 21.1. Main proteases involved in tumor invasion and metastasis

Type	Enzyme	pH range	Subtrates	Inhibitors
Serine	Plasminogen activators: tPA and uPA Plasmin	7–9	Activate plasminogen to plasmin Degrade some matrix proteins, such as fibronectin Degrades laminin, fibrin, fibronectin, and collagens Activates metalloproteinases	PAI-1 and 2 A2-antiplasmin A2-macroglobulin Fluorophosphates Amiloride
Cystein or thiol	Cathepsin B	3–8	Wide range; activates uPA and MMPs	N-ethylmaleimide
Aspartic or acid	Cathepsin D	2–7	Degrade endocyted proteins	Diacetones
MMPs	MMP-1 MMP-2 MMP-9 Stromalysins Matrisilins MT-MMPs	7–9	Interstitial collagens, basement membranes, proteoglycans, glycoproteins, and denaturated collagen (gelatin) Participate in the activation of MMP-2 on the cell surface	TIMP-1, -2, -3 and -4

laboratories to design specific targeting therapies. In consequence, the enzymology of uPA is particularly well understood.

Enzymology of uPA

Plasminogen activators uPA and tPA belong to the serine protease family. In contrast with other members of this family, such as trypsin, chymotrypsin, and elastase, which have a wide range of substrates, uPA and tPA present a highly restricted substrate specificity. Moreover, unlike other serine protease zymogens, uPA has high single-chain activity, equivalent to 0.2 percent of the active two-chain form of the enzyme. This single-chain activity is sufficient to induce pro-uPA autoactivation to the two-chain uPA active form [25] (Figure 21.3).

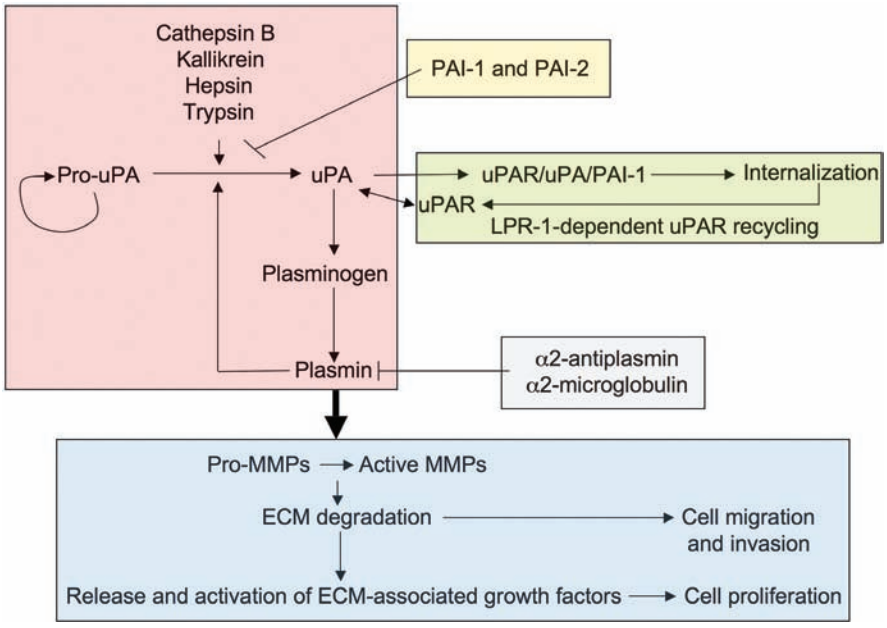

Figure 21.2. uPA activation and regulation. Pro-uPA enzymatic activation to uPA initiates a positive feedback mechanism converting the zymogen plasminogen into plasmin. This, in turn, can convert more pro-uPA into active uPA. Pro-uPA can also be autoactivated by its intrinsic enzymatic activity (pink). This positive feedback is blocked by two endogenous inhibitors, PAI-1 and PAI-2 (yellow). On uPA activation, downstream effectors such as MMPs become activated, allowing for ECM remodeling. In consequence, processes such as cell migration, invasion, and proliferation are favored in metastatic tumor cells (blue). A second level of regulation exists at the level of plasmin enzymatic activity inhibition by endogenous inhibitors that will block the activation of its downstream targets (gray). The formation of the uPAR/uPA/PAI-1 ternary complex induces the uPAR recycling and uPA/PAI-1 lysosomal degradation in a LPR-1-dependent process (green).

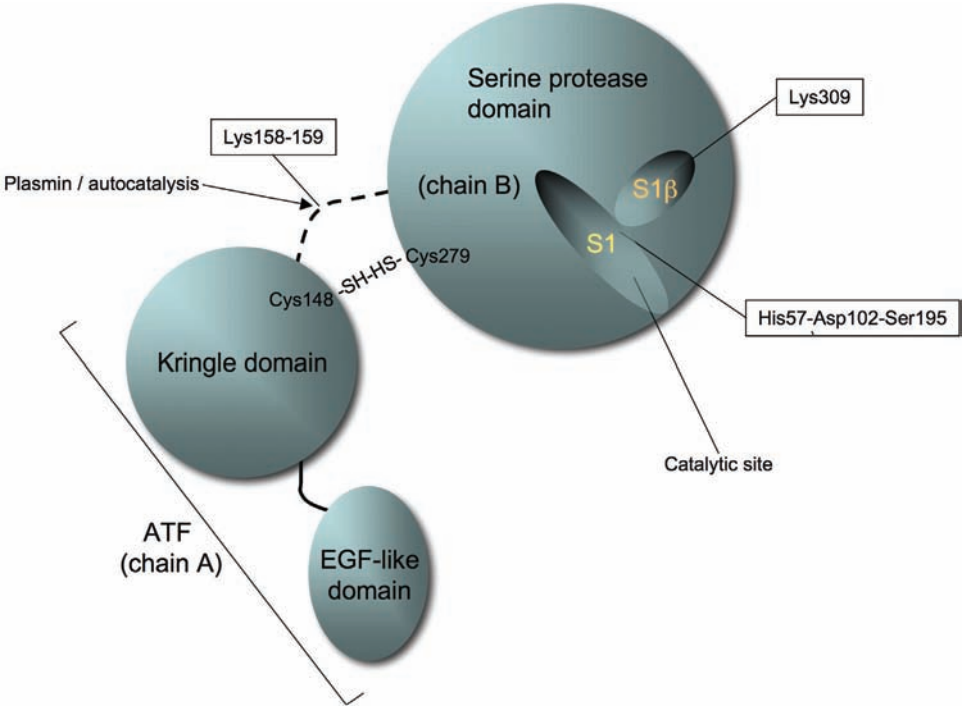

Figure 21.3. Two-chain uPA structure. After cleavage at the Lys158–159, uPA displays two chains (A and B) held together by the formation of a disulfide bond (SH–HS). The chain A contains the EGF-like and the Kringle domains, known as the amino-terminal fragment (ATF), responsible for uPAR binding. The serine protease domain contained in the chain B is responsible for uPA's enzymatic activity. The catalytic site is locate in a depression that has an S1 pocket, a common feature in other serine proteases; within this pocket, the His57-Asp102- Ser195 triad confers substrate specificity that is further increased by the presence of the S1β pocket located close to the catalytic site in a flexible loop. A point mutation in the Lys309, this flexible loop is known to be critical for the uPA enzymatic activity.

Pro-uPA is a 411-amino-acid multidomain glycoprotein of 55 kDa in humans (48 kDa in mice); its active form is generated by plasmin cleavage or autocatalysis at the center of a single chain at amino acids Lys 158–I159 (Figure 21.3). This leads to two smaller chains, A and B, held together by a single disulfide bond at Cys148–Cys279. Alternatively, cathepsin B, kallikrein, and hepsin [26, 27] can also cleave uPA zymogen, generating active uPA. The A chain of uPA (24 kDa, residues 1–158) has sequence homology to fibronectin, plasminogen, and prothrombin. This chain also has, at the N-terminal, a homology sequence to EGF (residues 10–43) followed by a kringle domain (residues 50–132), which together form the amino-terminal fragment (ATF) (residues 1–135). This region also includes the receptor-binding domain (residues 223–229) (Figure 21.3). The B chain (30 kDa, residues 159–411) contains the serine protease catalytic site and has homology to the S1 specificity pocket of the catalytic sites, containing the catalytic triad His57, Asp102, and Ser195, of other serine proteases such as thrombin, plasmin, and trypsin [28–30] (Figure 21.3). Within the uPA catalytic domain, lysine 300, which is located in the flexible loop (residues 297–313) of uPA, is essential for its catalytic activity. Mutations in this flexible loop, more specifically at proline 309, affect uPA enzymatic activity [25]. In general terms, the substrate specificity of an enzyme is dictated not only by the interaction of the enzyme catalytic domain with its substrate but also by secondary sites of interaction on the enzyme surface. In the case of uPA, an S1β pocket, which is close to the S1 specificity pocket of uPA, operates as a switch to open and close the access to the latter pocket. This confers increased substrate binding potency, contributing to a more efficient activation of plasminogen [25].

uPA Inhibitors

uPA activity is regulated by specific inhibitors that can be produced by the same cell that secretes uPA or by neighboring cells, such as fibroblast and endothelial cells [15]. These plasminogen activator inhibitors (PAIs) are known as PAI-1 and PAI-2 [31] (Figure 21.4). These are antiproteases that inhibit uPA and belong to the serpin (serin protease inhibitors) superfamily. PAI-1 is a 52-kDa (379 residues) single-chain glycoprotein that interacts covalently with uPA in a 1:1 ratio. PAI-2 exists as a circulating 60-kDa glycoprotein and

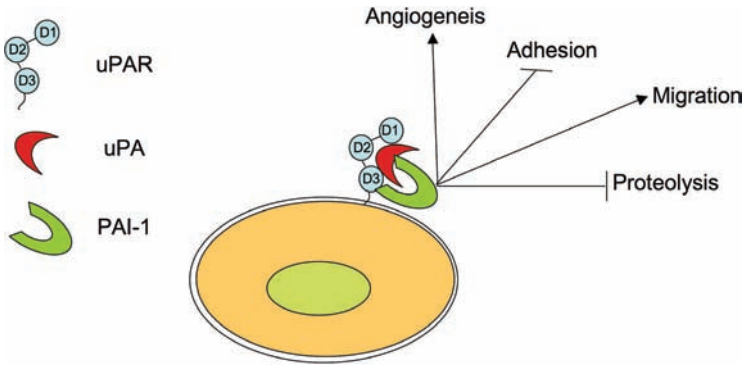

Figure 21.4. Opposing effects of PAI-1 on tumor cells. After uPA binding to uPAR, PAI-1 binds uPARs and uPA at the catalytic site to inhibit uPA enzymatic activity, reducing the ECM remodeling. Besides the inhibition of uPA activity, high levels of PAI-1 can exert paradoxical effects, such as inducing angiogenesis by the stabilization of blood vessels or blocking binding to the ECM component vitronectin, favoring cell migration.

a nonglycosylated intracellular form encoded by the same mRNA; its expression pattern is more restricted than PAI-1. It has been detected mainly in the placenta, monocytes, and epidermis; although its functions are not fully understood, it can also limit tumor invasion [32]. Besides its function as a protease inhibitor, intracellular PAI-2 also has a function in viral infections controlling cytopathic affects and apoptosis in infected cells [33].

Secreted uPA inhibition in normal and tumor cells is controlled mainly by PAI-1. This inhibitor binds to uPA in an irreversible fashion, binding the two-chain form of uPA [34]. To ensure that the protease activity is fully removed from the cell surface, the proteolytically inactive uPA-PAI complex is internalized (Figures 21.2, 21.3, and 21.4). This occurs upon binding of PAI to uPA-bound uPAR (also see the section "uPA Receptor"). This ternary complex is internalized in association with the α2-macroglobuin receptor/low-density lipoprotein receptor LPR-1 (α2M/LDL-receptor–related protein-1) [35–37]. The complex uPAR–uPA–PAI-1 is internalized in clathrin-coated vesicles. Then the uPA-PAI-1 complex is degraded by lysosomes, whereas uPAR and LRP-1 are recycled back to the cell surface from the endocytic compartment [38]. This tightly controlled mechanism is usually lost in cancer cells.

In this sense, contrary to what was anticipated, PAI-1 is not an inhibitor of invasion and metastasis. In fact, high levels of PAI-1 in tumors are linked with increased invasiveness and angiogenesis, and overexpression of PAI-1 is considered a marker of poor prognosis in many types of cancer [39] (Figure 21.4). This unexpected outcome of increased PAI-1 expression is a result of its ability to interact with other molecules besides uPA. PAI-1 is able to interact with vitronectin, a component of the ECM, competing with uPAR and integrins (e.g., $\alpha_v\beta_3$, $\alpha_v\beta_5$) for its binding at focal adhesion sites, which induces cell detachment and favors cell migration [15, 40, 41]. Most important, this paradoxical role of PAI-1 was resolved in part when it was found that PAI-1 could favor tumor angiogenesis. High levels of PAI-1 were linked to increased angionenesis, and induction of PAI-1 expression was the result of hypoxia in the tumor [42, 43]. It was found that PAI-1 was acting on the endothelial cells and stabilizing the blood vessels [44]. Thus, tumors that have high levels of PAI-1 have a more patent vasculature, minimizing leakiness and better oxygenating the tumor mass (Figure 21.4) [44–46]. This contributes to favoring aggressive growth and dissemination. Together, these findings highlight the importance of uPA and associated molecules, such as PAI-1, in determining the behavior of tumor cells. This is evidenced by proinvasive, proangiogenic, and motility-enhancing effects. In the following sections we will explore nonproteolytic functions of uPA and uPAR, as well as novel strategies to target this multifunctional cell surface complex.

uPA RECEPTOR

uPAR Expression and Proteolysis Scaffold Function in Tumors

Successful movement through tissue barriers requires that tumor cells be able to engage in controlled proteolysis that will dynamically remodel the ECM. To be fully equipped for these tasks, tumor cells must generate proteolytic activity. Cell surface associated proteases are probably the best candidates to perform the task of controlled, focused proteolysis in a complex in vivo environment (Figure 21.1).

The best-characterized example of specific localization of surface proteolysis is the uPA system, which involves uPA, its receptor uPAR, and plasmin. This mechanism has been shown to function as an enhancer of tumor cell invasion and metastasis [3, 15]. Three homologous domains that share similarity with the Ly6/CD59 family of proteins compose uPAR, which is linked to the plasma membrane by a GPI anchor (for a review, see [14] and Figure 21.1). Recently, new members of this family have been identified and may have a role in cancer [47, 48]. On the cell surface, uPA binding to uPAR enhances the conversion of plasminogen to plasmin by more than fortyfold, a property that allows tumor cells to eliminate barriers to movement [15]. This function may be further enhanced by the ability of uPAR to dimerize or oligomerize on the cell surface [14]. Further, in a nonproteolytic function, uPAR was found to serve as a receptor for the ECM molecule vitronectin, which has an important role in regulating

motility of normal and tumor cells [15, 40, 49] (Figure 21.1).

uPAR is overexpressed in tumor cells, where surface molecules can be in a 100- to 500-fold excess over their normal counterparts [50]. A similar overexpression is also observed for uPA [50]. In fact, similar signaling pathways and promoter regulatory elements control both uPA and uPAR gene expression. Numerous oncogenes and growth factor signaling pathways can induce these genes [50]. Regarding its role in favoring invasion and dissemination, uPA and uPAR have been detected in a small proportion of carcinoma in situ tumors but are almost always present in invasive and metastatic lesions [50]. Expression of uPA and uPAR in ductal carcinoma in situ of the breast predicts for early disease recurrence [51], suggesting that the acquisition of this protease and its receptor may precede tumor cell dissemination. It is not yet clear whether it is the lack of a specific protease or the underexpression of several crucial proteases that is responsible for keeping the in situ carcinoma cells confined to the epithelial compartment or whether undetected microinvasion and early dissemination occur in these conditions [52, 53] (Figure 21.1).

As indicated, conversion of plasminogen activation by uPA bound to uPAR occurs much more efficiently than for soluble uPA [15], and the massive overexpression of ligand and receptor vastly amplifies the ability of tumor cells to remodel their microenvironment. Plasmin can, in turn, directly or indirectly activate procollagenase 1 (proMMP-1) and prostromelysin 1 (proMMP-3), among other matrix metalloproteinases (MMPs), amplifying the proteolytic capacity and further favoring ECM degradation [15] and invasion (Table 21.1 and Figures 21.1 and 21.2). Clinical data support the correlation between expression of uPA and uPAR and acquisition of a malignant phenotype, because high levels of these proteins in primary tumor tissue are usually indicators of poor prognosis and can be used for choice of therapy in breast cancer [54, 55]. For example, the expression of both uPA and uPAR correlates with shortened disease-free survival in patients with breast, colon, and other tumors [54]. Thus, it appears that the acquisition of these proteases should confer the cells with an advantage to invade and disseminate to new anatomical locations.

uPAR Is a Powerful Signal Transducer in Cancer Cells

The cloning of the receptor for urokinase in 1990 [15, 56] opened a new era of research for the plasminogen activator field and allowed understanding of how the efficiency, localization, and focalization of plasminogen activation dependent on uPA occur in normal and pathophysiological conditions [15]. However, a completely unexpected role for uPAR (discussed later) was yet to be discovered, showing that uPAR, through the regulation of integrin function, could propagate mitogenic signals for powerful motility and proliferation of tumor cells [57] (Figures 21.1 and 21.5).

The first piece of evidence linking uPAR to the activation of discrete signaling pathways and the modulation by these signals of cell motility was provided ten years after its discovery [58]. Being a GPI-anchored molecule, uPAR can be localized to discrete surface domains composed of lipid rafts and caveolin-containing structures called *caveolae* [59]. This may further add to the topology of proteolysis and the signal transduction capacity of uPA/uPAR complexes. However, the massive surface overexpression of uPAR in caveolin-negative tumor cells suggests that this order of organization – and, therefore, proper regulation – may be lost in tumor cells [60]. It is now clear that uPAR is a receptor with a triple function: it can focus and localize plasminogen activation by uPA on cell surfaces, it serves as a non-integrin receptor for vitronectin, and it influences integrin function in *cis* and initiates intracellular signaling (for review, see [3, 57]). The fact that most human cancers overexpress uPA and uPAR fits well with the findings that blocking the uPA binding to uPAR can block tumor cells' invasion and development of metastasis [15]. Mitogenic effects were reported for uPA in culture, and uPA binding to cells could stimulate known signaling proteins, such as FAK, Src, ERK, p38, and MLCK, among others; these signals contribute to cell motility [3, 57]. However, the importance of uPAR signaling as a contributor to tumor cell proliferation in vivo remained unanswered.

Clinical evidence suggesting that uPAR could regulate proliferation – and, possibly, the transition from proliferative to quiescent/dormant behavior – came from studies on tumor cells in bone marrow in patients with gastric carcinoma [61] and other tumor types. In the gastric cancer studies, bone marrow samples were taken from patients before surgery and at six-month intervals thereafter. The samples were tested for the presence of tumor cells by double-staining with anticytokeratin and anti-uPAR antibodies. Bone marrow of patients containing cells that stained positively for uPAR in the original sample had increased tumor cell numbers in the follow-up samples. Such outcome was a predictor of a shorter disease-free survival [61]. Because the primary tumor had been surgically removed, the conclusion was that the presence of uPAR allows the bone-marrow–lodged tumor cells to proliferate.

Signaling by uPAR can also propagate prosurvival signals [62–66]. In brain tumors, inhibition of uPAR expression caused massive apoptosis of intracranial tumors [67]. This was shown to occur as a function of the prosurvival signaling through the AKT pathway. In this same tumor type, uPAR regulated local invasion into the normal surrounding tissue by

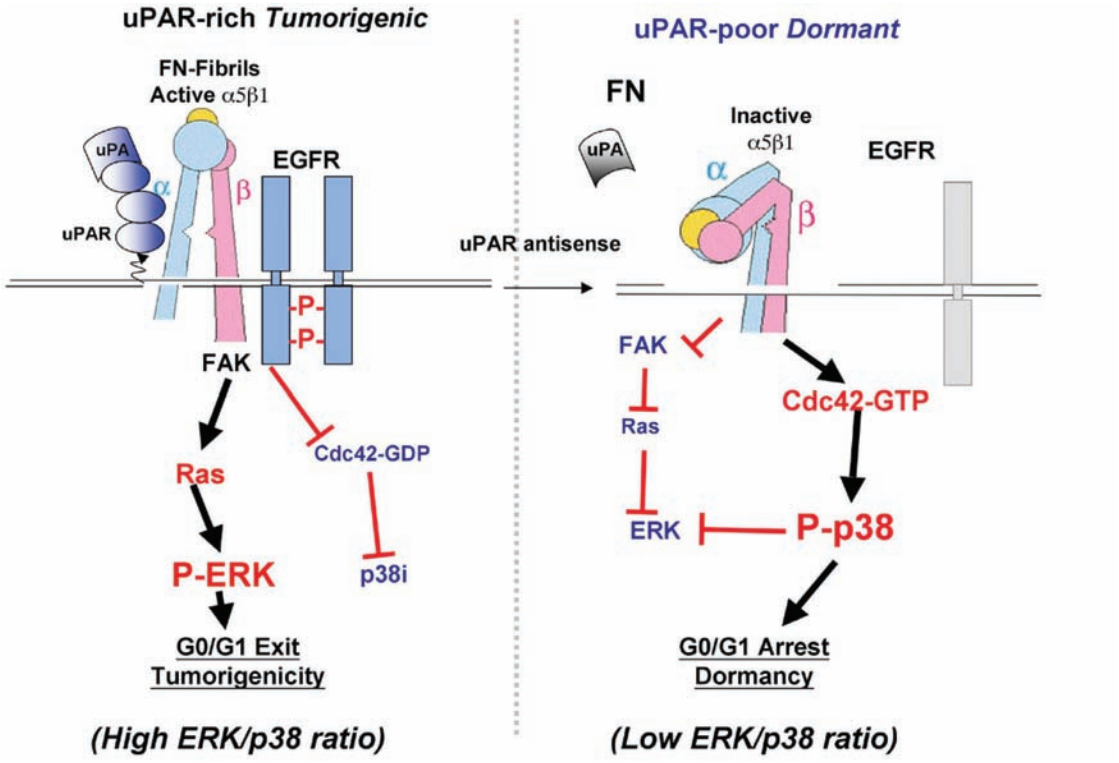

Figure 21.5. uPAR–integrin signaling in tumor growth. (Left) In uPAR-rich cells, uPA-bound uPAR frequently interacts with $\alpha 5\beta 1$ integrin, causing its activation. This leads to integrin-dependent recruitment of FAK/EGFR complex, which results in activation of Ras–ERK signaling. Concomitantly, this complex maintains Cdc42 inactive and prevents p38 activation. This results in a high-mitogenic ERK/p38 signaling ratio. Downregulation of uPAR or blocking of $\alpha 5\beta 1$ function (right) results in integrin inactivation, disassembly of the complex, inactivation of its intracellular signaling components, and reduced ERK activation. In contrast, Cdc42 becomes activated and induces p38 activation. Activation of p38 can impose an additional negative regulation on ERK signaling. This results in a low ERK/p38 ratio and induction of growth arrest. Blocking of FAK or EGFR activity can also induce a growth arrest; this is associated with strong inhibition of ERK activity (not depicted).

gliobastoma cells. Thus, in this tumor type, the prosurvival function of uPAR is also coupled to its proteolytic function [67].

Evidence for the mitogenic function of uPAR in tumors came from studies in which blocking the expression of this receptor in squamous carcinoma cells (HEp3) was achieved using antisense mRNA [68] (Figure 21.5). This resulted in the loss of malignancy of a human squamous carcinoma cell line [68], which was evidenced not only by a overwhelming decrease in the invasive properties of tumor cells, as was anticipated, but also by an unexpected and total loss of tumorigenicity [68]. More exhaustive studies [69] revealed that the loss of tumorigenicity was associated with the ability of uPAR-hypomorphic cells to form small tumor nodules in vivo that contained live dormant tumor cells; this was despite the presence of a normal vasculature [68, 69]. This dormant behavior was characterized by a dramatic reduction in proliferation and has been seen in several models [68–75]. The dormant behavior was also discovered for HEp3 cells that had a spontaneous downregulation of uPAR similar to the one achieved by the antisense technology [60]. Despite these major advances in tumors [76], the mechanisms by which this GPI-anchored receptor was signaling were unclear.

uPAR Coopts Adhesion and Growth Factor Signaling for Cancer Cell Mitogenesis

That blocking uPAR signaling could induce dormancy and serve as a therapeutic tool fueled our search for the mechanisms behind this phenomenon [60, 77, 78]. Work pioneered by the Chapman lab [79] had shown that uPAR interacts with integrins and regulates their function [59]. Subsequently it was shown that uPAR could engage in frequent interactions with the fibronectin (FN) receptor $\alpha 5\beta 1$ integrin [60]. This interaction was greatly diminished in human carcinoma cells expressing an antisense to uPAR or in cells from the same origin but that displayed a spontaneous downregulation of uPAR (Figure 21.5) [60]. uPAR activated integrin function, and cells overexpressing uPAR could bind efficiently to FN (Figure 21.5). Recent studies on uPAR and $\alpha M\beta 2$ integrin interaction further support the evidence that uPAR physically interacts with and regulates integrin active conformation [80]. In cells

in which uPAR was downregulated, although the total $\alpha5\beta1$ integrin levels did not change, their state of activation was decreased and their function as an adhesion receptor was substantially reduced [60]. Although in this and other systems uPAR transduced signals in an integrin-dependent manner, it has also been shown that signal transduction can happen independently of integrins [49, 81]. Still, the way in which this GPI-anchored receptor signals in the absence of an adaptor is still unclear.

Subsequently, it was found that in the HEp3 squamous carcinoma model, uPA activation of the ERK–MAPK pathway (a mitogenic signaling cascade) was strong in cells with high uPAR levels (Figure 21.5) [60]. In contrast, cells in which uPAR was downregulated displayed only a marginal activation of ERK. This was directly dependent on the ability of integrins to efficiently derive mitogenic signals from immobilized FN, as cells in which uPAR was downregulated were unable to "sense" FN and activate ERK (Figure 21.5) [60].

Integrins activate focal adhesion kinase (FAK) [82], which is hyperactivated in squamous cell carcinomas [83]. It was found that in cells in which uPAR was downregulated, there was decreased FAK phosphorylation at its major autophosphorylation tyrosine (Y397), basally or in response to adhesion to FN (Figure 21.5) [77]. ERK activation and proliferation induced by uPAR required FAK as expression of a dominant negative mutant of FAK (termed FRNK), which lacks the NH2 and central kinase domain of FAK but still interacts with integrin complexes [84, 85] strongly decreased the levels of active Ras and ERK (Figure 21.5) [77]. Accordingly, expression of FRNK also forced these uPAR-overexpressing human carcinoma cells into quiescence (Figure 21.5). Together, these results indicated that FAK was essential for mediating uPA/uPAR-$\alpha5\beta1$ signaling to the Ras-ERK module (Figure 21.5) [77].

It was shown that FAK could interact with both integrins and growth E receptors such as the epidermal- or the platelet-derived growth factor receptors [84, 86–88]. This allowed FAK to bridge and couple integrin and growth factor signaling. Additional studies showed that EGFR is a component of the uPA/uPAR/$\alpha5\beta1$/FAK complex required to couple uPAR signaling to the Ras-ERK pathway (Figure 21.5) [78]. Cells in which uPAR was downregulated displayed a dramatic downregulation of EGFR phosphorylation; when cells were plated on FN, only cells that had high uPAR levels were able to efficiently activate EGFR (Figure 21.5). Further, treatment of cells with sc-uPA (proteolytically inactive) was sufficient to strongly activate the EGFR–ERK pathway, but only in cells with high uPAR levels [78]. Interfering with FAK or with EGFR signaling by expressing FRNK, a dominant negative EGFR, or treatment with a compound (AG1478) that inhibits EGFR kinase abrogated EGFR and ERK activation (Figure 21.5) [78].

Thus, as illustrated in Figure 21.5, a complex comprising uPA, uPAR, $\alpha5\beta1$ FAK, and the EGFR is the minimal complex (uPAR-complex) required to transduce signals from uPAR to the ERK pathway [78]. This resulted in a more than fourfold difference in the level of active ERK in high-uPAR versus low-uPAR cells, a difference that could be clearly detected in vitro but, most important, in high- and low-uPAR cells inoculated in vivo [57, 60, 71, 77, 78, 89, 90]. Thus, cells with high uPAR that are able to form progressively growing tumor masses have a "constitutive" activation of ERK, a property that is lost in cells that express low levels of uPAR and that become dormant in vivo [60].

Consistent with the aforementioned regulation of mitogenic pathways by uPAR, it was shown that uPAR blockade in vivo results in a state of quiescence [60, 69, 78]. As early as twenty-four hours after in vivo inoculation, cells with high uPAR and active ERK levels showed a larger proportion of cells in the S-phase, whereas cells that were dormant displayed a G_0/G_1 arrest that was established as early as forty-eight hours after inoculation in vivo (Figure 21.5) [60, 69, 78].

CONSEQUENCES OF TARGETING uPA AND/OR uPAR IN TUMOR CELLS

Small Molecule Inhibitors of uPA and Their Potential Use in Treating Metastatic Disease

From more than two decades, it had been known that inactivation of uPA using specific antibodies results in a decrease in the invasive or metastatic capabilities of tumor cells. The seminal experimental work of Ossowski and Reich [91, 92] demonstrated that antibody treatment against tumor-derived uPA delays the onset of distant metastasis, suggesting that the protease is required during early stages of the process. Direct evidence was obtained in the chick chorioallantoic membrane model with the human squamous carcinoma HEp3, using rabbit antibodies that blocked the catalytic activity of human uPA but did not inhibit chicken uPA. When administered intravenously to embryos, the anti-uPA antibodies strongly inhibited metastasis to the lung [91]. Daily administration of anti-human uPA antibodies also inhibited local invasion of HEp3 tumors in nude mice. However, in this xenograft model, inhibition of tumor invasion did not lead to a reduced incidence of distant metastasis [93].

Blocking uPA activity – and, particularly, cell surface uPA – represents an attractive goal for the development of antiinvasiveness pharmaceuticals (Figure 21.1). Designing low-molecular-weight synthetic inhibitors with appropriate potency and selectivity for uPA represents an interesting approach to achieving this goal. Early efforts to inhibit plasminogen activation in experimental systems included known compounds

having anecdotal or unspecific antiproteolytic activity. Minocycline, EDTA, 1,10-phenanthroline, ε-aminocaproic acid, and aprotinin, among others, have been investigated [94, 95]. Regrettably, most compounds that block uPA also interfere with tPA or plasmin and thus are unsuitable for use as antiinvasive drugs because of the potential inhibition of fibrinolysis.

In 1987, the potassium-sparing diuretic amiloride (3,5-diamino-N-[aminoiminomethyl]-6-chloropyrazinecarboxamide) was reported to be a moderately potent selective inhibitor of uPA, having little or no inhibitory activity against tPA or plasmin [96]. Later on, more potent and highly selective competitive uPA inhibitors based on modifications of amiloride were synthesized [97, 98]. A variety of mono- and bicyclic aromatic guanidines and amidines were obtained; two compounds in this class, designated B428 and B623 (4-substituted benzo[b]thiophene-2-carboxamidines), were shown to block both free and cell-surface–bound uPA activity, as well as uPA-mediated degradation of fibronectin by HT-1080 human fibrosarcoma cells [97, 98]. Daily treatment with B428 or B623 markedly blocked the local invasion of a highly aggressive syngeneic mouse mammary carcinoma without overt toxic effects, although the compounds neither inhibited tumor-induced angiogenesis nor reduced the incidence of lung metastasis in this preclinical model [99]. It is possible that the existence of other fibrinolytic enzymes and activators of plasminogen [100, 101] may compensate for uPA inhibition.

Dose escalation of amiloride or B428 failed to eliminate pulmonary metastasis in a rat mammary cancer, suggesting that a maximum limit was attained for the inhibitory activity of compounds during metastatic spread [102]. Klinghofer et al. [103] have pointed out the importance of species-specificity of amidine-based competitive inhibitors of uPA, including amiloride and B428. They conducted a comparative study with diverse uPA inhibitors in both rodent and human enzymatic assays, reporting relevant differences between species. Thus, caution should be observed in the design and interpretation of in vivo studies with uPA inhibitors, especially when both murine and human components of the uPA/uPAR system are expressed, such as in xenograft tumor models [103].

Nevertheless, a combination of B428 with the antiestrogen tamoxifen [104] or the calcium channel blocker verapamil [105] efficiently blocked metastatic spread in different rodent models of breast cancer. Similarly, administration of amiloride together with celecoxib, a cyclooxygenase-2 inhibitor, reduced local recurrences and suppressed metastasis in a rat mammary carcinoma model, stressing the potential utility in combined adjuvant therapies [102, 106].

Partial inhibition of intravascular fibrinolysis by high doses of the most potent anti-uPA carboxamidine, B623, could enhance lung colonization by blood-borne metastatic cells [99]. B623 induced fibrin deposition followed by tumor cell aggregation in the presence of plasma and might, through this mechanism, increase metastasis [99]. In accordance, some authors have hypothesized that administration of an activator of fibrinolysis, such us uPA itself, might dissolve tumor-cell–associated fibrin presumed to be required for nesting of tumor emboli within the circulation [107]. These studies stress the critical importance of the balance that must be achieve in regulating tumor-cell–based proteolysis versus that in the host.

In this context, the effect of uPA inhibition on tumor progression may depend on a rather delicate balance between two opposing activities. If uPA increases cell shedding from the primary tumor, its inhibition may block tumor invasion. Conversely, if circulating plasminogen activators interfere with tumor cell trapping and lodgment in the vasculature of the target organ, uPA inhibition would neutralize this effect and thereby induce an increase in metastatic colonization. This view is consistent with the hypothesis that certain protease inhibitors may promote, as well as inhibit, metastasis [39] (see also the section "uPA Inhibitors"). These counterproductive effects might be salvaged by the fact that in the vast majority of cancers, dissemination has occurred by the time of diagnosis [53, 108]. Even in small "noninvasive" lesions, such as ductal carcinoma in situ (DCIS), dissemination is observed [53, 108]. Thus, if the targeting of uPA is done after the primary tumor has been removed and it is focused on limiting the expansion and local invasion of the residual cells, the effects on circulating tumor cells might be avoided.

Recently, the development of a set of small nonpeptidic diaryl phosphonate inhibitors was reported by Joossens et al. [109]. Some of these inhibitors showed a highly selective irreversible uPA inhibition with nanomolar potency. Preliminary studies with selected compounds demonstrated significant antimetastatic effects in a rat mammary carcinoma model without signs of acute toxicity. The authors claimed that selective, irreversible uPA inhibitors could be valuable in cancer therapy [109]. Other small molecules and peptide derivatives targeting uPA include different classes of arginine mimetics, such as amidinophenylalanine-, amidinobenzylamine- and 4-arylguanidine-based inhibitors (reviewed in detail in [110]). Some of these compounds are reaching clinical research, in an effort to develop antimetastatic prophylactic drugs for cancer patients. Compounds should be tested in combination with existing antitumor therapies in patients with suspected residual disease at high risk of metastasis. This is important because it is highly likely that the success of these therapies depends on the tumor burden. Targeting the uPA proteolytic

system in residual disease after debulking may enhance the chances of limiting secondary growths.

Targeting the uPA–uPAR Interface to Block Proteolytic and Signaling Functions of the uPA–uPAR Complex

Novel therapeutic strategies designed to target signaling properties of the uPA–uPAR complex may represent another interesting approach to treating metastatic and minimal residual disease. The receptor localizes uPA-dependent proteolytic activity on the cell surface and further propagates signals for motility and proliferation of tumor cells through the regulation of integrin function [57]. Interestingly, the anticatalytic compound B428 not only can inhibit tumor-associated proteolysis but also can interfere tumor cell adhesion and migration, suggesting than the compound may modify uPAR mobilization and uPA-dependent cell signaling [111]. These initial observations emphasized that uPA and its receptor are involved in attachment, proteolysis, and migration, and that the three processes of tumor invasion can be blocked by a synthetic uPA inhibitor.

Different groups have described the development of linear peptide antagonists of uPA–uPAR interaction [112, 113]. Some of these peptide compounds competitively inhibited human uPA binding in breast cancer cells and blocked intravasation of human HEp3 carcinoma cells in chick embryos [114, 115]. Novel cyclic peptide antagonists were also identified, showing reduction of the growth and spread of human ovarian cancer cells in nude mice [116]. In addition, a few small-molecule antagonists of uPAR have been reported, including N-substituted tripeptide derivatives, oligothiophenes, biphenyl-based peptidomimetics, and hydroxycoumaranone derivatives. Some of these compounds have nanomolar activity [110].

Targeting uPAR *Cis*-Interactions to Block Mitogenic Signaling in Tumors

The uPA–uPAR complex offers another possibility for targeting its signaling function for mitogenesis [57]. Recently, it was demonstrated that through a region comprising amino acids 240 to 248 in the third domain of uPAR, this receptor could interact and modulate integrin signaling [117]. An independent study found also that this region is important for regulating lateral interaction between uPAR and integrins [118]. In particular, Ser-245 and His-249 were found to be critical for this function, as Ala mutations of these residues caused a loss of the integrin-activating function [117, 118]. This signal was found to be important for tumor growth, as a mutant uPAR in Ser-245 was not able to strongly activate ERK signaling and to stimulate tumor growth in vivo [117].

Analysis of the recently identified three-dimensional structure of uPAR revealed that Ser-245 is on the opposite face from where uPA interacts with uPAR. This further supported the findings that peptide inhibitors comprising this region, or the receptor mutants themselves, have no effect on uPA binding to the receptor [117]. This has led to the screening of small-molecule inhibitors that target this region and disrupt both the integrin-interacting and mitogenesis-activating functions of uPAR (Ossowski et al., personal communication). These studies have also revealed the possibility of targeting with high specificity the binding of uPA to uPAR and the binding of uPAR to integrins. A region in domain II of uPAR was also found to be important for binding to the extracellular matrix protein vitronectin, without interference with uPA binding and with strong inhibitory effects on motility [41, 119]. Thus, it is possible that combining two, or all three, of these strategies will fully obliterate uPAR functions. As activation of mitogenesis and motility require uPA binding to the receptor, these inhibitory strategies might be synergistic. As mentioned in previous sections, given that uPAR and uPA are overexpressed in the vast majority of solid tumors, it is possible that these uPA–uPAR combination therapies might be highly beneficial for patients.

CONCLUSIONS AND FUTURE DIRECTIONS

The data summarized here show the exciting progress that has been made since the description of the plasminogen activators in the early 1970s and of uPAR in 1990 [7, 56]. These studies have highlighted the powerful biological roles of uPA and uPAR in cancer progression, and the detailed structural, as well as molecular and cellular, studies have allowed designing strategies to target the tripartite functions of the uPA–uPAR complex – namely proteolysis, motility, and survival/mitogenesis. The fact that uPAR knockout mice are not prone to gross developmental defects [120] suggests that this strategy might be specific for tumor cells and have few side effects.

Successful application of these new therapeutic molecules might depend not only on the potency of these inhibitors but also on a change of modality in treatment. As in the majority of anticancer strategies, it will be important to determine whether such targeted therapies might be more advantageous when targeting residual disease rather than using them for tumor debulking. As disseminated tumor cells are invariably the source of local recurrences and metastasis, targeting the uPA – uPAR complex in residual disease might be a more rational strategy.

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22 The Lymphatics: On the Route to Cancer Metastasis

Tara Karnezis, Ramin Shayan, Marc G. Achen, and Steven A. Stacker

The blood vascular and lymphatic systems are necessary for the flux of gases, liquids, nutrients, signaling molecules, and cells among tissues and organs. These two highly branched, treelike networks are interconnected through the largest lymphatic vessel, the thoracic duct, which drains lymph, the protein-rich interstitial fluid, into the blood circulation. Both networks contribute to homeostasis of a healthy individual, and their malformation or dysfunction contributes to the pathogenesis of many diseases, such as cancer [1].

The lethality of cancer is associated primarily with metastasis – that is, the spread of cancer cells from the primary site to lymph nodes and to distant organs [2]. In principle, tumor cells can spread within the body by three main pathways: direct invasion of surrounding tissues, via the blood system to distant organs (hematogenous spread), or via the lymphatic system to the sentinel lymph node,[1] distal lymph nodes, and distant organs (lymphogenous spread) (Figure 22.1).

Tumor cells induce the growth of new lymphatic vessels within and immediately around tumors and draining lymph nodes, enhancing immune cell trafficking to lymph nodes. Increased lymphatic vessel density in tumors is also associated with increased metastasis to lymph nodes [3]. The extent of lymph node metastasis is a major determinant for disease staging. Despite its clinical relevance, however, little is known about the mechanisms that govern spread via the lymphatic system. Tumor cell entry into the lymph node via the lymphatics was previously believed to be a passive process, whereby tumor cell uptake would be facilitated by the thin walls and lack of a physical basement membrane[2] barrier of lymphatics. Understanding the role of lymphatics in tumor spread has been hampered by the lack of markers that distinguish blood vessels from lymphatic vessels and by the lack of identified factors that contribute to lymphatic growth. However, in recent times, the discovery of lymphatic-specific molecular markers to identify lymphatic vessels from blood vessels and the lymphangiogenic growth factors, vascular endothelial growth factor (VEGF)-C and VEGF-D, and the development of animal models of cancer using these tools have indicated that lymphangiogenesis is associated with, and may be an active component of, the metastatic pathway [4–7]. The study of the lymphatic system is intriguing not only from a biological viewpoint but also because understanding the molecular regulation of lymphangiogenesis may provide targets for design of anticancer therapeutics that restrict the lymphogenous spread of cancer.

HISTORICAL OVERVIEW OF THE LYMPHATIC SYSTEM IN CANCER

The first description of the lymphatic system dates back to ancient Greece, where physicians noted lymphatic vessels during dissections of human and goat gastrointestinal systems. Hippocrates is known to have referred to lymph as "milky blood" [8, 9]. Lymphatic drainage from all areas of the body was described during the seventeenth century; it was also recognized that these lymphatic pathways merged into the thoracic duct as an organized system, and, from there, into the central venous system [10]. The means by which lymph enters the lymphatic system was partly addressed by Hunter (1746), who observed that the lymphatic capillaries were engaged in absorption, and Recklinghausen (1862) who established the concept of

[1] The *sentinel lymph node* is the first draining lymph node near a tumor.

[2] The *basement membrane* is a structure that supports and provides a physical barrier to overlying endothelia. Keratinocytes, glandular cells, and endothelial cells reside on basement membranes that consist of an electron-dense membrane called the *basal lamina*, about 300–700 Å in thickness, and an underlying network of reticular collagen fibrils, which average 300 Å in diameter. In addition to collagen, this supportive matrix contains intrinsic macromolecular components.

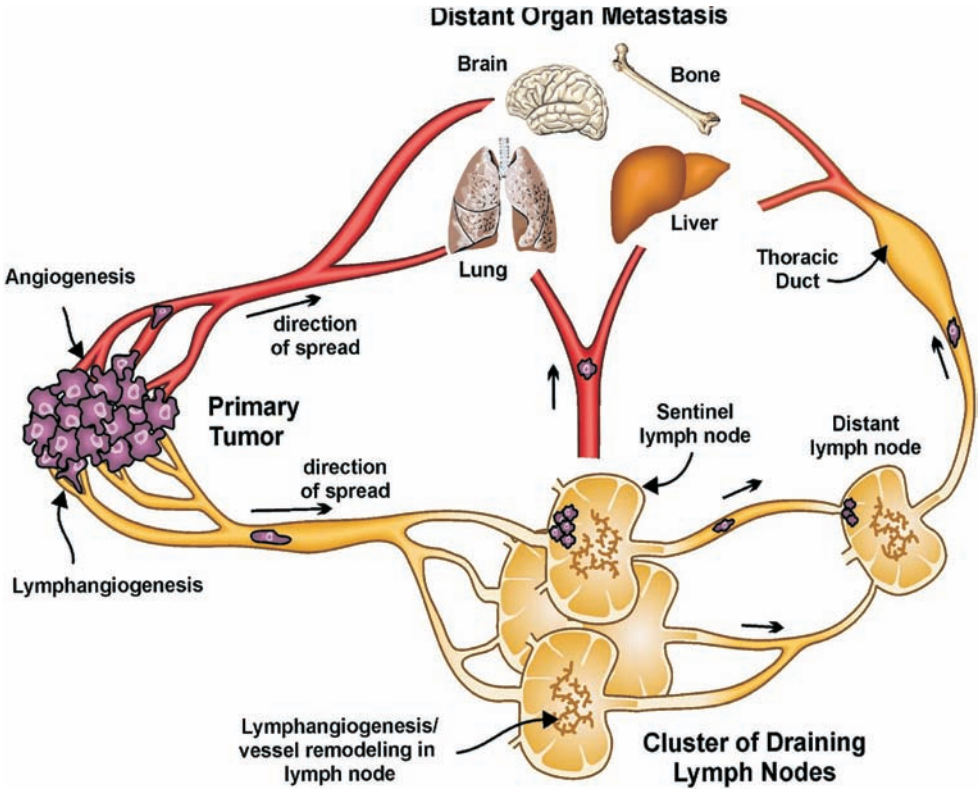

Figure 22.1. Schematic representation of the potential routes of metastasis. Tumor cells can spread via the lymphatic vasculature (yellow) or by the blood vasculature (red).

the "blind-ended" lymphatic vessel as opposed to the arterio-venous "loop" characteristic of the blood vascular capillary bed [10]. It was a further two hundred years before Sabin's embryonic pig injection studies would shed light on the venous origin of the lymphatic system [11]. We believe that it is important to define the nature of the primary tumor to differentiate it from metastatic lesions.

The importance of lymphatics in cancer spread was first noted in the sixteenth century, when it was observed that cancers of the breast that spread to the axillary[3] lymph node had significantly worse survival outcomes than those localized to the primary tumor[4] [12]. The idea of lymphatic injection using radioactive gold lymphoscintigraphy[5] and radiopaque dye was adopted in the mid- to late twentieth century to employ the technique of radiographic imaging for studying the lymphatic vasculature in living humans [13]. This methodology now forms the basis of modern lymphoscintigraphy and sentinel lymph node biopsy, as discussed by Andtbacka and Gershenwald in Chapter 45.

The next major clinical development regarding lymphatics in cancer occurred when clinicians began to use radioisotope injections to better understand which regional lymph node groups drain different parts of the body [14], essential information for identifying potential routes of cancer metastasis via the lymphatic vasculature. The late twentieth century saw the adaptation of lymphatic mapping for the prediction of each patient's lymphatic drainage from a specific tumor, and for identification of the sentinel lymph node [15].

[3] *Axillary lymph nodes* are lymph nodes located in the armpits. The medical term for armpit is *axilla*.

[4] A *primary tumor* is a tumor that is at the original site where it first arose. For example, a primary lung tumor is one that arose in the lung, as opposed to one that arose elsewhere and spread to the lung. The original tumor is sometimes called "the primary."

[5] *Lymphoscintigraphy* is a method used to identify the sentinel lymph node. A radioactive substance that can be taken up by lymph nodes is injected at the site of the tumor, and its movement is monitored on a computer screen. Once the lymph nodes that have taken up the substance are identified, they can be removed and examined to see if they contain tumor cells.

STRUCTURAL FEATURES OF THE LYMPHATIC SYSTEM

The intricate network of thin-walled vessels that constitutes the lymphatic vasculature begins within the superficial dermis of the skin as highly permeable, blind-ending sacs referred to as *absorbing lymphatic vessels* or *lymphatic capillaries* (Figure 22.2) [16, 17]. Unlike the blood capillaries, these lymphatic vessels are composed of a single thin, nonfenestrated lymphatic endothelial

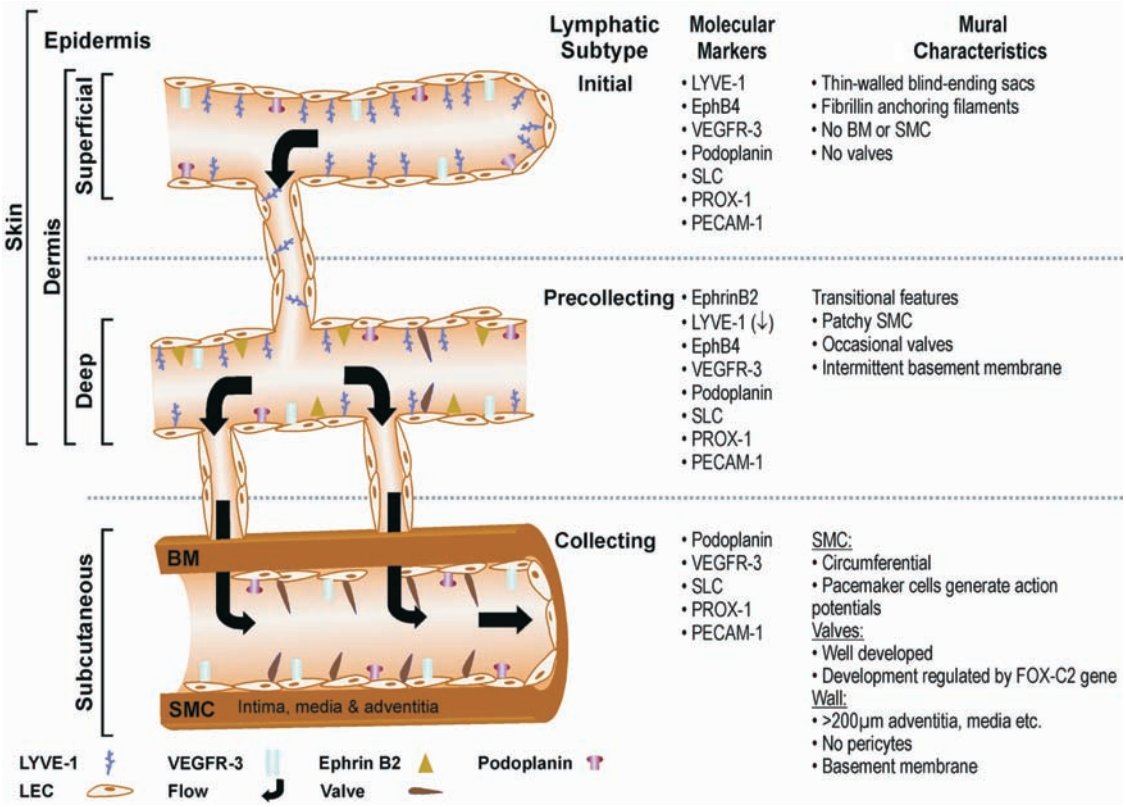

Figure 22.2. Schematic representation and molecular characteristics of lymphatic vessel subtypes found in the dermal and subcutaneous layers of the skin. Examples of markers that could be useful to distinguish lymphatic vessel subtypes are shown. Not all molecular markers are indicated on the lymphatic vessels.

cell (LEC) layer, which is not invested by pericytes or smooth muscle cells.[6] Characteristic overlapping cell junctions between LECs in lymphatic capillaries serve as valves. An increasing interstitial fluid pressure exerts a pulling force through the anchoring filaments, causing these junctions to open, allowing fluid uptake. After the fluid volume in the surrounding extracellular matrix is reduced, the anchoring filaments slacken, returning the LECs to their overlapping resting position [18]. Other methods of fluid absorption include intracellular trafficking through the LEC itself, a process referred to as *pinocytosis*[7] [19]. Recently, novel junctions between endothelial cells of initial lymphatics have been suggested to constitute an additional means of fluid absorption; these discontinuous button-like junctions ("buttons"), which differ from the regular, continuous cell junctions ("zippers") found in collecting lymphatics and blood vessels, represent specialized junctions that facilitate fluid uptake and are able to open and close without disrupting junctional integrity [20].

The lymphatic capillaries merge into the precollecting lymphatic vessels located in the deep dermis; these vessels are the initial drainage route of lymph (Figure 22.2). These precollecting lymphatics consist of segments that contain valves and are surrounded by a basement membrane and smooth muscle cells. These segments are interspersed by areas morphologically similar to the initial lymphatic capillaries [21]. The precollectors may have a role in lymph propulsion, rather than absorption of fluid, which is carried out by the initial lymphatic capillaries. The precollecting lymphatics, in turn, drain into the "collecting lymphatics," which are located in the subcutaneous tissue. These collecting lymphatic vessels have circumferential smooth muscle cells; regular intraluminal valves [17] have smooth muscle cell and pericyte investment and are generally more than 200 μm in diameter. The collecting lymphatics coalesce into lymphatic trunks, then into intrathoracic ducts. During the course of tumor spread, these

[6] A *pericyte* is a slender, relatively undifferentiated, connective tissue cell that wraps around vessels of the vasculature. As a relatively undifferentiated cell, it serves to support these vessels, but it can differentiate into a fibroblast, smooth muscle cell, or macrophage as well, if required. Smooth muscle cells are elongated, contractile cells that also associate with vessels. Both cells have been implicated in blood flow regulation.

[7] *Pinocytosis* is the ingestion of dissolved materials by endocytosis; the cytoplasmic membrane invaginates and pinches off, placing small droplets of fluid in a pinocytic vesicle. The liquid contents of the vesicle are then slowly transferred to the cytosol. Pinocytosis is used primarily for the absorption of extracellular fluids.

lymphatic vessels dilate in response to tumor-derived VEGF-C, thereby increasing the lymph flow and capacity to transport tumor cells to lymph nodes, and may become trapped and proliferate here or spread further to distal organs [22, 23].

DEVELOPMENT OF THE LYMPHATIC SYSTEM

Animal models have been an invaluable tool to study lymphatic development. Florence Sabin's pioneering injection studies of pig embryos led to the theory that the lymphatic system develops by "centrifugal sprouting" [11]. Based on ink injection experiments in pig embryos, she postulated that the two jugular lymph sacs develop from the anterior cardinal veins by endothelial budding. Because the mammalian lymphatic system originates from veins, its morphogenesis involves the acquisition of an LEC identity and separation from the blood vessel system typically achieved by the acquisition of markers with specificity for LECs (discussed later).

The first lymphatic vessels develop primarily from blood vessels, after the early arteries and veins are formed within the embryo [24]. In mice, these LECs sprout from the cardinal vein to form the primary jugular lymph sacs. Sox18, a member of the Sox family of transcription factors, triggers expression of another transcription factor, Prox-1, in a distinct cluster of endothelial cells (ECs) on the dorsal side of the cardinal vein [25]. Prox-1 is essential for lymphatic development and determines lymphatic fate by initiating the upregulation of lymphatic-specific genes, while suppressing certain blood vessel markers [18, 26]. These Prox-1–positive ECs also express low levels of the receptor tyrosine kinase VEGFR-3, the cell surface receptor that binds the lymphangiogenic growth factors VEGF-C and VEGF-D. Via VEGFR-3 activation, Prox-1 positive cells migrate toward a local VEGF-C gradient, leading to the initial sprouting of these committed ECs from the cardinal vein, forming the primitive lymph sacs [27]. In a centrifugal fashion, the lymph sacs sprout to form the primitive lymphatic plexus. By embryonic day (E) 15.5, the dermal plexus is formed and continues to develop after birth into the precollecting dermal layer, from which the primitive subepidermal lymphatic capillary plexus sprouts [24, 26, 28].

The recent discovery of genes that specifically control lymphatic development and lymphangiogenesis, and the identification of new lymphatic endothelium-specific markers, have facilitated key scientific advances and provided new insights into the molecular mechanisms that control lymphangiogenesis [26, 29–32]. These findings include the identification of specific genetic defects in certain hereditary diseases that are associated with lymphatic dysfunction and evidence that malignant tumors can directly activate lymphangiogenesis and promote lymphatic metastasis [33–36].

MOLECULAR MARKERS OF LYMPHATICS

The lymphatic and blood vasculatures were previously difficult to differentiate histologically. Markers that distinguish lymphatics from blood vessels unequivocally are essential. The identification of several markers that show different profiles of expression in blood vessels and lymphatics has facilitated detailed analyses of the development and pathologic role of the lymphatic vasculature.

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) is a 130-kDa member of the immunoglobulin superfamily [37] and is considered a pan-endothelial marker, widely expressed in both blood and lymphatic vessels. It has been used in combination with the basement membrane markers laminin and collagen type IV; vessels that were PECAM-1 positive but lacked basement membrane staining and red blood cells in their lumens were identified as lymphatic [31, 38, 39]. In addition, double staining with PECAM-1 and PAL-E has been used for the detection of lymphatic endothelium [40]. Tumor blood vessels and the high endothelial venules in lymph nodes are particularly PAL-E reactive, whereas lymphatic capillary ECs are unreactive [41]. The discovery of molecules that are specifically expressed by the lymphatic endothelium has enabled a more accurate and simplified lymphatic vessel identification. The main proposed lymphatic markers are VEGFR-3 (also known as *Flt4*), a cell surface tyrosine kinase receptor that binds the lymphangiogenic growth factors VEGF-C and VEGF-D [42–44]; podoplanin, an integral membrane protein expressed by cultured human lymphatic endothelial cells, which is one of the most highly expressed lymphatic-specific genes [45–50]; and LYVE-1, a homolog of the CD44 hyaluronan receptor, which is the lymphatic receptor for the extracellular matrix glycosaminoglycan hyaluronan (HA) [51, 52]. Figure 22.3 illustrates the use of some markers to distinguish blood vessels from lymphatics. Other proposed lymphatic markers include Prox-1, desmoplakin, the human β-chemokine receptor[8] D6, CCL21, CLEVER-1, and macrophage mannose receptor [53].

[8] *Chemokines* are a family of small proteins secreted by cells. Proteins are classified as chemokines according to shared structural characteristics such as small size (they are all approximately 8–10 kDa in size), and the presence of four cysteine residues in conserved locations. These proteins exert their biological effects by interacting with G-protein–linked transmembrane receptors called chemokine receptors, which are selectively found on the surfaces of their target cells. The major role of chemokines is to act as chemoattractants to guide the migration of cells. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration toward the source of the chemokine.

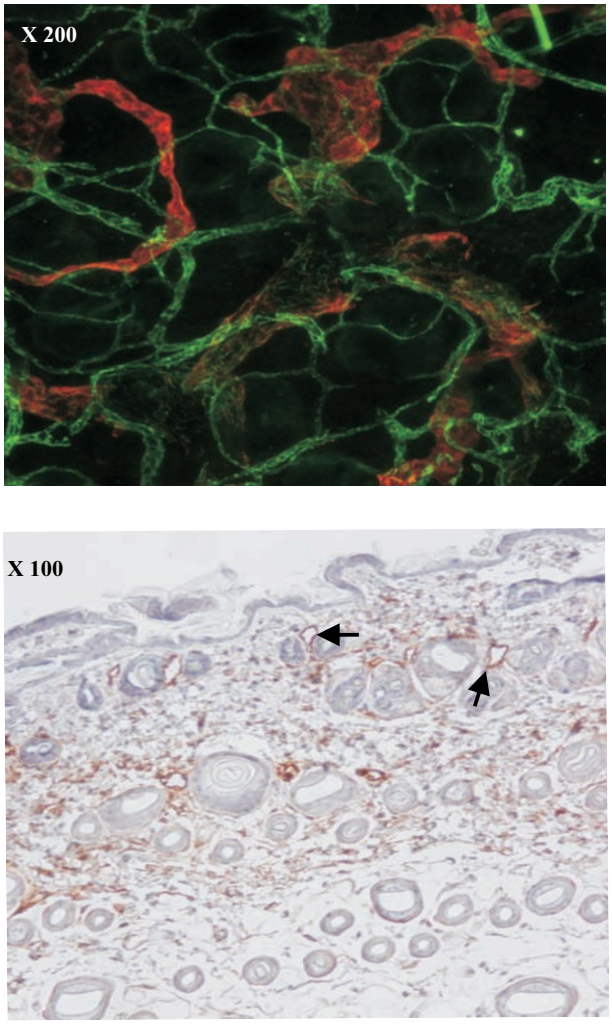

Figure 22.3. Markers used to differentiate blood vessels from lymphatic vessels. Top panel: Whole-mount staining of mouse ear for PECAM (green), indicating blood vessels, and LYVE-1 (red), indicating lymphatic vessels. Bottom panel: Staining of mouse dermis using podoplanin antibody to indicate lymphatics (arrow).

CHARACTERIZATION OF LYMPHATIC ENDOTHELIAL CELLS

Culturing and Purification of Lymphatic Endothelial Cells

Historically, pure cultures of LECs were first established by Johnston and Walker in the mid-1980s [54] from bovine mesenteric collecting lymphatic vessels. Lymphatic endothelium has since been cultured from a variety of species, including human, rat, and mouse [55]. Most of these studies have described isolation of LECs from large lymphatic vessels using crude mechanical methods of cell separation; because these vessels are supplied by a rich network of nutritive blood vessels, the purity of the isolated cell population remained in question [56].

The identification of lymphatic-specific markers that distinguish lymphatic endothelium from blood vascular endothelium has been a pivotal step in enabling researchers to purify LECs to homogeneity. By the development of techniques using these differences, LECs from microdermal cell preparations have been isolated by positive selection[9] using antibodies to podoplanin, VEGFR-3, and LYVE-1, and by a negative selection with antibodies to CD34, a marker for blood endothelial cells (BECs) [46, 47, 57, 58]. Purified LECs have been grown as a monolayer with a unique "cobblestone morphology," showing characteristic lymphatic-like overlapping cell junctions [17].

These studies demonstrated that LECs and BECs retain their differentiated phenotypes in culture. Both EC types were propagated and stably expressed VE-cadherin, CD31, and von Willenbrand factor, known endothelial cell markers. Molecules selectively displayed by LECs in vivo – podoplanin, LYVE-1, and VEGFR-3 – were strongly expressed by expanded LECs, but not by BECs, and the two EC types assembled vascular tubes with lumens in vitro in a strictly homotypic fashion [47]. These results show that LECs and BECs can be purified to relative homogeneity and constitute stable and specialized endothelial cell lineages all set with the potential to navigate leukocytes and, perhaps also, tumor cells into and out of the tissues.

LECs established by the different methods, however, were shown to exhibit some differences in gene expression [56]. These differences may be attributed to variations in source tissues (i.e., adult versus neonatal skin) or differences in culture conditions, or, alternatively, the different isolation strategies may select for specific subpopulations of LECs. For example, LECs isolated by VEGFR-3 selection may be partly contaminated with BECs, as VEGFR-3 can also be expressed by the blood

[9] In cell biology, mixed cell populations can be purified by magnetic-activated cell sorting (MACS) or by fluorescence-activated cell sorting (FACS). In MACS, the mixture of cells to be separated is incubated with magnetic beads coated with antibodies against a particular surface antigen, whereas in FACS, cells are incubated with fluoresently conjugated antibodies. FACS is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based on the specific light scattering and fluorescent characteristics of each cell. In MACS, cells expressing the desired antigen attach to the magnetic beads, after which the cell solution is transferred onto a column placed in a strong magnetic field. In this step, the cells expressing the antigen attach to the beads and stay on the column, whereas other cells (not expressing the antigen) flow through. With these methods, the cells can be separated positively or negatively with respect to the particular antigen(s). In positive selection, the cells expressing the antigen(s) of interest are washed out to a separate vessel and collected. In negative selection, the antibody used is against surface antigen(s) that are known to be present on cells that are not of interest. After administration of the cells/magnetic bead solution onto the column, the cells expressing these antigens bind to the column and the fraction that goes through is collected, as it contains almost no cells with undesired antigens.

vascular endothelium during different pathologies [59]. It remains to be determined which purification strategy and culture conditions allow for optimal preservation of the lymphatic endothelial phenotype in vitro. Recently, it was found that the gene expression profiles of cultured endothelial cells differed to some extent when compared with cells that had been isolated ex vivo [60, 61]. Nonetheless, this has not hindered the use of cultured endothelial cells because it is technically challenging to complete sets of experiments with very limited numbers of cells typically obtained by ex vivo methods.

Gene Profiling of Normal and Tumor-Associated Endothelial Cells

The purification of LECs from BECs has enabled gene profiling studies to be conducted in both normal and pathological settings [61, 62]. In the normal setting, the molecular signature of LECs appears to reflect their unique functional characteristics, providing novel insight into the molecular basis of lymphatic function [45, 46, 62]. Genes implicated in protein metabolism, sorting, and trafficking were found to be significantly upregulated in LECs compared with BECs [62]. In particular, genes with high representation were those encoding proteins that control specificity of vesicle targeting and fusion, such as members of the SNARE family, rab GTPases, AAA ATPases, and sec-related proteins,[10] indicating the existence of a robust vesicular transport system. The lymphatic endothelium is characterized by an abundance of membrane invaginations and cytoplasmic vesicles often observed by electron microscopy, yet their functional significance has not been established [19].

Intercellular clefts are considered to be a major pathway for the movement of fluid and proteins into the lymphatics. The data from these profiling studies suggest that, in addition to intercellular transport, transendothelial pathways may also be employed as a mechanism for entry of molecules into the lymphatics, raising the possibility that lymphatics may have the capacity to selectively remove molecules from the interstitium and thereby actively control the composition of lymph and interstitial fluid in vivo [62]. Other differences in gene expression between LECs and BECs include genes encoding proinflammatory cytokines and chemokines, as well as genes implicated in cytoskeletal remodeling and cell–matrix interactions, reflecting the differences in the organization of the actin cytoskeleton between the two cell types [45].

To gain insight into the process of hemotogenous and lymphogenous metastasis, an extensive profiling analysis was performed on ECs isolated from tumors to identify genes that were differentially expressed by tumor ECs. In the case of tumor angiogenesis, ECs from various tumors were purified using specific markers and were then compared with normal ECs [63]. It was found that normal and tumor blood endothelia were highly related, sharing many endothelial cell-specific markers. Of more than 170 transcripts predominantly expressed, 79 were differentially expressed, including 46 that were specifically elevated in tumor-associated endothelium. Several of these genes encode extracellular matrix proteins, but the majority were of unknown function. Most of these tumor endothelial markers were expressed in a wide range of tumor types, as well as in normal vessels associated with wound healing and corpus luteum formation [63].

Likewise, invasion of lymphatic vessels is a key step in the metastasis of primary tumors to the sentinel lymph node. To further understand this process, the RNA profiles of tumor LECs isolated from the vasculature of normal tissue and from highly metastatic tumors were compared. Many of the genes that were found to be differentially expressed were those that code for components of endothelial junctions, subendothelial matrix, and vessel growth and patterning [64]. The tumor LEC profile is distinct from that of normal and was characterized by elevated expression of such functionally significant molecules as the tight junction regulatory protein endothelial-specific adhesion molecule (ESAM), the transforming growth factor-beta coreceptor endoglin (CD105), the angiogenesis-associated leptin receptor, and the immunoinhibitory receptor CD200, and reduced expression of subendothelial matrix proteins including collagens, fibrilin, and biglycan [64]. Hence, gene profiles of blood and lymphatic endothelia purified from tumor and normal vessels are distinct at the molecular level, a finding that may have significant implications for the development of antiangiogenic and lymphangiogenic therapies.

MOLECULAR REGULATION OF TUMOR LYMPHANGIOGENESIS

VEGFs are critical regulators of blood and lymphatic vessel formation during both development and disease.

[10] The primary role of SNARE proteins is to mediate fusion of cellular transport vesicles with the cell membrane or with a target compartment (such as a lysosome).The Rab family of proteins is a member of the Ras superfamily of monomeric G proteins. Rab GTPases regulate many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. These processes make up the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled. AAA or AAA+ is an abbreviation for **A**TPases **A**ssociated with diverse cellular **A**ctivities. These proteins are involved in a range of processes, including protein degradation, membrane fusion, microtubule severing, peroxisome biogenesis, signal transduction, and the regulation of gene expression. The characteristic of AAA proteins is the coupling of chemical energy by ATPase, provided by ATP hydrolysis. Sec-related proteins are membrane components of the secretory pathway and protein-secreting ATPase complex, also known as *translocon*, responsible for the secretion of proteins into the extracellular milieu.

The VEGF family members are secreted, dimeric glycoproteins of approximately 40 kDa. In mammals, five VEGF ligands (VEGF-A, -B, -C, -D, and placenta growth factor [PlGF]) have been identified thus far, with structurally related proteins existing in parapoxvirus (VEGF-E) and snake venom (VEGF-F). These VEGFs mediate their effects by binding in an overlapping pattern to three receptor tyrosine kinases:[11] VEGF receptor (VEGFR)-1, -2, and -3 (Figure 22.4).

Structurally, the VEGFs have intrachain and interchain disulfide bonds among eight cysteine residues in conserved positions and form homodimers. The crystal structures of VEGF-A, VEGF-B, and PlGF have been solved to date [65–67]. The bioactivity of VEGF family members appears to be regulated by proteolytic processing, enabling specific interactions with different types of receptors [68]. The VEGFRs belong to the receptor tyrosine kinase superfamily and contain an extracellular domain of ~750 amino acids organized into seven immunoglobulinlike folds, followed by a single transmembrane region, a juxtaposed membrane domain, a tyrosine kinase domain, and a C-terminal tail (Figure 22.4). Guided by the binding of ligands, the VEGFRs are able to form both homodimers and heterodimers, which are then accompanied by activation of the receptor tyrosine kinase activity, leading to autophosphorylation of the receptors; phosphorylated receptors then recruit interacting proteins and induce specific signaling cascades [68].

VEGF-C and VEGF-D have been identified as specific lymphangiogenic factors, acting via VEGFR-3, which are expressed on LECs [69]. Mice deficient in VEGF-C fail to develop a functional lymphatic system, and transgenic expression of soluble VEGFR-3 results in pronounced lymphedema [27, 48]. In animal tumor models, VEGF-C and VEGF-D increase tumor-associated lymphangiogenesis and lymphatic metastasis [34–36], and expression levels of VEGF-C and/or VEGF-D have been shown to correlate with metastasis in a large number of human tumor types [70]. Much of our understanding of the development and growth of lymphatic vessels has been a result of the discovery of molecules that promote lymphatic vessel growth; the VEGF-C/VEGF-D/VEGFR-3 signaling axis is believed to play a pivotal role in the control of lymphangiogenesis during development and disease. The components of this pathway are highlighted in the following section. In addition, non-VEGF family growth factors that have been shown to be involved in the developmental and pathological formation of lymphatic vessels are also described.

[11] *Receptor tyrosine kinases* are single transmembrane domain proteins with an intracellular tyrosine kinase domain that, when activated upon ligand binding, transfers a phosphate group from ATP onto a tyrosine residue. Phosphorylation of proteins by kinases is an important mechanism in signal transduction for regulation of enzyme activity. Receptor tyrosine kinases are particularly important because some are promising therapeutic targets for the treatment of cancer.

VEGF-C/VEGF-D/VEGFR-3 SIGNALING AXIS

VEGF-C

The first lymphangiogenic growth factor to be discovered was VEGF-C [71, 72]. VEGF-C expression does not appear to be regulated by hypoxia but is increased in response to proinflammatory cytokines [73–75]. VEGF-C is synthesized as a proprotein that undergoes subsequent proteolytic processing. The mature form of VEGF-C consists of dimers of the central VEGF homology domain (VHD) and contains binding sites for VEGFR-2 and VEGFR-3 (Figure 22.4) [4, 76]. Unprocessed VEGF-C binds to VEGFR-3; the stepwise proteolytic processing of VEGF-C generates several forms that have sequentially increased affinity for both VEGFR-2 and VEGFR-3 [4]. As VEGFR-2 can be broadly expressed on endothelia, and VEGF-C can be expressed in a range of tissues, the synthesis of VEGF-C as a proprotein may prevent unnecessary angiogenesis induced via VEGFR-2 and allows VEGF-C to signal preferentially via VEGFR-3, which is restricted to the venous endothelium during later stages of development and adult life [17]. In certain circumstances, proteolytic processing would release mature VEGF-C, which signals via both VEGFR-3 and VEGFR-2, to promote angiogenesis and lymphangiogenesis [73].

VEGF-C-deficient mice lack lymphatic vessels, resulting in prenatal death from fluid accumulation in tissues [27]. In these animals, endothelial cells commit to the lymphatic lineage but are unable to sprout to form lymphatic vessels. The lack of sprouting in these mutants could be rescued by VEGF-C or VEGF-D but not VEGF-A, indicating VEGFR-3 specificity [27]. The recent discovery of a lymphatic system in *Xenopus* tadpoles and in developing zebrafish provided additional models in which to examine lymphangiogenesis [77–79]. Using the zebrafish model, researchers demonstrated that lymphatic development was mediated via the VEGF-C/VEGFR-3 axis in species other than mammals [80].

VEGF-C has been shown to stimulate the migration of endothelial cells and to induce vascular permeability and endothelial cell proliferation. In blood vessels, these biological effects are thought to be mediated predominantly by VEGFR-2 signaling, although VEGFR-3 can be upregulated on BECs and contribute to angiogenesis [81]. The lymphangiogenic effect of VEGF-C is thought to be mediated predominantly via VEGFR-3 signaling, although VEGFR-2 can be expressed on LECs at low levels and could play a role in lymphangiogenesis [4, 82]. Because the fully processed form of VEGF-C

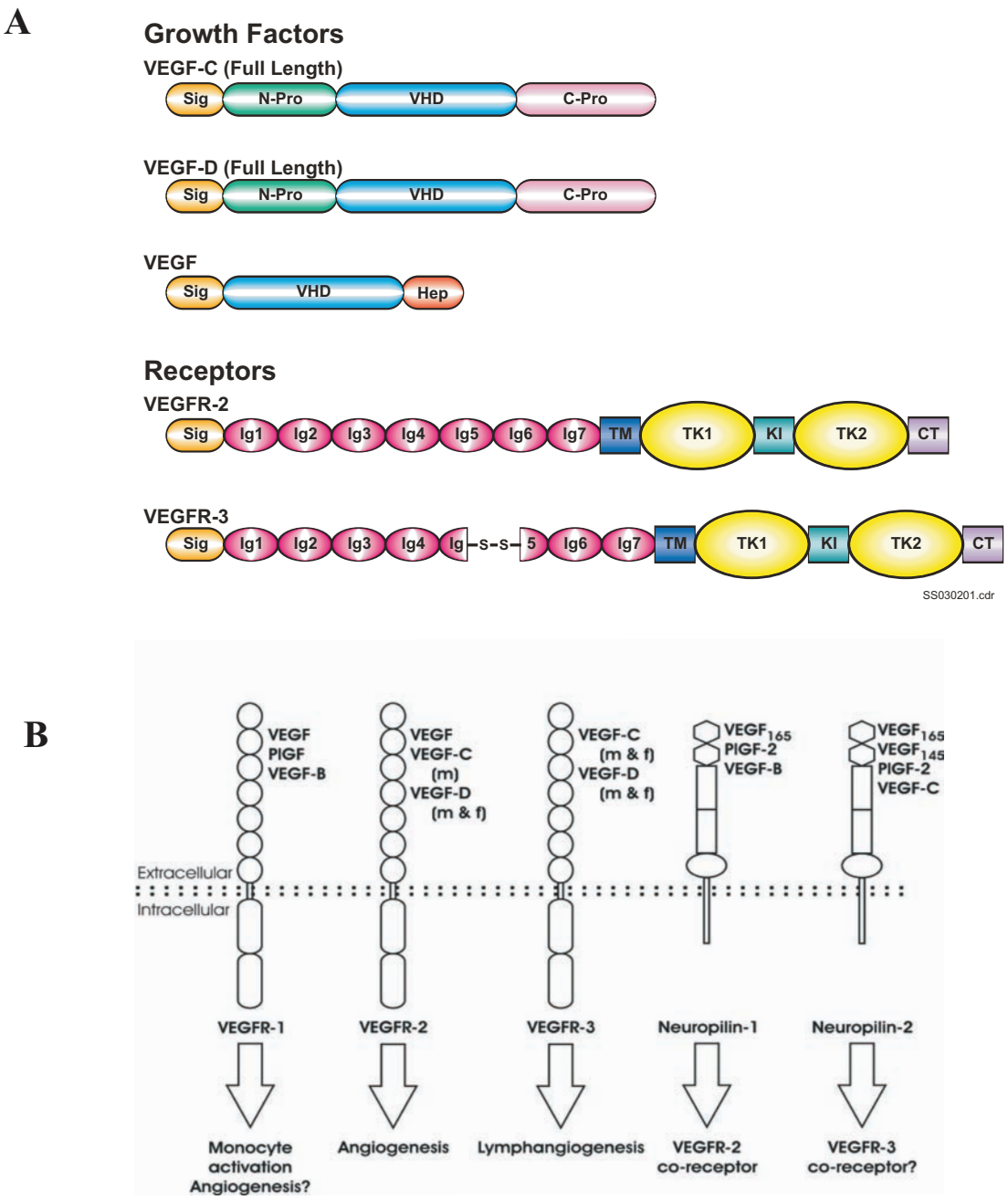

Figure 22.4. Structure and interaction of the VEGFs with receptors. (**A**) The VEGFs are secreted homdimeric proteins that contain a hydrophobic amino-terminal signal sequence for secretion (sig). All members contain a central region known as the VEGF homology domain (VHD). VEGF-C and VEGF-D are the two members implicated in lymphangiogenesis; VEGF-A is shown for comparison. The receptors for VEGF-C and -D are VEGFR-2 and VEGFR-3. These receptors are structurally related and consist of an extracellular domain with seven immunoglobulinlike domains (Ig), a transmembrane domain (TM), a split tyrosine kinase domain (TK) with a kinase insert sequence (KI), and a cytoplasmic tail (CT) at the carboxy-terminus. (**B**) VEGFs bind to three receptors, VEGFR-1, VEGFR-2, and VEGFR-3. Mature and full-length forms of VEGF-C and VEGF-D are denoted "m" and "f," respectively. VEGFR signaling is modulated by different coreceptors, such as the neuropilins. Upon ligand binding, the proposed function is listed.

activates VEGFR-2 on blood vessels, VEGF-C can regulate both physiological and pathological angiogenesis, as observed in mouse cornea and limb ischemia [83, 84]. In addition, it can induce lymphangiogenesis in various models [75, 85] and promote lymphatic vessel enlargement when overexpressed in the skin [72] and was found to promote a dose-dependent enlargement and tortuosity of veins, which, along with the collecting lymphatic vessels, were found to express VEGFR-2 [86].

VEGF-D

The second lymphangiogenic growth factor to be discovered was VEGF-D, which was first named "c-fos-induced growth factor" [5]. The mature form of VEGF-D, consisting of dimers and the central VHD, shares a 61 percent amino acid sequence identification with VEGF-C [87]. VEGF-D can be proteolytically cleaved at the N- and C-terminal regions of the VEGF homology domain. The processing of VEGF-D is required to produce a growth factor that binds both VEGFR-2 and VEGFR-3 with high affinity [88]. The enzymes responsible include members of the proprotein convertase (PC) family and the serine protease, plasmin, which has been shown in vitro to cleave both the N- and C-terminal propeptides from the human VEGF-D [89, 90]. The capacity of VEGF-C and VEGF-D to promote tumor growth was inhibited when the proteolytic cleavage sites were abolished, demonstrating that processing of these proteins is important for their biological effects [91, 92; Harris and Achen, unpublished).

Interestingly, in mice, VEGF-D binds only to VEGFR-3, implying that VEGF-D may have a somewhat different function in mice and in humans [93]. Unlike VEGF-C, a specific role for VEGF-D in murine lymphatic development has not yet been demonstrated [27, 94]. However, in *Xenopus* tadpoles, it was shown that VEGF-D may have a "modifier" role in lymphatic development, in particular in LEC migration [95]. Although these findings are yet to be validated in mammals, the mechanisms guiding the development of the lymphatic system seem to display a high degree of conservation within higher vertebrates [96].

Viral-mediated delivery of the mature form of human VEGF-D induced predominantly angiogenesis in rat muscle but both angiogenesis and lymphangiogenesis in rat skin, suggesting that the biologic effects of VEGF-D may depend on the abundance of blood vessels and lymphatics expressing the receptors for VEGF-D in a given tissue [97]. The mature form of VEGF-D promoted both angiogenesis and lymphangiogenesis in rabbit muscle, whereas the full-length form was specifically lymphangiogenic in this model system, indicating that differently processed forms of VEGF-D may be used to generate distinct biological responses relevant to different clinical conditions [98]. Importantly, VEGF-D was found to promote lymphangiogenesis and metastatic spread via the lymphatics in a mouse tumor model [36]. In human breast cancer samples, tumor vessels that were positive for VEGF-D protein were also positive for VEGFR-2 and/or VEGFR-3 but negative for VEGF-D mRNA, indicating that VEGF-D secreted by tumor cells subsequently associates with the endothelium via VEGFR-2- and/or VEGFR-3-mediated uptake, thereby promoting tumor angiogenesis, lymphangiogenesis, and metastatic spread by a paracrine[12] mechanism [99].

VEGFR-3

VEGFR-3 is a highly glycosylated cell surface receptor tyrosine kinase of approximately 180 kDa. Its cDNA was cloned from human erythroleukemia cells and placental libraries [100]. Two alternatively spliced isoforms of VEGFR-3 have been described, which differ in the length of their cytoplasmic domains and possibly in their signaling properties [101]. Analysis of gene knockouts has implicated VEGFR-3 in the formation and maintenance of the lymphatics (as discussed earlier).

VEGFR-3 forms homodimers or heterodimers with VEGFR-2 in response to binding of proteolytically processed VEGF-C [102]. Importantly, the dimerization partner directs the use of potential phosphorylation sites that may reflect different substrate specificities of kinases [68]. VEGFR-3 was also shown to mediate activation of particular pathways that might be important during embryonic and tumor vessel development [48]. In addition, signal transduction by VEGFR-3 is also modulated by coreceptors such as the neuropilins, namely neuropilin-2. The crucial role of this interaction has been shown by the phenotype of *Neuropilin-2*$^{-/-}$ mice, which fail to form normal lymphatic vessels and capillaries [103].

Expression of VEGFR-3 correlates with lymphatic metastasis in some prevalent forms of human cancer. For example, it has been reported that the presence of VEGF-D and VEGFR-3 in endometrial carcinoma may be a prognostic indicator for lymph node spread [104]. Expression of VEGFR-3 by LECs in human prostate cancer is also thought be important for metastatic spread of tumor cells to the lymph nodes [105].

It is plausible that the relative expression levels of VEGFR-2 and VEGFR-3 in blood vascular and lymphatic endothelia may influence whether VEGF-C or VEGF-D elicit predominantly angiogenic (via VEGFR-2 activation) or lymphangiogenic (driven by VEGFR-3) effects. The dissection of VEGFR-2 and VEGFR-3 signaling pathways in terms of angiogenesis and lymphangiogenesis is complicated by observations that VEGFR-3 is involved in maintenance of tumor blood vessels, and that VEGFR-2 expression is detectable on lymphatic endothelial cells [47, 59, 81, 106]. Furthermore, other positive and negative regulators of angiogenesis and lymphangiogenesis may modulate the biological consequences of VEGF-C and VEGF-D expression [53].

[12] *Paracrine signaling* is a form of cell signaling in which the target cell is near the signal-releasing cell.

VEGF-A/VEGFR-2 Signaling

VEGF-A has been identified as the predominant tumor angiogenesis factor in many human and experimental murine cancers, acting via VEGFR-1 and VEGFR-2, expressed on endothelial cells lining blood vessels [107]. Recently, it has been shown that lymphatics, similarly to their blood vessel counterparts, also express VEGFR-2, one of the receptors for VEGF-A [46]. VEGF-A expression in various tumors has led to increased lymphangiogenesis. For example, when transgenic mice overexpressing VEGF-A in the skin were subjected to a chemically induced skin carcinogenesis regime, active proliferation of VEGFR-2–expressing tumor-associated lymphatic vessels, as well as tumor metastasis to the sentinel and distant lymph nodes, were observed [3]. Likewise, overexpression of VEGF-A in murine fibrosarcomas induced the growth of lymphatic vessels, with metastases in lymph nodes frequently detected [108], and neutralizing VEGF-A was shown to enhance lymphatic vessel density and lymph node metastasis in an orthotopic breast tumor model [109] but not in other tumor models [110, 111]. The variable effect of VEGF-A on tumor lymphangiogenesis in animal models may depend on the level of VEGF-A expression, the splice variant of VEGF-A being expressed in the model, or the abundance of VEGFR-2 on nearby lymphatic vessels.

OTHER SECRETED GROWTH FACTORS

In addition to the VEGF family members, other secreted growth factors have been shown to induce lymphangiogenesis in either a VEGFR-3–dependent or –independent manner and, in some cases, to promote lymph node metastasis. A comparative analysis of the gene expression of purified LECs versus BECs revealed that LECs express significantly higher levels of hepatocyte growth factor receptor (HGFR). Whereas little or no HGFR expression was detected by lymphatic vessels on normal tissues, HGFR was strongly expressed by activated lymphatic vessels in inflamed skin. Transgenic or subcutaneous delivery of HGF, the ligand for HGFR, promoted lymphatic vessel formation in mice, whereas systemic blockade of HGFR inhibited lymphatic function [112]. HGF was found to promote peritumoral lymphangiogenesis when expressed in a transgenic model of breast tumorogenesis, although lymph node spread was not observed [113].

Platelet-derived growth factor (PDGF)-BB was shown to have lymphangiogenic activity in vitro and in vivo; expression of PDGF-BB in a mouse tumor model induced lymphangiogenesis and promoted lymph node metastasis [114]. The insulinlike growth factors (IGFs)-1 and -2 have also been demonstrated to promote lymphangiogesis in vivo [115]. More specifically, activation of IGF-1 receptor in a Lewis cell carcinoma model increased expression of VEGF-C and lymph node spread, suggesting that this pathway can serve as a positive regulator of VEGF-C and lymph node metastasis [116]. With regard to expression, IGFs are expressed in many human tumors, and their expression is correlated with metastatic spread and poor prognonsis [117]. Likewise, fibroblast growth factor (FGF)-2 stimulated both angiogenesis and lymphangiogenesis and caused an upregulation of VEGF-C. The effect of FGF-2 can be ablated by treatment with anti-VEGFR-3 antibodies [118]. These lymphangiogenic growth factors, in addition to the VEGFs, may serve as new targets for therapeutics designed to inhibit pathological lymphangiogenesis.

LYMPHATIC VESSELS AND LYMPHANGIOGENIC GROWTH FACTORS IN CANCER

A common finding for several solid tumors, including carcinomas, is the spread of tumor cells through the lymphatic network. By actively inducing the growth of "neolymphatics," tumor cells gain accessibility to the lymphatic network and, ultimately, to regional lymph nodes [55, 119]. The detection of tumor cells in local lymph nodes is a significant factor in tumor staging and for designing treatment protocols [70]. It has become clear that peritumoral lymphatics,[13] which are often enlarged, are associated with the metastatic propensity of human tumors, whereas the importance of intratumoral lymphatics[13] has remained in doubt. Although intratumoral lymphatics have been detected in human cancers, including cervical and ovarian cancer and melanoma, it was believed that these vessels would be collapsed, because of the high physical pressure within tumors, and therefore would be nonfunctional [120]. The functional significance of intratumoral lympahtics in metastatic spread remains in question [121].

Lymphatic vessel density has been shown to be prognostically significant in several malignancies [122, 123]. In addition to lymphatic vessel density being shown to be predictive of lymph node metastasis, the lymphangiogenic growth factors themselves have also been demonstrated to be important prognostic indicators in several key human tumors [70]. For example, in malignant melanoma, lymphatic vessel density – in particular, peritumoral lymphatics – and expression of the lymphangiogenic growth factors were important in determining which tumors metastasize to regional lymph nodes [124, 125].

There is ample clinical evidence for expression of VEGF-C in human tumors. VEGF-C expression has been detected in breast, colon, lung, thyroid, gastric,

[13] Lymphatics associated with tumors are often referred to as *peritumoral lymphatics*, those that are located at the tumor periphery, or *intratumoral lymphatics*, those that are located within the tumor.

and squamous cell cancers, and in mesotheliomas, as well as in neuroblastomas, sarcomas, and melanomas. The expression of VEGF-C correlates with lymph node metastasis and poor survival outcomes, as seen for head and neck squamous cell carcinoma and thyroid and lung carcinomas [126–129]. For example, in gastric carcinoma, VEGF-C was shown to be predictive of enhanced lymphatic vessel density, lymphatic invasion, and metastasis and poor prognosis [130–133].

The presence of VEGF-D in tumors has also been investigated. VEGF-D expression was shown to be significantly higher in breast carcinomas that were associated with increased lymph node metastasis, was independently related to a reduction in disease-free survival, and was predictive of both lymph node metastasis and direct peritoneal spread [134]. In uterine endometrial carcinoma, expression of VEGF-C correlated with tumor invasion via the lymphatics and lymph node metastasis and, in addition, the presence of VEGF-D and VEGFR-3 was associated with reduced patient survival [104, 135]. Colorectal carcinoma is a major cause of adult morbidity and mortality, despite public awareness and screening measures. Immunohistochemical staining using lymphatic-specific markers has shown that increased lymphatic vessel density is positively correlated with metastasis to regional lymph nodes and metastasis to the liver [136]. In particular, VEGF-C and VEGF-D expression is associated with lymph node metastasis and poor disease outcome, and VEGF-D was considered an independent prognostic indicator of poor disease-free and overall survival in patients with colorectal carcinoma [137, 138]. Interestingly, in lung adenocarcinoma and head and neck squamous cell carcinoma, VEGF-D expression was inversely correlated with lymph node metastasis [126, 127]. The reason why VEGF-D is associated with metastasis in some tumors but not in others remains unclear [70].

Overall, a causal relationship exists between increased tumor lymphatic density and tumor cell dissemination to lymph nodes, reflecting a possible correlation between the level of the lymphangiogenic growth factors VEGF-C and VEGF-D in tumors with the likelihood of metastatic disease. It is therefore feasible that lymphatic vessel density and the presence of the lymphangiogenic growth factors may make a clinically useful addition to traditional histological analysis, for prognostication and in identifying patients who could be "upstaged" to a staging category in which more aggressive adjuvant treatment is required.

ROLE OF LYMPHANGIOGENESIS IN CANCER SPREAD

Although the presence of lymphatic vessels in clinical samples is suggestive of their relevance in metastatic disease, the question as to whether they are an active component of the metastatic pathway remains unresolved. The discovery of lymphangiogenic growth factors and their cognate receptors has enabled the development of rodent models, providing compelling evidence that tumor lymphangiogenesis facilites lymphatic metastasis. For example, a xenograft[14] model based on the MCF-7 human breast cancer cell line demonstrated that VEGF-C expression led to increased peritumoral and intratumoral lymphatics, which strongly correlated with increased rates of lymph node metastasis. A similar finding of increased intratumoral and peritumoral lymphatic vessels and an increase in regional lymph node spread was observed in an alternative VEGF-C–overexpressing orthotopic[15] model of breast cancer [35]. Significant angiogenesis did not occur in either setting, presumably because of the predominant production of the unprocessed form of VEGF-C that preferentially binds to and activates VEGFR-3 (i.e., inducing lymphangiogenic signaling).

In contrast, a mouse xenograft tumor model, based on cells expressing human VEGF-D, exhibited not only enhanced tumor lymphangiogenesis and lymph node metastasis but also an increase in angiogenesis and solid tumor growth rate, which could be inhibited with a neutralizing antibody to VEGF-D [36]. The differences between the tumor angiogenic properties of VEGF-C and VEGF-D in these tumor models might have been due to different degrees of proteolytic processing of these growth factors in different models [70]. In a transgenic[16] mouse model of pancreatic cancer, overexpression of VEGF-C in the β-cells of the pancreatic islets increased lymphangiogenesis around the primary tumor and enhanced spread to the draining lymph nodes [34]. In a similar model system, VEGF-D induced peritumoral lymphangiogenesis, with lymphocyte accumulations and hemorrhages, and lymph node and lung metastases [139]. Importantly, VEGF-C-induced lymphangiogenesis and lymph node spread were abrogated when a soluble form of VEGFR-3 was produced by transfected tumor cells or delivered systemically, further supporting the role of the lymphatics in tumor spread [33, 140]. VEGF-A was able to promote

[14] *Xenotransplantation* (*xeno-* from the Greek meaning "foreign") is the transplantation of living cells, tissues, or organs from one species to another, such as human to immunocompromised mouse. Such cells, tissues, or organs are called *xenografts*.

[15] Cells, tissue, or organ grafts may be transplanted to their normal position in the recipient and are then known as *orthotopic*. In an orthotopic model of breast cancer, breast cancer cells are transplanted to the chest cavity of recipient mice.

[16] A transgenic mouse contains additional, artificially introduced genetic material in every cell or specific cells (e.g., skin). This often confers a gain of function – for example, the mouse may produce a new protein, but a loss of function may occur if the integrated DNA interrupts another gene. Transgenic mice are used to model human diseases that involve the overexpression or misexpression of a particular protein.

tumor lymphangiogenesis, as well as angiogenesis, apparently signaling via an upregulated VEGFR-2 on LECs when squamous cell carcinoma was induced in a transgenic mouse overexpressing VEGF-A in the skin [3].

Collectively, data generated from animal models have highlighted the importance of the lymphatic system and the lymphangiogenic growth factors in metastasis. Overall, lymphangiogenic growth factors might enhance metastasis by increasing the number of lymphatic vessels in and around the tumor, resulting in an increase in the contact surface area between the invading cancer cells and the lymphatic endothelium. In addition, activation of the lymphatic endothelium by tumor-cell–secreted factors may change the adhesive properties of the lymphatic endothelium, promoting tumor-cell–LEC interactions or leading to an increase in lymphatic vessel size, thus facilitating entry of tumor cells into the lymphatics [18]. VEGF-C or VEGF-D secreted by tumor cells might also increase vascular permeability, or have important effects on the tumor interstitial fluid pressure, which may promote tumor cell entry into the lymphatics, as well as into the veins [18, 70, 141].

TARGETING THE VEGF-C/VEGF-D/VEGFR-3 PATHWAY FOR CLINICAL BENEFIT

Regardless of advances in surgery, chemotherapy, and radiotherapy, the prognosis of many cancers remains poor, highlighting the requirement for new antimetastatic therapies. Therapeutic approaches for targeting the signaling of soluble growth factors, such as VEGF-C and VEGF-D, via their cognate receptor tyrosine kinases, such as VEGFR-3, include monoclonal antibodies, soluble receptors, small-molecule inhibitors, peptide drugs, and antisense techniques. Such approaches targeting VEGF-C/VEGF-D/VEGFR-3 have the potential to block the lymphogenous spread of cancer as well as the contribution that this signaling axis makes to tumor angiogenesis, tumor growth, and hematogenous metastasis [142]. The use of humanized anti-VEGF-A monoclonal antibody (bevacizumab [Avastin]), designed as an antiangiogenic agent, for the treatment of metastatic colorectal carcinoma has proved successful [143, 144]. Analogous to VEGF-A blockade to reduce tumor angiogenesis, an approach to block VEGF-C and/or VEGF-D binding to VEGF receptors would be promising for the inhibition of tumor lymphangiogenesis and lymphogenous metastasis, as well as for restricting tumor angiogenesis. A neutralizing VEGF-D antibody (designated VD1), which blocks the interaction of VEGF-D with VEGR-2 and VEGF-3, inhibited angiogenesis, lymphangiogenesis, and metastatic spread to lymph nodes in a mouse xenograft tumor model [36, 145].

An alternative approach to neutralizing antibodies is a soluble version of the extracellular domain of VEGFR-3 that would sequester VEGF-C and VEGF-D. Regression of tumor-induced lymphatic vessels was achieved through the administration of soluble VEGFR-3 [140, 146]. Further evidence of the therapeutic potential of soluble VEGFR-3 was provided by transgenic expression of soluble VEGFR-3 in mouse skin, which inhibited fetal lymphangiogenesis and induced regression of lymphatic vessels that were already formed, though the blood vasculature was unaffected [48]. Adenoviral-mediated delivery of the soluble receptor domain blocked the growth of peritumoral lymphatic vessels in a mouse model of breast cancer and inhibited lymph node spread, but not lung metastasis, in a lung cancer model [140]. In addition to VEGFR-2, VEGFR-3 was found to be highly expressed on sprouting blood vessels, and treatment of tumors with a combination of blocking VEGFR-2 and VEGFR-3 antibodies resulted in additive inhibition of angiogenesis and tumor growth. The presence of VEGFR-3 on both tumor blood vessels and lymphatic vessels makes it an attractive target for inhibiting not only lymphangiogenesis but also angiogenesis, two important contributors to tumor growth and dissemination [81].

Another approach for inhibiting tumor lymphangiogenesis would be targeting the processing of the lymphangiogenic growth factors. Angiogenesis and lymphangiogenesis induced by tumors expressing VEGF-C and/or VEGF-D could be inhibited by targeting the enzymes responsible for activating these proteins, such as plasmin and members of the proprotein convertase family of proteases. [90, 91, 147, 148]. The enzymes responsible include the PCs and the serine protease, plasmin [89]. One possibility could be the use of monoclonal antibodies to VEGF-C and VEGF-D that block access of the proteases to these growth factors.

Small-molecule inhibitors that enter the cell and inhibit the tyrosine kinase activity of VEGFR-2 and VEGFR-3 have already been developed. The potential of such approaches has been illustrated by studies showing that inhibitors of the VEGFR-2 tyrosine kinase block tumor angiogenesis in several animal models [149]. Clinically approved small molecule inhibitors of VEGFR-2 and VEGFR-3 include sorafenib (Nexavar), which has been used in the treatment of advanced renal cell and hepatocellular carcinomas, and sunitinib (Sutent), which may offer targeted treatment in select tumors such as carcinoma of the lung, renal cell carcinoma (RCC), and gastrointestinal carcinoma [150, 151]. Other small-molecule inhibitors that target VEGFR-2 and VEGFR-3 pathways, also currently involved in clinical cancer treatment trials for cancer, include XL999, CEP-7055, PTK 787/ZK 222584, and BAY 43–9006 [152–153].

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although angiogenesis has been studied extensively, lymphangiogenesis is still a relatively new field in vascular biology. Recent studies have identified several new LEC-specific molecules, and based on gene knockout studies, many of these molecules, such as PROX-1, VEGFR-3, and podoplanin, are also functionally important for the lymphatic vasculature. The identification of molecules that are involved in the formation of lymphatic vessels, lymphatic-specific markers, and techniques for purifying and culturing LECs has enabled an understanding of the role lymphatics play in development and disease. Furthermore, the discovery of growth factors VEGF-C and VEGF-D, as well as other lymphangiogenic mediators, and the signaling pathways that specifically promote lymphangiogenesis has led researchers to better understand the significance of lymphatic vessels in facilitating tumor cell dissemination. Studies of blocking the VEGF-C/VEGF-D/VEGFR-3 signaling axis have shown that targeting this axis leads to inhibition of tumor lymphangiogenesis and of the metastatic spread of tumor cells via the lymphatics.

Although VEGFR-3 and its ligands have been studied extensively during embryonic and growth-factor–driven lymphangiogenesis, as well as in tumor models, knowledge of the function of the VEGFR-3 signal transduction system in other physiological and pathological processes is only superficial. Moreover, it is apparent that other signaling pathways are likely to contribute to lymphangiogenesis and it would be of significance to understand the interplay among these pathways. It is unclear at present what the contribution of lymphatic vessel subtypes is in lymphatic function and, more importantly, in disease states such as cancer. Analysis of a profile of lymphatic markers combined with morphological, structural, and molecular distinctions may facilitate the study of the biology of individual lymphatic vessel subtypes. Understanding the complexities of lymphatic development, anatomy, and pathophysiology in terms of lymphangiogenic growth factors and signaling cascades may yield novel therapeutic targets for the treatment of human pathologies such as cancer.

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23 Introduction to Clinical Research

Nancy E. Davidson

It is axiomatic that the most critical and life-threatening quality of many cancers is the ability to metastasize. Similarly, the biggest challenge for treatment of many malignancies is the successful management of metastatic disease. Indeed, the presence or absence of distant metastases is the single most important determinant of survival for most patients with cancer, as manifested by the prominent role that metastases play in the current staging system for cancer.

The past several decades have seen a wholesale increase in our understanding of the basic biology of the metastatic cascade, as discussed in Chapters 1–22 in this volume. In addition, improved therapy for the primary cancer can, paradoxically, open the opportunity for symptomatic metastatic disease to become manifest in some cases. It is also clear that the widespread availability of enhanced imaging capabilities means that today it is easier to diagnose metastatic disease – and, perhaps, at a seemingly earlier time. Finally, despite the commonality of certain mechanisms for metastases, there is much that is disease specific in both the biology and treatment of metastatic cancer. Think for a minute about the predilection of prostate cancer for the bone and ocular melanoma for the liver. But there is also much that is cross-cutting, such as the biology and treatment of bony metastases from several primary tumor types.

In the following pages, experts from a variety of disease orientations summarize findings and strategies in several important cancer types. From these chapters, tailored to individual cancer types, come several unifying themes. First is the understanding that many types of cancer acquire the ability to metastasize at a very early time in their natural history, perhaps well before the primary tumor is detectable by conventional means. This finding is at the core of some current controversies about the utility of screening for early detection for some cancers. A second theme is our concern about whether the metastases faithfully recapitulate the primary tumor, or even resemble each other with regard to biological characteristics and sensitivity to systemic treatments. A third theme revolves around our uncertainty about whether all metastases are created equal – the important concepts of stem cells, dormancy, and heterogeneity must be more fully elucidated to answer this question.

Practical implications for the patient and the health care provider (and society) also follow naturally. What is the role of early detection for malignancies? What is the role of early diagnosis of metastatic disease after the seemingly successful treatment of a primary cancer? Are the treatments for a primary cancer also useful for a metastasis, or can metastasis-specific interventions be envisioned? Finally, is prevention or eradication of metastasis a requirement for the successful treatment of a malignancy, or can we imagine that control of metastatic disease can be achieved for some patients through the use of chronic therapy with acceptable toxicity? These are the questions for the next decade of the twenty-first century in cancer biology and medicine.

24 Sarcoma

Chand Khanna and Lee Helman

PATTERNS OF METASTATIC SPREAD, ORGAN SPECIFICITY, AND TIMING OF RECURRENT DISEASE

Sarcomas are a large and highly heterogeneous family of cancers that share presumptive origins from the mesoderm or endoderm; however, the precise cell of origin for many sarcomas remains unclear [1]. Indeed, an increasingly attractive hypothesis in the field suggests that sarcomas may arise from mesenchymal stem cells [2]. The heterogeneity seen in the family of tumors described as sarcomas may therefore begin as a product of distinct populations of mesenchymal stem cells (defined by maturity, lineage differentiation, and tissues of origin) that are permissive to a variety of oncogenic events. For many sarcomas, specific cancer-associated genes have been defined [3]. These include sarcoma-specific translocations that result in oncogenic fusion genes believed to be necessary for malignant transformation [1, 4, 5]. In sarcomas in which translocations are not present, a complex karyotype is often present, in which driving oncogenic events have been more difficult to define. The biological diversity of the sarcoma family predicts their clinical diversity (Figure 24.1). Sarcomas can be seen in all ages, including pediatric, adult, and geriatric patients. Sarcomas may develop in any organ system and in all anatomic locations. Not surprisingly, and pertinent to this chapter, sarcomas as a family are also associated with a diversity in metastatic biology, and in their responses (or lack of response) to various treatment modalities. As is the case with most solid tumors, the development of metastatic disease in sarcoma patients is the most common cause of death. Patients with sarcomas require new therapies directed at preventing or treating metastasis to attain improved outcomes [6].

Timing of Metastatic Progression

For most sarcomas, the timing of metastatic spread is believed to be early in the course of the disease. This presumption is based on the fact that despite early, successful, and complete management of the primary tumor for many patients, the risk of distant metastasis remains high. For example, after complete surgical success, the risk of metastasis in patients with synovial sarcoma is 57 percent at fifteen years [7]. An argument for the development of late metastasis is also reasonable in some forms of sarcoma because tumor size and surgical cure can be independent predictors of outcome and reduced metastatic risk. A clinical scenario that supports late metastatic progression comes from abdominal liposarcoma [8]. In the majority of cases, these tumors develop in the abdomen proper or in the retroperitoneal space. Invasion into surrounding tissues often prevents surgical cures. If surgery is successful, the risk for distant metastasis is 54 percent at five years. If surgery is not successful, however, this risk rises to as high as 90 percent, suggesting an ongoing and late development of metastasis in more than 40 percent of patients [8].

Route of Metastasis

The spread of sarcomas to distant sites may occur by the hematogenous or lymphatogenous routes, or by local seeding. The hematogenous route is likely the most common among sarcomas. For osteosarcoma, a pediatric cancer of the bone, the propensity of hematogenous spread is nearly absolute. Indeed, the TNM clinical staging for these cancers does not include lymph node (N) designations [9, 10]. The lymphatogenous route of metastasis is also seen in many sarcomas. Rhabdomyosarcoma – in particular, alveolar

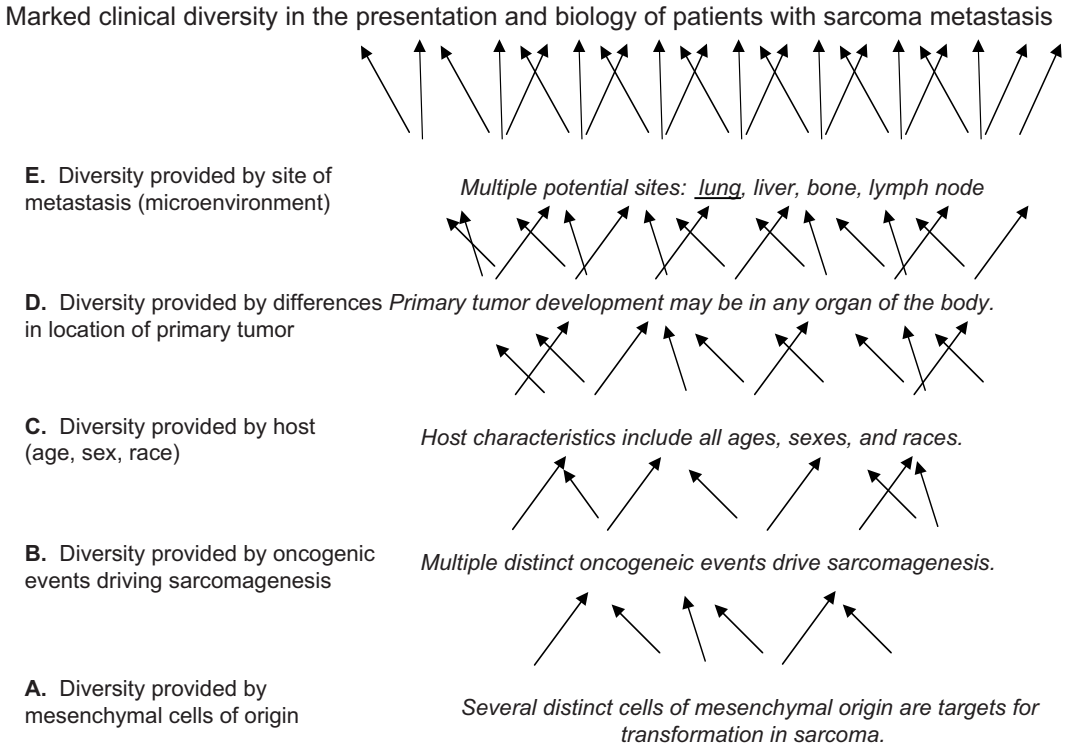

Figure 24.1. The presentation and biology of metastasis in sarcoma patients is characterized by marked diversity. This diversity is explained by heterogeneity in all determinants of metastatic progression, including the tumor, host, and microenvironment. (A) Sarcomas arise from mesenchymal cells. These mesenchymal cells vary based on their lineage, state of differentiation, and tissue of origin. (B) These target cells are receptive or permissive to a vast array of potential oncogenic driving events that are responsible for sarcomagenesis. For the most part, these driving events are not well defined. In some cases, sarcoma-specific translocations that result in oncogenic fusion genes are present and are clearly linked to malignant transformation. (C) Unlike many cancers, sarcomas can be seen in all ages of patients (i.e., host), including pediatric, adult, and geriatric patients. They may develop with nearly equal frequency in males and females and, as a family, can afflict individuals of all racial backgrounds. (D) Sarcomas may develop initially in any organ system and accordingly are influenced by several distinct microenvironments. (E) The most common site of metastatic development for sarcomas is the lung; however, sarcoma metastases may be seen in nearly any organ, including liver, bone, lymph node, and brain. Accordingly, the diversity in metastatic presentation, biology, and therapy for sarcoma patients is predictable.

rhabdomyosarcoma – is classically associated with lymphatogenous spread, with metastasis development occurring in successive lymph nodes within a chain [11]. Malignant fibrous histiocytoma, a sarcoma that may develop in any age of patient and in many primary tumor locations, can disseminate by both lymphatogenous and hematogenous routes. Finally, direct extension or seeding metastasis into third space sites, such as the abdominal peritoneum and omentum or pleural space, may also occur following the development of malignant sarcoma effusions or rupture of intracavitary lesions. For example, tumor rupture in angiosarcoma or leiomyosarcoma is linked to intraabdominal recurrence despite the fact that these patients may be free of gross disease following surgery [8].

Sites of Metastasis

The fact that sarcomas develop at a large diversity of primary tumor sites and that the routes of metastatic progression are numerous predicts the many anatomic sites to which sarcomas may metastasize. The most common locations for metastasis are similar to those for other cancers and include lymph node, liver, lung, and bone. There are no sarcoma-specific or unique sites for metastasis.

Dormancy

Despite the presumed initiation of the process of metastasis early in the course of disease, in most sarcoma patients the clinical manifestation of metastasis at secondary sites may not occur for long periods of time. The period of metastatic dormancy may last for five, ten, or even twenty years in some patients. A review of patterns of metastatic recurrence supports the presence of a prolonged dormancy period in several sarcoma histologies, including osteosarcoma, Ewing sarcoma, and others [12]. The overrepresentation of pediatric sarcomas in a list of cancers associated with dormancy may merely reflect the fact that younger patients have a greater opportunity for longer dormancy than adult

and geriatric patients do. It is also reasonable that the phenotype of dormancy in these pediatric cancers is connected to features of their intrinsic biologies.

Dormancy in sarcoma is as poorly understood as in other cancer histologies [13]. First, it is unclear where in the body these dormant metastatic cells exist. Recent studies support an attractive hypothesis that the bone marrow may be a site in which dormant metastatic cells reside. Support for this hypothesis includes the identification of osteosarcoma cells in the bone marrow of osteosarcoma patients [14]. As bone marrow infiltration is rarely a clinical outcome of progression in osteosarcoma, the finding of cancer cells in the marrow is quite interesting. This hypothetical model reasons that cells would disseminate from the primary tumor to the bone marrow early during oncogenesis. The metastatic cells persist during the period of dormancy in the bone marrow and then subsequently emerge and colonize distant secondary sites at the end of their dormant state. The presumed mesenchymal stem cell origin for sarcomas and their potential proclivity for trafficking to the bone marrow provide additional, albeit circumstantial, support for such a hypothesis. The determinants that result in a break in dormancy are also poorly understood. Experimental data suggest a link between bursts in angiogenesis and breaks in dormancy in sarcoma and other cancer models [15]. The causes for such bursts or the clinical scenarios that may be linked to these events are not understood.

PROBLEM OF METASTASIS IN SARCOMAS

The earliest treatment of most solid tumors included surgery alone. In some sarcomas complete surgical cures can be attained (e.g., wide resection of a low-grade soft tissue sarcoma), whereas for others, surgical cures are less common (e.g., gastrointestinal stromal tumors). For most sarcoma patients, the presence of microscopic metastasis that develops even before the patient presents with a primary tumor is the principal clinical problem that will limit long-term opportunities for cure. The benefit of adjuvant chemotherapy following surgical management of the primary tumor, most notably in pediatric sarcomas, supports the effectiveness of conventional chemotherapy in the treatment of microscopic disease [16, 17]. However, the fact that some proportion of patients will still develop metastatic recurrence following adjuvant chemotherapy suggests the biological resistance of microscopic disease in some patients. The mechanisms for resistance of these microscopic metastatic lesions to conventional chemotherapy, given before, during, and after primary tumor management, are unclear. It is reasonable to assume that these microscopic cells are dormant, and that they reside outside the cell cycle, and are therefore are transiently resistant to cytotoxic therapies. These metastatic lesions (or cells) may also have inadequate vascularization for sufficient drug delivery. Several additional explanations for the resistance of these microscopic metastases to conventional therapies have been suggested; nonetheless, a significant problem limiting patient survival is caused by these microscopic metastases that progress to form gross metastatic lesions (after a variable period of clinical dormancy).

At the time of clinically detectable metastatic recurrence, the use of systemic therapy is necessary for the management of most sarcoma patients. However, in some sarcomas, depending on the pattern and location of metastasis, surgery is considered to be a first-line treatment [18]. Indeed, for osteosarcoma patients at the time of initial recurrence, pulmonary metastasectomy is an effective stand-alone treatment in 25 percent of patients. The biological basis for the unique opportunity to manage osteosarcoma metastases with surgery is not understood. Unfortunately, if repeated recurrence does occur, the benefit of surgery alone as a treatment strategy predictably wanes. For most sarcoma patients, metastatic lesions are problematic because they are or will become resistant to most conventionally available treatments. It is unclear whether these metastatic lesions are inherently more resistant to conventional chemotherapeutics or whether they acquire resistance over time. Irrespective of the cause, new treatments are needed not only to prevent or block the process of metastatic progression but also to better treat established metastatic lesions.

STATE-OF-THE-ART DIAGNOSTIC/PROGNOSTIC ASSESSMENT

Establishing a definitive diagnosis of a specific sarcoma can be relatively difficult as compared with the more common cancer types. For some sarcomas, clear histological features, such as the striated histostructure of skeletal muscle in rhabdomyosarcoma, the vascular channeling patterns of angiosarcoma, or the presence of osteoid in osteosarcoma, can provide a definitive diagnosis [19]. However, a histological assessment alone is not sufficient for a definitive diagnosis in many sarcomas. This may be the result of the relative paucity of clearly distinguishing histological features and also may be because most aggressive sarcomas demonstrate a poorly differentiated phenotype that can be shared by many sarcoma family members. In such instances, immunohistochemical staining is necessary to further define a sarcoma diagnosis. In addition, the tools of the molecular pathologist have not only increasingly provided clarity in the diagnosis of specific sarcomas but also have identified subgroups of sarcomas previously considered to be the same disease [5].

For translocation-positive sarcomas, the presence of a fusion oncogene or oncoprotein has been useful in diagnosis. PCR primers that uniquely target the breakpoint of the translocations are now conventionally used in the diagnostic workup of patients. Further definitions of sarcoma family members are likely to emerge from additional molecular screening of sarcomas. Recent studies have demonstrated the ability of gene expression signatures derived from distinct sarcomas to be useful in the prospective diagnosis of patients [20]. These approaches are not yet part of the gold standard assessment but do provide a view of the future opportunity for molecular pathology in sarcoma diagnosis [21].

The histological grade is of prognostic value in many sarcomas and can be used to predict the risks for local and distant recurrence in patients [22, 23]. Histological grade is based in part on mitotic rate, necrosis, cellular differentiation, and stromal components [24]. For example, patients who have soft-tissue sarcoma with low-histological-grade tumors rarely have metastases develop, whereas patients with high-histological-grade tumors will have a 40 percent risk of metastatic recurrence in five years [25]. Unfortunately, the assessment of prognosis provided by histological grade is not refined and is not relevant for many sarcoma family members [26]. For the most part, the definition of risk for metastatic progression in sarcoma patients is difficult to determine and requires additional clinical research. Candidate gene/protein studies have identified potential biomarkers linked to progression that may also be useful therapeutic targets. Reliable predictors of metastatic risk would allow identification of those patients for whom surgery alone may be curative; those for whom conventional adjuvant therapies should be used; and those at highest risk, for whom clinical imperatives for novel therapeutic approaches (i.e., investigational studies) are required.

The clinical staging of sarcoma should include an assessment of the histological grade, tumor size, and tumor location [25]. The assessment of distant disease relies on conventional imaging studies including CT scan, MRI, and, in some cases, PET scan. The presence of established metastatic disease in sarcoma patients is a highly predictive and negative prognostic indicator [27–29]. For example, survival in patients with gastrointestinal leiomyosarcoma is 75 percent when patients present with localized disease, whereas survival is less than 12 percent in patients with established metastasis [30]. For sarcomas with risk for lymph node metastasis, surgical resection and evaluation of abnormal lymph nodes is often considered. De facto resection of sentinel lymph nodes can be included in the staging and management of sarcomas [31]. The use of novel imaging approaches as a means to detect the metastatic burden and to provide an early assessment of therapeutic response is a current area of active translational and clinical investigation. Indeed, the therapeutic benefit of the c-KIT targeting agent, imatinib (Gleevec), in the treatment of patients with gastrointestinal stromal tumors (GIST) was evident by PET scan within twenty-four hours of treatment [32, 33]. These "metabolic responses" are predictive of objective tumor regressions that can be seen in subsequent weeks or even months.

BIOLOGIC TARGETING OF CURRENT DRUGS AND SPECIFIC GENES/RECEPTORS

It is unfortunate that the process of metastases (i.e., the verb) and the resulting metastatic lesion (i.e., the noun) have the same name. The verb and the noun describe very different biological and clinical conditions that should be considered as distinct entities [34]. Although they are clearly related, the verb refers to a set of stepwise cellular processes that result in the dissemination of tumor cells from the primary tumor site to distant secondary sites. The process of metastasis includes tumor cell migration, invasion, entry into the circulation, and the eventual arrest and extravasation at distant secondary sites. The noun refers either to microscopic metastatic cells or to the gross metastatic lesion at the secondary site of a patient. The focus of most basic research in the field of metastasis is the process of spread – that is, the verb. This focus overlooks the fact that patient mortality is the result of the metastatic lesions (the noun). Therapeutic targets within these established lesions are likely to be quite distinct from the therapeutic targets associated with the process of metastasis or the primary tumor. As suggested previously, neither the process nor the metastatic lesions themselves are adequately treated currently with available treatments. The search for sarcoma-specific targets through the study of genetic aberrations or gene expression studies has led to important advances in the treatments of specific sarcomas. The best examples of molecular aberrations in sarcomas that are valuable therapeutic targets include those that are mutated and driving of the malignant phenotype [35–38]. The overexpression and mutation of c-kit in GIST is emblematic of such a driving mutation, in which therapeutic agents that target the c-kit kinase domain (i.e., imatinib, sunitinib) have dramatically changed long-term outcomes in patients. Continued efforts are under way to develop improvements on the therapeutic success seen in the GIST targeting of c-kit – namely, a need exists to effectively treat GIST patients at the time of their first relapse, a problem likely encountered by most GIST patients treated with imatinib [39, 40].

In terms of sarcoma-specific targets, consistent recurring translocations found in some sarcomas provide a unique opportunity for drug development and

targeting [1]. These recurring translations commonly result in fusion oncoproteins that act as the driving oncogenic event for that given cancer. For the most part, experimental targeting of these translocation events (oncogenes) will reverse the malignant phenotype of these tumors or cells [41]. Adding value to the credentials of these sarcoma targets is the fact that, by definition, these cancers are expressed only in tumor cells and not in normal cells. Several efforts are under way to identify agents that may successfully target these sarcoma-specific oncogenic events. These strategies must overcome the conventional definition of these targets as being nondruggable because of their functions as transcription factors.

In sarcomas in which defining translocations are not found, a bizarre and complex karyotype is often present. This complexity of this karyotype has complicated efforts to identify driving and causal mutations for oncogenesis and progression/metastasis. The increasing ability for detailed and robust next-generation sequencing strategies may yet uncover recurrent translocations or other mutations in sarcomas that have eluded discovery to date. In the absence of such consistently defined genetic aberrations, the therapeutic targeting for these sarcomas may be based on common clinical features of the disease. Despite the diversity and heterogeneity of the cancers that are included in the sarcoma family, a number of biological motifs (i.e., growth factor signaling paths, angiogenic phenotype, and mesenchymal stem cell origin) remain consistent in many sarcomas. These motifs have provided and will provide a basis from which to consider new therapeutic approaches directed against the metastatic progression of these cancers.

Not unlike other cancer types, recent interest and success have been seen in sarcoma patients treated with multitargeted kinase inhibitors. The complexity and heterogeneity of molecular alterations even within sarcoma subfamilies may strongly argue for such “dirty” approaches to targeted therapy. Similarly, the targeting of angiogenesis may be appropriate in these patients because of its common association with the growth and progression of sarcomas [42]. Several antiangiogenic and vascular targeting agents have been assessed in clinical trials in sarcoma patients [43]. The complexity of the angiogenic phenotype suggests a strong likelihood that single-agent inhibitors of a single component of this angiogenic phenotype (i.e., VEGFR inhibition) will not be sufficient to control metastatic progression alone. Furthermore, the hypothetical lack of resistance associated with antiangiogenic therapy has not proved itself to be true in the clinic, in more complex preclinical models of cancer, and in patients [44, 45]. Collectively, and not surprisingly, combinations of antiangiogenic agents, or combinations of antiangiogenic agents with other treatments, will be necessary.

The insulin-like growth factor (IGF)-I pathway has been linked to the development and progression of many sarcomas. The growth and development of the adult mesenchymal tissues are largely the result of induced growth hormone release of IGF-I (primarily from the liver) and its interaction with the IGF-I receptor present on mesenchymal cells. Proliferation and survival of normal and malignant mesenchymal cells have been linked to activation of the IGF-I pathway. However, amplification or activating mutations in the IGF-I receptor have not been found in sarcomas to date. Detailed sequencing studies are under way in sarcoma patients and tumor tissues to better identify mutations in the IGF-I receptor and other parts of the IGF-I signaling pathway. Nonetheless, both preclinical and clinical studies suggest the importance of the IGF-I receptor and the IGF-I pathway in sarcoma.

Targeting of the IGF-I ligand in cancer has shown promise in several cancer types [46]. Recent opportunities to target the IGF-I receptor have been made possible by using humanized and fully human antibodies that target the IGF-I receptor and small molecule inhibitors directed against the IGF-I receptor kinase [47]. A number of therapeutic antibodies, targeting the IGF-I receptor, are in various stages of preclinical and clinical development in cancers, including sarcoma, and have been associated with single-agent activity in preclinical models [48]. An even more exciting development has been the evidence of activity for these agents in patients with Ewing sarcoma in early human clinical trials [49]. These dramatic responses in sarcoma patients suggest a unique dependence or an addiction that these cancers have for the IGF-I receptor pathway. Additional agents targeting downstream components of the IGF-I receptor pathway, including inhibitors of PI3 kinase and Akt kinase, have been studied and will increasingly enter human trials. The combination of multiple agents that target the IGF-I pathway may be useful to optimally block this signaling cascade in sarcomas.

c-MET is the receptor for hepatocyte growth factor (HGF). Aberrant signaling resulting from either c-MET or HGF overexpression has been linked to the development of cancer in murine models [50]. Furthermore, preclinical studies in vitro and in vivo support the role of c-MET signaling in cancer progression, and specifically in metastasis [51]. c-MET has been shown to be expressed in sarcoma primary tumors and in metastatic lung nodules [52]. It is likely that several metastatic processes are linked to c-MET signaling, including cell motility, invasion, proliferation, and survival [50]. Because c-MET is a growth factor receptor with an intracellular tyrosine kinase activity, the development of small-molecule inhibitors of c-MET has been possible. Several potent and both highly specific and less specific (dirty) tyrosine kinase inhibitors of

c-MET have been developed and have been shown to be active against metastatic progression in preclinical models [53]. The inhibition of c-MET has been effective in suppressing metastatic phenotypes in osteosarcoma cells and in preclinical models [54]. Recent data suggest a unique dependence or addiction for c-MET signaling in metastatic lesions that is not observed in the primary tumor [51, 55]. The expression of c-MET in sarcoma and the evidence linking its inhibition with a suppressed metastatic phenotype are encouraging factors. The evaluation of this agent in an appropriate and informative patient population will be necessary to prevent a false-negative (type II error) result from these studies.

The mammalian target of rapamycin (mTOR) is a critical node in a signaling pathway that connects many growth factor receptors through intermediaries, including AKT and MAPK, to the translational machinery of the cell. As a result, mTOR is able to convert signals that sense the nutritional and stress status of a cell (i.e., in the cell's microenvironment) into specific proteins that can manage the stress. Cancer cells are highly dependent on the targets of mTOR-mediated translation, specifically by way of cap-dependent translation through eIF4E/4EBP1. Many of the presumed translational targets of mTOR have been connected to cancer, including c-myc, VEGFR, HIF, and TGFβ. However, the specific mechanism of antitumor activity for mTOR inhibition or mTOR inhibitors in cancer remains unclear.

That mTOR may be an important target for sarcoma is also supported by the importance of mTOR in mesenchymal stem cells. [56] Rapamycin, an approved agent useful in the setting of immunosuppression for transplant, directly inhibits mTOR1 and, as such, prevents downstream expression of mTOR targets. The chronic use of rapamycin in all probability results in a functional inhibition of both mTOR1 and mTOR2 [57]. Rapamycin and newly developed analogs (rapalogs) have been evaluated in preclinical and human clinical studies in a number of cancers, including sarcoma [58]. Preclinical studies have shown that rapamycin and its blocked ester (rapalog, CCI779) reduced metastases in a murine model of osteosarcoma [59]. Early human clinical data with rapalogs support the therapeutic value of this target in many sarcoma histologies [58]. Indeed, these responses have supported the initiation and recent completion of a Phase III study of the rapalog, ridaforolimus, in patients with soft tissue sarcoma. Given the multiplicity of effects related to the inhibition of mTOR (i.e., immunosuppression and anticancer), it is likely that optimal schedules for treatment will be required for the complete success of these agents to be seen.

Recent studies, particularly in IGF-I–driven cancers, have shown that phosphorylation of AKT occurs following mTOR inhibition by rapamycin or rapalogs. The clinical consequences of AKT phosphorylation in this setting are unclear; however, these observations have been part of the rationale to combine mTOR inhibitors with agents that may act at or upstream of AKT (e.g., IGF-I receptor inhibition). Novel small-molecule inhibitors that block the mTOR kinase and inhibit both mTOR1 and mTOR2 do not appear to be linked to this feedback effect. Further therapeutic advantages related to the combined inhibition of mTOR1 and mTOR2 have been suggested [57, 60].

Heat shock protein 90 (Hsp90) is a molecular chaperone of specific "client" proteins that are linked to oncogenic and metastatic phenotypes of cancer [61]. In many cases, Hsp90–client protein interactions protect these proteins from degradation. It is believed that physiologic protection of specific cancer-associated proteins has emerged as a means to overcome short-term cellular stressors. In cancer, specifically in metastatic lesions, these cellular stressors are experienced chronically. It is theorized that the ability of metastatic cancers to survive the process of metastases is the result of their management of these stressors. Thus, it is reasonable that metastatic cancer cells are more highly dependent on heat shock protein protection of client proteins than normal tissues or even primary tumors. Preclinical data support the fact that Hsp90 inhibition results in impaired cell growth, apoptosis, and suppression of angiogenesis, presumably through degradation of client proteins previously protected by Hsp90 [62]. The classic Hsp inhibitor is geldanamycin (GA), a benzoquinone ansamycin. GA, its analogs, and its derivatives have entered early-phase human studies and have shown reasonably low toxicity at exposures that can disrupt Hsp90 stabilization of client proteins [62]. Many of these client proteins, such as IGF1R, AKT, and c-MET, have specific relevance to sarcoma. Newer-generation inhibitors of Hsp90 are under preclinical and clinical development in several histologies including sarcoma. Improvements in these agents include specific binding of Hsp90, enhanced competition with client proteins, a reduction in toxicity, and improved pharmacokinetics.

FUTURE DIRECTIONS AND OPPORTUNITIES

Some will question, because many of the cellular processes associated with metastasis (i.e., the verb) have already occurred when a sarcoma patient presents, whether the therapeutic targeting of these events is too late. Our understanding of the events and the timing of events leading to metastatic progression are far from complete; accordingly, such speculation should not guide therapeutic decision making. For example, it is unclear whether microscopic cells that leave the primary tumor early in the development of sarcoma move directly to the secondary site (i.e., the lung) and reside

there for long periods of time (dormancy) or whether these cells transit from the primary tumor to "protected environments" in which they undergo dormancy and then subsequently move through all the steps of metastasis on their route to the secondary site. Indeed, if metastatic cells emerge from "protected sites" after dormancy, then most of the steps associated with the metastatic cascade will occur following a patient's presentation and, as such, are able to be appropriately targeted in therapy. Similarly, it is a reasonable assumption that the cells capable of completing the complex set of events required for metastasis will continue to metastasize to yet distant sites. If metastasis begets metastases, then all the steps associated with the metastatic progression will occur even after a patient presents with gross metastasis. Shortcomings in our understanding of the biology of metastasis preclude any speculation on the value of agents that target metastatic processes. Rigorous preclinical studies and innovative clinical trial designs are necessary to allow the appropriate assessment of these agents in patients who are at risk for or who already have metastases.

Although the list of targets available for the potential treatment of sarcoma patients is long and provides a basis for optimism, several factors, both related and unrelated to the biology of these aggressive diseases, must be addressed before improvements in long-term patient outcomes will occur. The number of patients diagnosed with sarcoma each year is small. Because of this limited number, very few Phase III trials including new agents are launched for sarcoma patients each year. Accordingly, creativity in the design of studies and in the use of the available data is needed. The design and prioritization of agents for study in clinical trials must be provided through a well-conceived preclinical translational path. Finally, it is necessary that we improve on current methods to prospectively identify the patients with the highest risk for metastasis following conventional treatment. Correct prediction of poor prognosis will allow the evaluation of novel treatments in these high-risk populations.

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25 Neuroblastoma

Nai-Kong Cheung and Brian H. Kushner

Neuroblastoma (NB) originates from neural crest precursors in the adrenal medulla, the sympathetic ganglia in the neck, mediastinum, retroperitoneum, or pelvis.[1] It is the most common extracranial pediatric solid tumor and the most common neoplasm among infants. More than 90 percent of the more than 700 cases diagnosed yearly in the United States are in children younger than five years of age.

NB has a notorious reputation among solid tumors of childhood because of its massive and widespread tumor burden. Stage for stage, however, this embryonal neoplasm of the sympathetic nervous system has become one of the most curable pediatric solid tumors. In fact, more than 90 percent of patients with localized NBs, including those with tumors spreading to regional lymph nodes, will survive, often with little or no cytotoxic therapy. Cure rates of metastatic NB exceed 90 percent in infants (usually treated with low-dose chemotherapy) and approximately 25 percent in toddlers. In contrast to NB, osteomedullary metastases result in less than a 5 percent chance of cure for other pediatric solid tumors. The long-held view that many cases spontaneously regress or mature into asymptomatic ganglioneuromas was confirmed by the findings of urine catecholamine screening programs in infants.[2] These programs yielded 50 percent to 100 percent more cases than the unscreened population. Most patients identified had the low-risk forms of the disease, and the incidence of high-risk disease was not reduced.

Environmental causative factors have not been identified, nor has NB been significantly associated with any other disease or condition. Recurring aberrations of specific chromosomal regions and genetic loci are distinguishing features of tumor cells in about 50 percent of patients (typically with high-risk disease) but are rare among those with low-risk disease. The presence of these chromosomal abnormalities, including involvement of the *MYCN* proto-oncogene, and chromosomal aberration of 1p, 2p, 11q, 14q, and 17q, conveys prognostic insights that have refined the risk-based management of the remaining 15 percent to 20 percent of NB patients. Despite the radically different chromosomal composition between clinical subgroups, a model of tumorigenesis has been proposed to encompass all forms of this disease, centered on a common precursor and a common tumor-initiating mutation (Figure 25.1).[3] Different inherited tumor-initiating chromosomal regions (e.g., 2p, 4p, 6p, 12p, and 16p) have also been implicated in the formation of NB, although none with clear pathogenetic explanations, and their effects are often diluted by interfamily variance and incomplete penetrance.[4,5]

The diagnosis of NB is made by either the characteristic histopathological findings or the presence of tumor cell clumps (syncytia) in the bone marrow, as well as elevations in urinary vanillylmandelic acid (VMA), homovanillic acid (HVA), or other catecholamines.[6] For evaluation of the primary site, computed tomography (CT) is traditionally the standard for defining soft tissue masses and associated adenopathy, although magnetic resonance imaging (MRI) is increasingly used. For distant disease, nuclear imaging by ^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) (an analog of catecholamine precursors) is the study of choice,[7] although bone scan is useful for distinguishing cortical bone from bone marrow involvement. MRI remains the gold standard for the assessing epidural or leptomeningeal lesions. Although plain X-rays are useful screening studies for lytic lesions, MRI and nuclear scintigraphy remain the final arbiters of active disease. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) is increasingly used as a confirmatory study for recurrent or metastatic NB.[8]

METASTATIC NEUROBLASTOMA

More than 50 percent of all NBs establish metastatic sites early during tumorigenesis, well before their

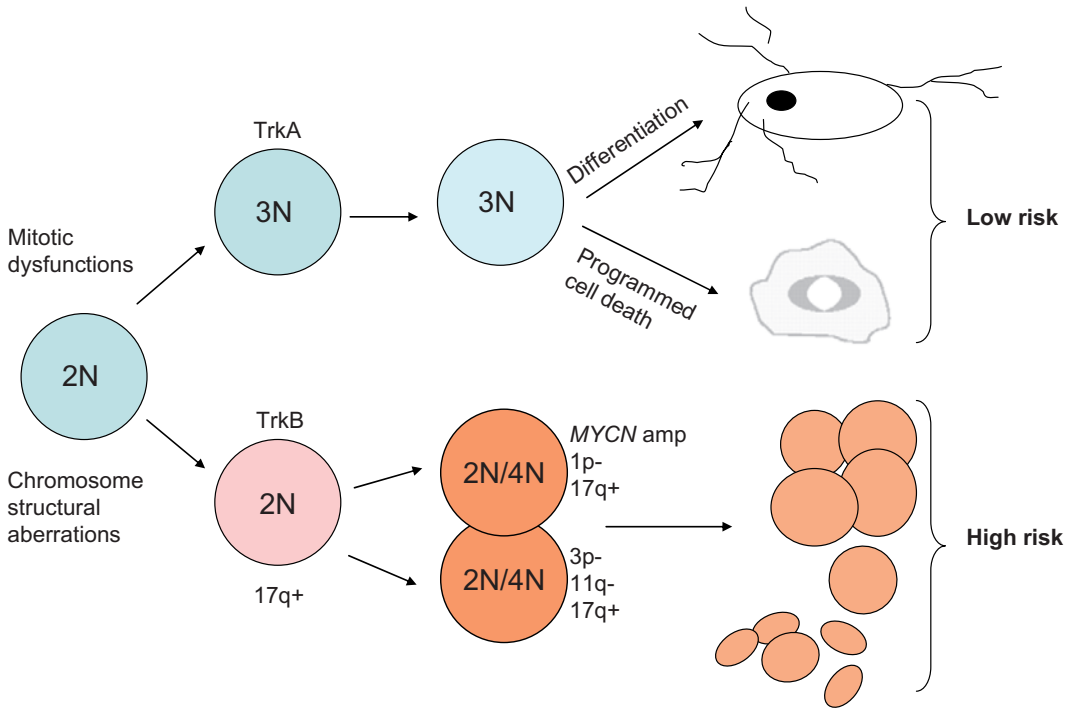

Figure 25.1. Schematic development of major subtypes of neuroblastoma. A genetic derangement in a diploid (2N) precursor of the sympathetic nervous system leads to triploid (3N) cells with whole chromosome gains, or to diploid or tetraploid (4N) cells with chromosomal structural changes (– indicates loss, + indicates gain). Low risk means excellent prognosis with little or no therapy. High risk means poor outlook despite multimodality therapy, although infants with metastatic bone/bone marrow disease lacking *MYCN* amplification do well with modest doses of chemotherapy (and are better classified as being at intermediate risk).[183]

clinical detection. They present as stage 4 disease. This high prevalence of major metastases to bone and bone marrow contrasts with that for other solid tumors. Unlike other solid tumors, NBs that present at the local–regional stage (stages 1, 2, and 3) rarely evolve into metastatic stage 4 disease. Hence, the clinical rationale of adjuvant therapy to prevent distant spread does not generally apply to this tumor. NB metastasizes by hematogenous and lymphatic routes. Nevertheless, lung and central nervous system (CNS) metastases are extremely rare at diagnosis. Unique among cancers, the spontaneously regressing small subset of widespread stage 4S NB differs distinctly from the lethal stage 4 tumors (Table 25.1).[9,10] Stage 4 disease typically arises in children older than 18 months of age, with metastases to bone marrow, cortical bone, and lymph nodes, whereas the predominant distant sites in stage 4S (which is limited to infants) are liver and skin, with less common and less extensive bone marrow and no cortical bone involvement.[16]

Although stage 4S disease tends to regress spontaneously with minimal to no therapy, classic stage 4 NB requires cytotoxic, and often aggressive, multimodality therapy. The marked differences in outlook hold true irrespective of metastatic sites. Thus, hepatic lesions in a child impart a grave prognosis over and above metastases to other organs. However, the same findings have no adverse prognostic significance in an infant with stage 4S disease. Even leptomeningeal metastases, which are highly lethal in stage 4 patients, have been reported to regress spontaneously in the stage 4S setting.[11] Children whose NB is stage 4 by virtue of distant lymph node metastases only (i.e., no liver or osteomedullary metastases) have also been noted to be more curable.[12]

The decisive modifier on the prognosis of metastases is the age of the patient at diagnosis.[13,14] This is most strikingly evident by the ready curability using modest doses of chemotherapy against even massive osteomedullary metastatic disease in infants.[13] Moreover, localized NB in young patients rarely ever relapses in distant sites, but similar tumors in adult patients will eventually all metastasize, although much more insidiously than in children, and will ultimately involve the same sites, such as bone marrow, cortical bone, and lymph nodes. The effect of the "soil" (host) on the aggressiveness of the "seed" (tumor) may become apparent as young patients survive to an older age.[15,16] This organ-specific tropism of NB with near opposite programs of sustained growth versus regression, clearly modified by the age of the host, has remained its most intriguing biology (Table 25.2).

At disease progression or recurrence, bone and bone marrow are the most common metastatic sites, often

TABLE 25.1. Sites of metastases (percent) in patients with stage 4 versus stage 4S neuroblastoma at diagnosis and at first recurrence

	Stage 4		Stage 4S	
Disease localization	Initial	1st recurrence	Initial	1st recurrence
Bone marrow	87.3	35.2	61.5	19.2
Bone	66.1	46.6	0.0	15.1
Lymph nodes	18.6	8.9	0.0	7.7
Liver	17.4	7.5	76.0	38.5
Skin	2.8	0	12.5	7.7
Intracranial/cerebral	9.1	19.0	0.0	15.4
Lung/pleura	4.7	3.1	0.0	0
Paratesticular	1.0	0	2.6	11.5
Ovary	0.3	0	0.0	0
Isolated local recurrence		17.0		26.9
Isolated metastatic recurrence		58.1		30.7
Combined local and metastatic recurrence		24.9		42.4

Berthold F and Simon T. Chapter 7: Clinical Presentation. Cheung NK and Cohn S (editors). *Neuroblastoma*. Springer. 2005.

after patients have achieved clinical remission. With more effective local control in the past decade, recurrence in the primary site is less of an issue.[17,18] For individual patients, the site of first recurrence depends on the initial biology and the preceding treatment. For example, in patients with only soft tissue metastases, NB at recurrence tends to be restricted to soft tissue sites. Another example is the development of lung metastases following the infusion of autologous stem cells often contaminated with NB. More recently, with the use of intensive induction chemotherapy plus anti-GD2 monoclonal antibody (mAb) for consolidation, the incidence of bone marrow relapse has decreased, whereas metastatic relapse in the CNS appears to be more prevalent. The distribution pattern of metastases after first-line therapy appears to depend on how well the systemic disease is under control. It is highly likely that sanctuary sites, where chemotherapies and biologic therapies cannot reach, provide havens for late relapses.

The clinical gravity of relapse is attenuated by the increasingly sophisticated and sensitive methods of detection. With the introduction of ^{123}I-MIBG and extensive bone marrow testing, asymptomatic relapse

TABLE 25.2. Outstanding features of neuroblastoma

- ≥50% of patients present with distant metastases, especially to bone and bone marrow (≤20% for other solid tumors)
- Tumor burden of metastatic disease is typically massive.
- Metastatic sites are established early during tumorigenesis, well before their clinical detection.
- Local–regional stage NB rarely evolves into metastatic stage 4 disease.
- Prognosis of metastasis is significantly influenced by patient age.
- Metastasis and treatment resistance are independent events.
- MIBG is a sensitive and specific nuclear imaging of metastatic disease.
- Molecular markers are available for sensitive and specific detection of minimal residual disease (MRD) in the bone marrow.
- An extensive array of prognostic indicators is available for increasingly precise definition of risk groups.
- Gene signature of metastatic neuroblastoma is complex.
- Biologics (13-*cis* retinoic acid, monoclonal antibodies) can be effective therapy for MRD beyond standard surgery, chemotherapy, or radiotherapy.

is now readily identified, typically in the bone marrow.[19,20] In these studies, ^{123}I-MIBG demonstrated superior sensitivity over ^{131}I-MIBG scan, bone scan, or computed tomography/magnetic resonance imaging (CT/MRI).[20] Asymptomatic patients whose monitoring includes ^{123}I-MIBG rather than ^{131}I-MIBG scans have less extensive relapse because of early intervention, accounting for their significantly longer survival regardless of whether measured from diagnosis or from time of relapse.[20] Either reason is possible, although CNS relapse is not exclusively seen in patients receiving biologics or specific chemotherapeutic agents.

Recurrences in brain parenchyma can be asymptomatic but classically cause headache, emesis, and/or focal neurological deficits.[21,22] The problem usually emerges within nine months after completion of chemotherapy, often without evidence of systemic recurrence. Despite aggressive multimodality treatment, CNS relapse is typically followed by new parenchymal or leptomeningeal and systemic spread, with a rapidly fatal course. A novel treatment program is showing encouraging promise in reversing this historically dismal outcome (discussed later).

PROGNOSTIC FACTORS

Low- and intermediate-risk NBs, which include localized and metastatic disease, are highly curable. Their hallmark is complete regression, or less commonly, maturation into benign ganglioneuromas, either spontaneously or after modest doses of chemotherapy. In contrast, cure rates of high-risk NB have only been 20 percent to 30 percent, despite aggressive multimodality therapy.[14,23–25] Among the many clinical and biologic prognostic factors,[1,10] age at diagnosis (more than eighteen months) and metastatic stage are the most important. The impact of metastatic site is greatly influenced by the age of the patient at diagnosis and the age at time of disease recurrence. For example, bone marrow metastases in infants often regress spontaneously; low-dose chemotherapy will result in a cure rate of more than 90 percent. In contrast, such bone marrow metastasis is lethal in more than 70 percent of older children, and it is rarely curable in adult patients. Similar statistics apply to bone metastasis in infants versus children and adults.

The specific sites of metastases also have prognostic importance. Thus, CNS, lung, or liver metastases in stage 4 disease have the worst outcome, whereas metastases isolated to the lymph nodes, with absence of osteomedullary involvements, is generally more curable. High-risk patients often have high serum levels of lactate dehydrogenase (LDH >1500 U/L) and ferritin, as well as unfavorable urinary VMA:HVA ratios. *MYCN* proto-oncogene amplification (30% of NB tumors) is associated with failure to respond, disease recurrence, and CNS metastases. It typically occurs with 1p loss of heterozygosity (LOH) and 17q gain, whereas unbalanced (unb) 11qLOH and 3p deletion (del) are strongly associated with, and predictive of, relapse among *MYCN*-nonamplified NB.[1] Tumor diploidy in young patients (up to 15–18 months old) and high expression of the TrkA neurotropin receptor (also known as NTRK1) are both favorable indicators, whereas high expression of TrkB (also known as NTRK2) is common with poor-prognosis NB. Few studies have examined the molecular signatures of primary versus metastatic NB, except the level of *MYCN* amplification, which appeared similar between primary and metastatic sites.[26]

TREATMENT OF METASTATIC NEUROBLASTOMA

Dose-Intensive Induction Chemotherapy

Classic high-risk NB is usually massive in the primary site, with widespread metastases. The goal of initial treatment is rapid reduction of the entire tumor burden. Various combinations of cyclophosphamide, ifosfamide, doxorubicin, cisplatin, carboplatin, etoposide, and topotecan are used with dose-dense or high-dose-intensity strategy (i.e., the same or higher total dose given over a short induction period).[23,27,28] Three to five cycles of high-dose chemotherapy[29] and radiotherapy (2100–3000 cGy) have substantially reduced recurrence at primary sites to less than10 percent.[17,18] Control of systemic metastases is a much more daunting challenge. Myeloablative chemotherapy, sometimes combined with total body irradiation (TBI, 1000–1200 cGy), has been widely used since the 1980s, with a survival advantage reported in two of three multicenter randomized studies.[24,30–32] Tandem, triple, allogeneic, and combined ^{131}I-MIBG/autologous transplants are being explored in clinical trials, some with encouraging results.[33] TBI is used less often, in part because of late toxicities.[34,35]

Antibody Therapy Targeting Differentiation Antigens

Biologic therapies targeting chemoresistant NB attempt to destroy the seed as well as to modify the soil. In particular, chemoresistant bone marrow disease forebodes a lethal outcome.[36] Several mAbs have been developed to target NB-associated disialoganglioside GD2.[37–39] GD2 is an adhesion molecule widely expressed among melanomas, small-cell lung cancers, bone and soft tissue sarcomas, retinoblastomas, and brain tumors. It is rarely expressed in normal tissues except neurons, skin cells, and pain fibers. The murine

IgG3 anti-GD2 3F8 mAb has undergone testing in a wide range of clinical settings.[37,40–49] A recent COG randomized study[40] found a significantly better progression-free survival with the posttransplant use of the anti-ganglioside G_{D2} chimeric mAb ch14.18 combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-2 in alternating cycles, as developed in a Phase I trial.[41]

Scintigraphy using ^{131}I-3F8 showed selective uptake in NBs with a high tumor-to-nontumor ratio.[42] 3F8 mediates antibody-dependent cellular cytotoxicity (ADCC) by human granulocytes and mononuclear cells.[50,51] Chimeric or human IgG antibodies interact preferentially with FcγR3A allelotype bearing valine at position 158 on lymphoid cells, which was associated with superior clinical outcome following treatment with rituximab.[52,53] Mouse IgG antibodies prefer the FcγR2A-R131 over the -H131 allelotype, resulting in differential mitogenic potency and cytokine release[54,55] and differential outcome following 3F8 treatment of NB.[49] 3F8 also induces complement-mediated cytotoxicity of NB cells,[56] which lack decay-accelerating factor[57] and CD59.[58] Complement deposit on NB cells enhances ADCC through the iC3b receptor (Mac-1, CR3, CD11b/CD18, or αMβ2- integrin) on leukocytes.[59–61] There is also strong preclinical evidence that mouse IgG3 enhances the immunogenicity of vaccines, inducing potent long-lasting protective immunity.[62]

Anti-GD2 mAbs have achieved clinical responses in small Phase I and Phase II studies,[37,39,44,63,64] and, as consolidative adjuvant therapy, yielded encouraging results in a large study of 3F8 at Memorial Sloan-Kettering Cancer Center (MSKCC),[46] though not initially for ch14.18 in a German cooperative group study.[65] Results with the randomized COG study (mentioned earlier) represent a major advance in regard to support for the advantage of this kind of immunotherapy in the treatment of high-risk NB.[40] At a follow-up of 2.1 years, immunotherapy was superior to standard therapy with regard to rates of event-free survival (66 ± 5% vs. 46 ± 5% at 2 years, P = 0.01) and overall survival (86 ± 4% vs. 75 ± 5% at 2 years, P = 0.02 without adjustment for interim analyses).[40] Several factors favor combined use with GM-CSF, as done by the Children's Cancer Group[66] and at MSKCC.[48] Thus, whereas standard therapy for high-risk NB causes prolonged T-cell lymphopenia, granulocyte and monocyte production is only transiently suppressed,[67] and GM-CSF induces neutrophilia and eosinophilia,[68] while priming granulocytes and monocytes–macrophages for greater antineoplastic cytotoxicity.[51,69–74]

The importance of the route of GM-CSF administration was highlighted in a recent report, in which 3F8/GM-CSF was tested in eighty patients with chemoresistant NB in the bone marrow as detected by histology and/or MIBG scan. The 36 percent (SE + 10%) three-year progression-free survival (PFS) of fifty-four patients treated with subcutaneous GM-CSF [ClinicalTrials.gov NCT00072358] was significantly better than the 12 percent (SE+7%) for twenty-six patients treated with two-hour intravenous infusions of GM- CSF (p = 0.003) [ClinicalTrials.gov NCT00002560]. Significantly better PFS (p = 0.004) was noted for the R/R and H/R forms, as compared with the H/H form, of FCGR2A, the Fcγ receptor found on myeloid but not lymphoid cells. Eighty percent of patients with NB in bone marrow achieved histologic complete response (CR), and about 40 percent achieved MIBG CR. Common toxicities were pain and urticaria, and treatment was on an outpatient basis.

In the most recent update of the clinical utility of 3F8, 157 patients with high-risk NB in first remission treated on consecutive 3F8 protocols from 1991 through 2007 were analyzed. At diagnosis, ninety percent of patients were more than eighteen months old with bone marrow ± bone metastases. Forty-five percent had *MYCN*-amplified NB. All had standard-dose intensive induction therapy. Patients received (1) 3F8 (+/– targeted radiotherapy with ^{131}I-3F8) [ClinicalTrials.gov NCT00002634, NCT00040872] instead of autologous stem cell transplant (SCT); (2) 3F8 plus intravenous (iv) GM-CSF [ClinicalTrials.gov NCT00002560]; or (3) 3F8 plus subcutaneous (sc) GM-CSF [ClinicalTrials.gov NCT00072358]. Long-term PFS past five years among group 1 was 40% ± 8%. PFS was similar when treatment included 20 mCi/kg of ^{131}I-3F8. In group 3, PFS improved to 61% ± 7%, whereas for group 2, PFS was 51% ± 7%. All three groups had better PFS than historical control when SCT was the standard of care.[75] Patients who developed anti-idiotypic (Ab2) and anti-antiidiotypic (Ab3) antibodies appeared to have survival advantage,[47] consistent with an active antitumor immunity modifying the soil for metastasis.

Radioiodinated 3F8 for Systemic NB

^{131}I-3F8 targets selectively to NB primary tumors and metastatic sites in lymph nodes, bone marrow, and bone with superior sensitivity when compared with ^{131}I-MIBG.[81] The radius of radiation effect is about 800 μm, and the optimal tumor size for achieving a curative dose with ^{131}I-3F8 is ~2 mm. Because of the lack of extramedullary toxicities in a phase I study, ^{131}I-3F8 at a dose of 20 mCi/kg was added to a multimodality program for high-risk NB patients (n = 35). Toxicities included self-limited pain, fever, and rash, followed by myelosuppression that required bone marrow rescue. Other than hypothyroidism, no extramedullary toxicity was observed. With continued follow-up (6 –10 years from diagnosis), overall survival for NB patients newly diagnosed at more than eighteen months of age treated with ^{131}I-3F8 was around 40 percent.[75,76] There were no

unexpected late effects (including secondary leukemia) among children treated on this regimen.

Compartmental radioimmunotherapy (RIT) by intrathecal administration of radiolabeled mAb has the advantage of delivering high doses of radiation to the affected areas while reducing radiation exposure to bone marrow, blood, and other organs.[77] A phase I study using intraventricular (intra-Ommaya, IO) administration of escalating doses of ^{131}I-3F8 in patients with leptomengineal (LM) disease has recently been completed.[78] Toxicities included self-limited headache, fever, and emesis. Maximum tolerated dose was 10 mCi. Three of thirteen assessable patients achieved objective radiographic and/or histologic responses.

Anti-B7-H3 Antibody for Compartmental RIT

mAb 8H9 is a murine IgG1 against cell surface antigen 4Ig-B7-H3, which is present on most solid tumors.[79] 8H9 can be radiolabeled with ^{124}I or ^{131}I, retaining immunoreactivity. In a Phase I study of ^{131}I-8H9, treatment doses from 10 to 60 mCi have not yet encountered dose-limiting toxicity. Calculated mean radiation dose to the cerebrospinal fluid was 36.3 (range 12.8–106) cGy/mCi; mean blood dose was 2.5 cGy/mCi.[80] In a retrospective analysis of forty-eight patients with recurrent NB metastatic to the CNS, fifteen received IO-RIT with ^{131}I-3F8 or ^{131}I- 8H9 after surgery, chemotherapy, and craniospinal irradiation. Additional treatments for systemic disease included 3F8/GM-CSF immunotherapy, 13-cis-RA, and temozolomide. Thirteen of these fifteen patients remain free of CNS NB six to fifty-eight months after the CNS event, with eleven in complete remission. One patient died of infection at twenty-two months with no evidence of disease at autopsy, and one of lung and bone marrow metastases at fifteen months. In contrast, thirty-one of thirty-three patients who received conventional treatment alone for CNS relapse have died, median time to death was 5.8 months for those with systemic and CNS disease and 11.5 months for those with isolated CNS relapses from the CNS event. The IO-RIT-salvage regimen for CNS metastases was well tolerated by young patients, despite their prior history of intensive cytotoxic therapies. This therapy has the potential to radically increase survival with better-than-expected quality of life.[81]

MIBG Therapy

MIBG is a guanethidine derivative structurally resembling norepinephrine. Both specific and passive mechanisms promoted MIBG uptake by NB. When labeled with ^{123}I, MIBG is ideal for tumor imaging, and when labeled with ^{131}I, MIBG is suitable for therapy. ^{131}I-MIBG has been extensively evaluated as a single agent in single- and double-infusion studies and dose-escalation trials,[82–88] with documentation of excellent activity against chemoresistant as well as newly diagnosed NB,[89] although complete responses are rare. ^{131}I-MIBG therapy is well tolerated, with side effects limited to myelosuppression (often necessitating stem cell support), hypothyroidism, and sialoadenitis.[90] Not surprisingly, the radioactivity may be leukemogenic.[91,92] Investigations continue into how best to combine ^{131}I-MIBG therapy with conventional or myeloablative chemotherapeutic or other kinds of (radiomimetic) agents in order to augment antitumor activity with acceptable toxicity.[93–98] Patients going through induction for high-risk NB are now having large numbers of peripheral blood stem cells collected, taking into account their possible need at some future time for salvage treatments such as ^{131}I-MIBG therapy.

Differentiation Therapy Using Retinoids

Vitamin A or retinol (primarily from the diet in humans) is critical in normal neural crest development. Intracellular retinol is metabolized to all-*trans* retinoic acid (ATRA), which then activates a number of nuclear receptors that heterodimerize and regulate gene transcription.[99,100] ATRA treatment of NB cells was accompanied by a decrease in the expression[101] and transcription[102] of the *MYCN* gene and increases in the cyclin dependent kinase inhibitor p27,[103,104] followed by G1 -arrest and morphologic differentiation.[101,105] 13-*cis*-RA at low doses did not improve survival,[106] but in a randomized Phase III trial,[30] the three-year PFS for patients treated with high doses was 46% ± 6%, compared to 29% ± 5% for the untreated control ($P = 0.027$). Retinoids have also been shown to sensitize NB cells to T cells by modulating MHC class I antigen presentatation.[107] A promising improvement is N-(4-hydroxyphenyl) retinamide or fenretinide (4-HPR), which inhibits RA-resistant NB growth in vitro.[108,109,110] Intravenous and easy-to-ingest oral formulations of 4-HPR are in clinical trials.[111]

Target and Pathway-Specific Strategies

Gene Signature of High-Risk Neuroblastoma Metastasis

At the chromosomal level, losses of 1p and 11q, unbalanced gain of chromosome 17q, and *MYCN* amplification tended to be associated with metastatic disease. The mechanism by which high *MCYN* amplification (and expression) favors metastasis is not known; protein kinase C (PKC), c-fos and NFKB are probably involved. PKC phosphorylates a number of growth factor receptors that stimulate NB growth, such as insulin-like growth factor receptor (IGFR), epidermal

growth factor receptor (EGFR), and c-Met, the receptor for hepatocyte growth factor (HGF).[112] *MYCN* also downregulates neural cell adhesion molecule (NCAM), thereby promoting NB spread.[113] *MYCN* expression is often associated with upregulation of *Twist*, a transcription factor that regulates epithelial–mesenchymal transition, promoting cell motility and metastasis.[114] While *MYCN* drives cell proliferation, *Twist* counters its proapoptotic activity by inhibiting the ARF/p53 pathway.[115,116] The *nm23-H1* and *nmn23-H2* genes are nucleoside diphosphate kinases (NDPKs) required for the synthesis of nucleoside triphosphates (NTP) other than ATP. NM23-H1 and H2 are up-regulated in NB both by gene dosage (17q gain) and transcriptional activation (MYCN overexpression).[117,118] High expression of human nm23-H1 is usually associated with a decreased metastatic potential[119] except in prostate cancer, non-Hodgkin lymphomas, and NB, in which a high expression is associated with an adverse outcome. Resistance to anoikis ("detachment-induced apoptosis") has been suggested to be a prerequisite for cancer cells to metastasize.[120] TrkB overexpression in NB increases HGF expression and its receptor c-Met, matrix metalloproteinases (MMPs), and serine proteases (including urokinase and tissue plasminogen activators) to promote cell motility and metastasis.[121] Loss of caspase-8 and unligated integrins have also recently been linked to increased metastatic potential.[122,123]

Stromal-derived factor (SDF)-1/CXCL12 is expressed by bone marrow stromal cells and by osteoblasts, and promotes bone metastasis in prostate cancer.[124] NB cells express the CXCR4 chemokine receptor for SDF-1 [125] providing the navigator toward the bone marrow.[126] SDF-1 upregulates integrins such as VLA2, VLA3 and VLA6, CD56, c-kit, TNF-α, VEGF, interleukin (IL)-8, and GM–CSF, which may also enhance tumor cell proliferation and survival in the bone marrow microenvironment.[127] CXCR4 expression is upregulated by the liver and suppressed by adrenal stroma, probably through cytokines IL-5 and IFN-γ.[128] However, CXCR4 on NB cells isolated from patient marrow may not be functional.[129]

AMD3100 is a peptide that blocks CXCR4 and has been shown to be safe in patients with non-Hodgkin lymphoma and myeloma.[130] It may have the potential to modulate NB metastasis. NB cells also express the chemokine receptor CCR2 that interacts with the monocyte chemoattractant protein (MCP)-1, secreted by bone marrow stromal and endothelial cells.[131] Similar to marrow metastasis, the mechanism of NB metastasis to bone is complex. It involves the RANK/RANKL axis, IL-6, BDNF, PTHrP, and inflammatory cytokines besides tumor-stroma interactions.[132]

TrkB. Clinically, despite initial sensitivity of high-risk NB to chemotherapy, chemoresistance eventually emerges. The basis of this phenomenon is multifactorial, governed by traditional chemoresistance factors such as drug efflux pumps[133] and mutations in TP53.[134,135] In addition, drug-resistant NB cell lines have increased expression of BDNF[136] and TrkB.[137] TrkB has also been implicated in the resistance to anoikis and metastasis.[120] Recent studies have identified several targets of the TrkB pathway: Trk tyrosine kinases, PI-3-kinase, and Akt and its downstream members. A drug targeting Trk tyrosine kinases (CEP-751) has shown preclinical efficacy against NB mouse xenografts,[138] and is currently in a NB clinical trial. Furthermore, a number of compounds targeting the PI-3-kinase pathway are in pre-clinical or clinical development. Such agents may enhance the toxicity of chemotherapeutic agents against aggressive NB.

MDR and MRP. Multidrug-resistance–associated proteins (MRPs) are a family of transport proteins for cellular detoxification. Of these, MRP1, MRP2, and MRP3 have been shown to confer resistance to a variety of natural products and anticancer drugs,[139] including the vinca alkaloids, anthracyclines, epipodophyllotoxins, camptothecin-class topoisomerase I inhibitors, glutathione and glucuronide conjugates. MRP4 (ABCC4) regulates resistance to the nucleoside analogs 6-mercaptopurine and thioguanine, antiretroviral compounds,[140] and irinotecan and its active metabolite SN-38.[141] Similar to MRP1, it is overexpressed in high-risk NB and correlates with poor clinical outcome, as well as *MYCN* gene amplification and expression.[133] Various attempts to modulate the activities of these transporters using reversal agents have had limited clinical success.

p53 pathway defects. p53 is a key regulator of cell cycle checkpoints and apoptosis, which, upon activation by cellular stress, particularly DNA damage, binds DNA in a sequence-specific manner to activate the transcription of a large number of genes, including *p21, MDM2, BAX*, and *NOXA*.[142] MDM2 expression is induced by p53 and functions as a ubiquitin ligase targeting p53 for proteosome-mediated degradation by forming an autoregulatory feedback loop.[143] Amplification of *MDM2* suppresses p53 activity by increasing its degradation. $p14^{ARF}$ activates the p53 pathway by directly binding to and antagonizing the E3 ubiquitin ligase activity of MDM2.[144] Inactivation of $p14^{ARF}$ increases MDM2 levels, which in turn inactivates p53. Although p53 gene mutation is found in around 50 percent of human malignancies,[145] it is rare (<2%) in NB obtained at diagnosis.[135] However, p53/MDM2/$p14^{ARF}$ pathway defects are observed in NB cell lines derived at relapse,[151] and in nearly 50 percent of fresh tumors during the disease course. In vitro studies demonstrate that p53/MDM2/$p14^{ARF}$ pathway defects confer drug resistance to NB. Strategy to reactivate the p53 pathway is a promising approach to reverse drug resistance. Small-molecule p53 activators (e.g., nutlin3) have

clinical potential.[147] Selective checkpoint kinase (e.g., Chk1) inhibitors may also have utility in enhancing the efficacy of DNA-damaging agents, especially when the p53 pathway is defective.

Anaplastic lymphoma receptor tyrosine kinase (ALK). ALK is a tyrosine kinase transmembrane receptor with homology to neurotrophin receptors and the MET oncogenes, and restricted expression to the developing nervous system.[148] Many human cancers activate ALK signaling through fusion transcripts from chromosomal translocation events.[149] Human NB cell lines express ALK transcripts and ALK protein.[150] ALK was recently identified as a molecular target in NB by screening cell lines with pharmacological antagonists of the ALK kinase domain.[8,151–155] Activating mutations can also be somatically acquired in up to 12.4 percent of sporadic cases. ALK is a potential target for a subset of NB.

Angiogenesis

Multiple angiogenic pathways are active in high-risk NB tumors.[156] Significantly higher expression levels of VEGF, VEGF-B, VEGF-C, basic fibroblast growth factor, Ang-2, transforming growth factor alpha, and PDGF-α were found in advanced-stage tumors. Expression of PDGF-α was significantly associated with patient survival. Several drugs have potential against NB. Retinoids such as fenretinide,[157] TNP-470,[158 159] thalidomide,[160] and endostatin[161,162] have shown activity in preclinical testing, and anti-VEGF strategies using bevacizumab[163] or VEGF-TRAP[164] have also shown promising results. In a recent Phase I trial of bevacizumab in children, treatment was well tolerated. However, no objective responses were found.[165] Given the redundancy in angiogenic pathways exploited by NB, targeting multiple pathways or combination with chemotherapy or radiotherapy will likely be necessary for clinical responses.

Lymphocyte-Mediated Therapy

Cell therapy using natural killer (NK) cells and T-cells
NK cells express CD16, the low-affinity FcγR3 receptor required for binding mAb (e.g., 3F8 or ch14.18) and triggering NK-mediated ADCC. NK cells bear activating receptors (e.g., DNAM-1, NKG2D, NKp46, and NKp30) whose ligands are expressed on NB cells.[166] Human NK cells are effective against NB xenografts in NOD/scid mice.[167] They are licensed to kill if they lack killer inhibitory receptors (KIRs) for specific class I molecules,[168–170] consistent with the alloreactivity advantage after HLA-mismatched transplantation for the treatment of acute myelogenous leukemia.[171–173] Among children with high-risk NB undergoing autologous stem cell transplantation plus 3F8 immunotherapy, improved overall and progression-free survival are associated with the absence of one or more HLA class I ligands for the patient's NK cell inhibitory KIR receptor. These results suggest that NK tolerance is modified after ASCT, and that KIR-HLA genotypes may influence mAb- based immunotherapy. Activating KIR may also contribute to NB NK susceptibility. To bypass the low expression of HLA antigens on NB, T cells can also be retargeted using antibody-based chimeric receptors, and early clinical results look encouraging.[174,175]

Vaccines. Preclinical studies have shown that whole-cell vaccines engineered to express multiple transgenic immunostimulatory molecules are potent stimulators of the immune system. Using NB cell lines transduced with a combination of IL-2 and lymphotactin (LTN) (to enhance chemotaxis), antitumor response was seen in a Phase I clinical trial.[176] Using autologous neuroblastoma tumor cells, a similar strategy was tested in seven patients, with tolerable side effects. Injection site biopsies revealed infiltration of $CD4^+$ and $CD8^+$ lymphocytes, eosinophils, and dendritic cells. Peripheral blood lymphocytes, when assayed in vitro, showed increased tumor recognition.[177] More recent studies using GD2 mimics as vaccines have also shown promising preclinical results.[178]

Immunocytokines. Cell-mediated cytotoxicity has been highly effective against tumors in vitro and in animal models. Immunocytokines[179,180] have shown remarkable success in activating and redirecting effectors to human tumors. Most of these studies have focused on NK, NKT or T cells,[180] and granulocytes.[60] Antibody-IL-2 immunocytokine can eradicate metastatic murine NB while inducing long-term antitumor immunity.[179,180] Following initial successes with IL-2 immunocytokine, constructs containing other cytokines have also been tested with encouraging results.[180] These include IL-12, tumor necrosis factor, and lymphotoxin. More recently, the combination of a plasmid DNA vaccine and IL-2 immunocytokine in the mouse model was shown to be more effective than when either one was administered alone.[181] In a Phase I study, hu14.18-IL2 caused immune activation/modulation as evidenced by elevated serum levels of soluble IL-2 receptor a (sIL2Ra) and lymphocytosis, but there were no measurable complete or partial responses.[182]

FUTURE DIRECTIONS

If the current direction of success in NB treatment is sustained, one can envision cure rates beyond the 85 percent mark for local–regional or 4S NB with minimal or no cytotoxic therapy, where risk-appropriate treatments are applied to the remaining 15 percent. Infants with stage 4 NB may be spared some of the cytotoxic therapy while maintaining their cure rates of greater

than 90 percent. However, the less than 25 percent long-term survival rate for high-risk stage 4 NB, despite highly toxic therapies, is highly unacceptable. The most common reason for failure is chemoresistance of both soft tissue disease (e.g., retroperitoneal, liver, CNS, and lung) and osteomedullary metastases. Although some of these difficult cases involve gross resistant disease, the majority are microscopic residuals.

Directing novel and effective drugs at the tumor cell ("seed") is an obvious solution, but therapies focused on the tumor microenvironments ("soil") at the time of minimal residual disease should also be explored. However, given the complex gene signatures of metastatic and chemoresistant NB, multiple pathways in the "seed" will need to be targeted. Likewise, for the "soil," drug strategies directed at multiple pathways will most likely be necessary. It is encouraging that mAb specific for a single antigen (ganglioside GD2) can eliminate chemoresistant NB to produce long-term survival. With a better understanding of the host immune capability and immunogenomics (e.g., FcγR polymorphism and KIR mismatch), patients with responder genotypes can be identified up front. To bypass nonresponder genotypes, mAb may need to be genetically modified and cell therapy using appropriately mismatched NK cells applied. When this is combined with the use of metronomic low-dose chemotherapy that is not destructive to the immune system, and pathway-specific small molecules that synergize with standard chemotherapy to reduce myelotoxicity and organ damage, the ultimate cure for metastatic NB may be in sight.

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26 Retinoblastoma

Ira J. Dunkel and David H. Abramson

PATTERNS OF METASTATIC SPREAD, ORGAN SPECIFICITY, TIMING OF RECURRENT DISEASE, AND COMPLICATIONS CONFRONTED WITH METASTASIS

Retinoblastoma is the most common type of primary intraocular pediatric tumor. The incidence of retinoblastoma is approximately 1 in 20,000 live births, and it is estimated that there are about 300 to 350 new cases each year in the United States.

There are two forms of retinoblastoma. The most common form of retinoblastoma, known as *unilateral disease*, presents with a single tumor focus at a median age of about twenty-four months. Patients may also present with multiple tumor foci, usually in both eyes (*bilateral disease*), at a median age of about twelve months. The recognition of the earlier presentation of bilateral disease led to the classic two-hit hypothesis of retinoblastoma etiology [1]; in 1986 the "hit" was discovered to be a mutation in the *RB1* gene on chromosome 13q14 [2]. It is currently believed that all patients with bilateral disease have a germline mutation in *RB1*, whereas most patients (about 85%) with unilateral disease have a sporadic mutation in the tumor cells only [3]. DNA sequencing to identify the mutation can be performed at specialized laboratories and is clinically useful to identify survivors of unilateral disease who may be at risk for other cancers later in life (secondary malignancy) and/or transmission of the disease to their offspring, to determine which relatives or offspring of survivors inherit the mutation and need to be carefully clinically screened, and to allow survivors with a germline mutation the option of considering preimplantation genetic diagnosis and in vitro fertilization to avoid the risk of transmitting the mutated *RB1* to their offspring [4].

In most industrialized nations, retinoblastoma usually presents with localized intraocular disease that may threaten the eye and vision, but metastatic disease is uncommon (<5% of cases). However, metastasis remains a common and usually fatal problem for retinoblastoma patients in the poorest nations [5].

There are different mechanisms for retinoblastoma spread outside the eye. The tumor may grow contiguously through the wall of the eye (the choroid and sclera) into the orbit, or it may grow back through the optic nerve and invade the brain (Figure 26.1). In the case of spread to the brain, retinoblastoma may enter the subarachnoid space and spread along the leptomeninges via the cerebrospinal fluid. It may also spread hematogenously, resulting in metastases to the bone, bone marrow (Figure 26.2), and/or liver. Notably, even though the eye has only limited lymphatic drainage (through the conjunctiva), in rare cases retinoblastoma may spread to produce regional nodal disease [6].

The presenting signs and symptoms of metastatic retinoblastoma are quite variable and depend on the affected site(s). When retinoblastoma metastases occur, they are typically clinically evident within a few months of intraocular primary disease diagnosis. In patients who have previously undergone enucleation (surgical removal of the eye), orbital recurrences are often discovered upon parental observation that the prosthesis no longer fits well. More extensive orbital disease may present as a visible mass. Bone disease may present with pain, and bone marrow disease may present with abnormally low blood counts; however, metastases at those sites and in the liver may be asymptomatic and discovered only on evaluation of extent of the disease. Central nervous system (CNS) signs and symptoms may include headache, irritability, emesis, and/or focal neurological signs.

STATE OF THE ART IN DIAGNOSTIC/PROGNOSTIC TESTING

Patients in whom metastatic retinoblastoma is suspected should be thoroughly evaluated to assess the

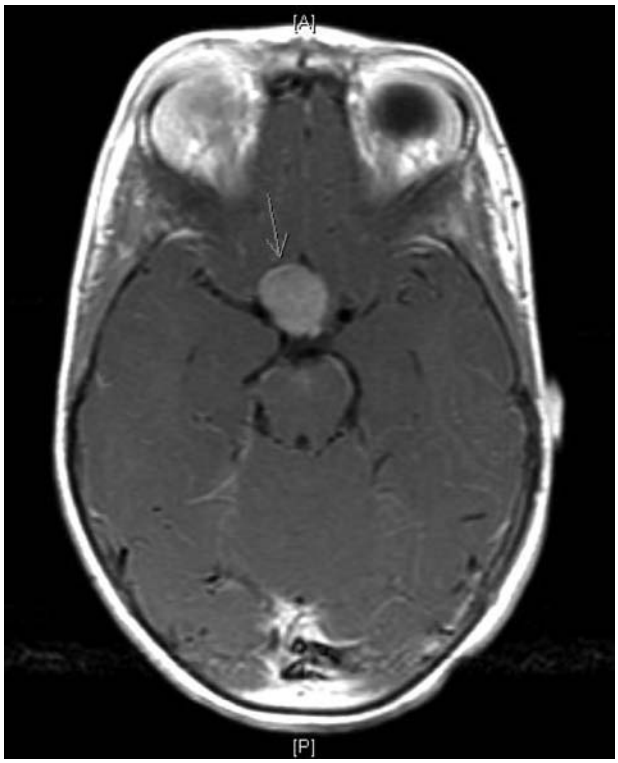

Figure 26.1. A brain MRI scan (axial T-1 post-gadolinium contrast injection) demonstrating metastatic retinoblastoma.

anatomic sites at risk for metastasis. We recommend that the evaluation should include:

- Brain and orbit MRI with and without contrast
- Lumbar puncture for CSF cytology
- Spine MRI with and without contrast (if CNS disease is strongly suspected or appropriate focal neurological signs present)
- Abdominal CT with IV contrast
- Bone scan
- Bone marrow aspirate and biopsy

CURRENT TREATMENT PARADIGMS FOR PRIMARY AND METASTATIC RETINOBLASTOMA MANAGEMENT

Although the crucial role of *RB1* mutations in retinoblastoma etiology has been well described, this understanding has not yet translated into any clinical therapeutic approach. Currently, patients with retinoblastoma are treated with some combination of surgery, radiation therapy, and chemotherapy.

Children with unilateral disease usually present with extensive intraocular disease, limiting the visual potential of the eye. Enucleation (surgical removal of the eye) is performed in the large majority of cases and is usually curative.

Children with bilateral disease historically have had an excellent chance of surviving the primary disease with treatment consisting of enucleation and/or external beam radiation therapy. However, in patients with germline *RB1* mutations, there is a significant risk of secondary malignancy later in life. This risk is further increased with the use of external beam radiation to treat the primary cancer. In a recently published update of a large cohort of retinoblastoma survivors, it was shown that the 963 patients with germline *RB1* mutations had a 36 percent chance of developing another cancer up to fifty years after their original diagnosis [7]. Radiation therapy further increased the risk by 3.1-fold [7]. Age at the time of radiation therapy also appears to be important: irradiation at less than twelve months of age has been associated with a greater risk of secondary malignancies, whereas risk was similar in patients one year of age or older when irradiated and patients who never received irradiation [8].

In the 1990s, recognition of the risks associated with radiation therapy motivated many groups to use carboplatin-based chemotherapy instead, both in an attempt to improve eye salvage and to avoid the use of external beam radiation therapy. Several recent publications have summarized the literature to date regarding the use of chemotherapy in primary retinoblastoma [9]. Studies show that multiagent regimens (usually vincristine, carboplatin, and etoposide, with or without cyclosporine A) in conjunction with focal therapy (laser, cryotherapy, and/or plaque brachytherapy) have been associated with approximately a 90 percent chance of radiation- and enucleation-free survival for eyes with less advanced disease (Reese-Ellsworth group 1–3) and approximately a 30 percent chance for eyes

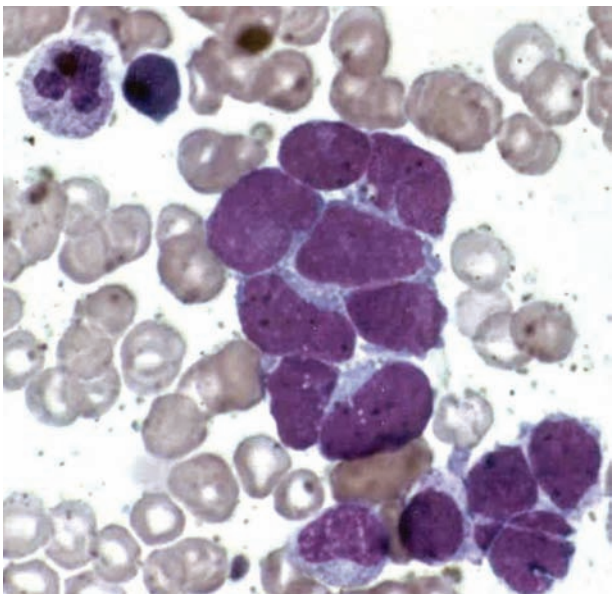

Figure 26.2. A bone marrow aspirate demonstrating metastatic retinoblastoma. The metastatic cells are large and undifferentiated, and aggregate together.

with more advanced disease (Reese-Ellsworth group 4–5) [9].

Importantly, patients with metastatic retinoblastoma have a poor prognosis when treated with conventional therapy. For example, two recent reports from Argentina and Brazil revealed that only one of forty patients survived following treatment with conventional-dose chemotherapy and radiation therapy [6, 10]. However, it appears that patients with metastatic retinoblastoma may be cured when the regimen is intensified to include high-dose chemotherapy with autologous stem cell rescue (ASCR). Institut Curie investigators treated eleven patients with metastatic retinoblastoma not involving the CNS with high-dose carboplatin, etoposide, and cyclophosphamide followed by ASCR and noted that five of the eleven (45%) demonstrated event-free survival [11].

Our group at the Memorial Sloan-Kettering Cancer Center previously reported four patients with metastatic retinoblastoma who promptly responded to a vincristine, platinum agent, cyclophosphamide, and etoposide (plus doxorubicin in one case) induction regimen, were treated with a high-dose carboplatin, thiotepa, and etoposide with ASCR regimen, and were all event-free survivors [12]. We subsequently presented updated data and noted that seven of ten patients with metastatic retinoblastoma not involving the CNS were event-free survivors at a median of eighty-four months post-diagnosis of metastases [13]. Two relapsed with CNS involvement at seven and ten months after the initial diagnosis of metastatic disease (prior to high-dose chemotherapy). These two failures were associated with treatment delays owing to fungal infection ($n = 1$) and insurance denial ($n = 1$); the patients later died of progressive tumors. One patient relapsed with CNS involvement sixteen months after the diagnosis of metastatic disease and later died of a progressive tumor. The remaining seven patients survived event-free 16 to 130 months after the initial diagnosis of metastatic disease.

Subsequently, other groups from Germany, Memphis, Los Angeles, and Japan published small studies using various high-dose regimens. The overall results appear promising, with approximately two-thirds of patients in those studies achieving event-free survival [14–17]. Overall, these studies indicate that high-dose chemotherapy with ASCR is associated with improved survival in patients with metastatic retinoblastoma not involving the CNS. Additionally, the inclusion of thiotepa in the regimen may be associated with a lower risk of CNS recurrence (the most likely site of failure) because of excellent CNS penetration of that agent [18]. Fewer data are available regarding the prognosis of patients with retinoblastoma involving the CNS (metastases or trilateral disease) treated with high-dose chemotherapy and ASCR, but such an approach appears promising in that setting as well [19–20].

FUTURE DIRECTIONS AND OUTLOOK IN RETINOBLASTOMA MANAGEMENT

In 2008, the Children's Oncology Group (COG) opened a treatment protocol (COG ARET 0321) that will attempt to confirm the promising results discussed previously in a multiinstitutional and international setting. Patients with regional extraocular retinoblastoma (orbital disease, regional nodal disease, and/or optic nerve margin positivity) will receive aggressive conventional chemotherapy and involved-field external beam radiation therapy. Those with distant metastatic disease and those with trilateral retinoblastoma will receive aggressive conventional induction chemotherapy; have autologous stem cells harvested; receive high-dose carboplatin, thiotepa, and etoposide with ASCR; and, depending on their response to induction, will be considered for involved-field external beam radiation therapy. The protocol also includes a request that primary and metastatic tumor tissue samples be submitted for biological analyses. By analyzing those samples, we hope to identify molecular targets that may allow us to investigate targeted agents for metastatic retinoblastoma in the future.

A significant problem is that the treatment approach being used in COG ARET 0321 requires a great deal of advanced technology and is very expensive. This unfortunately prohibits its use in many of the poorer nations where most patients with metastatic retinoblastoma live. New therapeutic approaches that can help treat those patients are urgently needed.

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27 Primary Brain Tumors and Cerebral Metastases

Matthew C. Tate and Mitchel S. Berger

Metastatic brain tumors (MBT) are the most common tumor type encountered in the brain. As survival of patients with primary tumors has improved over time, the incidence of MBT has increased. In this chapter, the basic biological and clinical aspects of metastatic spread to the brain are presented, including an overview of the epidemiology of MBT and recent advances in tumor biology particularly relevant to brain metastasis. Therapeutic strategies for MBT, including surgery, radiation, and chemotherapy, are reviewed, with an emphasis on application of randomized clinical trial data to practice guidelines. Finally, systemic metastases of primary brain tumors are discussed. Although relatively rare in comparison to metastases of systemic cancers to the brain, the frequency of extraneural metastases is increasing as therapy for primary brain tumors improves, thus warranting consideration.

INTRACRANIAL METASTASES

Epidemiology

Brain metastases are the most common intracranial tumor seen in adults, with approximately 10 percent of adults with cancer developing symptomatic brain metastases [1]. In the United States, the estimated prevalence of brain metastases is 200,000 cases per year [2], with an incidence of 8.2 per 100,000 persons [3]. The three most common primary cancers that metastasize to the brain are lung (38%), breast (19%), and melanoma (13%). Among these, melanoma demonstrates the highest likelihood of metastasis, with a rate approaching 50 percent in patients diagnosed with the disease. Table 27.1 lists the percentage of patients with symptomatic intracranial metastases at the time of death and the percentage of patients with brain metastases at autopsy in various primary tumor types. Importantly, metastatic tumors of the brain appear to be increasing in frequency over time, likely as a result of improved survival owing to more effective treatment of the primary cancer, as well as increased detection as a result of advances in neuroradiology.

Distribution of metastases in the brain roughly parallels cerebral circulation. Approximately 85 percent of metastases are found in the cerebral hemispheres, typically in watershed areas between major cerebral arteries. Ten to 15 percent of metastases are found in the cerebellum, and about 3 percent are found in the brainstem [4]. In terms of the number of metastatic foci at time of diagnosis, 53 percent of patients have single metastases [5], and 70 percent have three or fewer metastases [4].

Biology of Brain Metastasis

The process of metastasis begins with the growth of a solid tumor, partly owing to an imbalance of cell proliferation and apoptosis. As the tumor expands, a subset of cells invades the vasculature and is transported to distant organs. Approximately 0.01 percent of circulating tumor cells are able to evade circulating immune system components, invade the blood–brain barrier (BBB), and survive at a distant site [6]. Once established, the micrometastasis expands in a process that requires disruption and cooperation of the host site. The complex processes of establishing a macrometastasis require reorganization of the extracellular matrix, recruitment of new vasculature, and import of supporting cells from the native host tissue. In this section, these processes are discussed, with a particular focus on brain-specific phenomena and their relationship to clinical MBT.

For systemic tumors to reach the arterial circulation and thus have the potential to reach the brain, they must either (1) grow in the lung and enter the pulmonary venous system (primary lung cancer); (2) cross a patent foramen ovale and thus bypass the pulmonary vasculature; or (3) traverse pulmonary capillaries.

TABLE 27.1. Distribution of symptomatic brain metastases vs. brain metastases found at autopsy as a function of underlying primary tumor.

Primary tumor	% of patients with symptomatic brain metastasis at time of death	% of patients with brain metastasis at autopsy
Melanoma	50	72
Lung	24	34
Prostate	22	31
Breast	21	30
Urinary system	16	23
Leukemia	16	23
Non-Hodgkin lymphoma	11	16
Colorectal	5	7
Female genital tract	5	7
Pancreas	5	7

Adapted from DeAngelis et al. [8].

These limitations help to explain both the high incidence of concomitant lung metastases at the time of intracerebral metastasis diagnosis and the propensity of primary lung cancers to metastasize to the brain. Two mechanisms that promote eventual intracerebral metastasis have been proposed. First, given that a large portion of cardiac output is devoted to brain perfusion, circulating tumor cells in the arterial circuit have a relatively high probability of entering the cerebral circulation. Second, certain brain microenvironments are particularly suited for deposition and survival of certain primary tumor cell types. This is referred to as the "seed and soil" hypothesis, with the "seed" (primary tumor cell), though being "planted" at multiple sites, surviving only in the appropriate "soil" (host site of metastasis). For example, metastatic pelvic tumors, although distributed to all brain areas, survive preferentially in the cerebellum. Additionally, p75 neurotrophic receptor-expressing melanoma cells show an increased capacity to establish intracerebral metastases, as compared with non–p75-expressing melanoma tumor cells [7].

With advances in targeted primary tumor chemotherapy, another factor promoting selective metastasis to the brain that is becoming increasingly relevant is the "sanctuary" afforded to the tumor cells by the BBB. Chemotherapeutic agents are unable to cross the BBB even though systemic disease is well controlled, thereby affording relative protection to the metastatic tumor cells within the brain and "selecting" for intracerebral metastases [8]. However, protected intracerebral metastases avoid exposure to high levels of first-line chemotherapy agents; as MBT grow, the BBB becomes leakier, and it is plausible that recurrent intracerebral tumors may still be quite responsive to first-line chemotherapy regimens.

Clinical Issues Related to Intracerebral Metastases

Symptoms

The neurologic symptoms resulting from intracerebral metastases are similar to those observed for primary brain tumors. The most common symptom is headache, seen in approximately 25 percent of affected patients. Other common findings include focal weakness, seizures, cognitive changes, and ataxia. In comparison with primary brain tumors, metastatic lesions demonstrate a higher degree of peritumoral edema; thus, symptoms tend to progress more rapidly in patients harboring brain metastases. This relatively high level of edema also explains why symptoms of MBT often respond better to corticosteroids compared with similar symptoms in patients with primary brain tumors [8]. Acute alterations in neurologic function are often secondary to intratumoral bleeding and/or seizure activity. Interestingly, and for reasons that remain unknown, patients with multiple MBT may have simultaneous intratumoral bleeding at several foci.

Imaging and Diagnostic Considerations

The diagnostic procedure of choice when considering the diagnosis of MBT is MRI with and without gadolinium. In the case of a single metastatic lesion seen on CT, an MRI scan should be obtained to evaluate for any additional metastatic foci. Metastatic lesions on MRI are located at gray–white junctions, have a higher degree of edema, and are more regular in shape compared with primary brain tumors; these lesions also uniformly enhance (smaller lesions) or rim-enhance (larger lesions). Of note, the rim-enhancement

of MBT seen on MRI is typically larger than that of intracranial abscesses and is more regular than what is seen in primary tumors [8]. Advanced imaging techniques are often helpful in distinguishing metastatic lesions from other lesions on the differential diagnosis. For example, diffusion-weighted MRI demonstrates a hyperintense core in abscesses versus hypointensity in metastases. An important question that often arises is the distinction between recurrent tumor and treatment effect; MR spectroscopy is a technique that can aid in that distinction. In primary or metastatic lesions, choline:creatinine and choline:NAA ratios are elevated relative to those in the normal brain, whereas in treatment-induced changes such as radiation necrosis, spectroscopy demonstrates lipid-associated but not choline peaks.

Although diagnosis of a metastatic brain tumor is often relatively easy in the context of a known primary tumor and characteristic MRI features, there are certain clinical scenarios that merit further discussion. In the absence of a known primary tumor (negative chest/abdomen/pelvis CT, negative serum markers), a single brain lesion should be addressed surgically to establish a diagnosis. In a trial conducted by Patchell et al., 11 percent of patients with a known history of cancer and a single brain lesion were found to not have metastatic disease on pathologic diagnosis [9]. For brain lesions observed in the context of primary tumors that rarely metastasize to the brain but are known to suppress the immune system, such as Hodgkin's disease, intracerebral infection should be strongly considered. Also, patients undergoing chemotherapy for solid tumors are at an increased risk for intracerebral infections [8]. Other conditions that can mimic metastases on MRI include primary brain tumors, demyelinating disease, infarction, radiation necrosis, granulomatous lesions, and hemorrhage. Thus, depending on the particular clinical scenario, it is important to include these conditions in the differential diagnosis, as each would direct a different treatment strategy.

Symptom Management

Corticosteroids

Corticosteroids are a mainstay of symptomatic treatment in metastatic brain tumor patients, as many of the neurologic symptoms arise as a result of peritumoral edema and resultant elevation of intracranial pressure. Dexamethasone is typically the corticosteroid of choice because of its long half-life, lower mineralocorticoid cross-reactivity, and milder cognitive side effects [10]. Although MBTs are known to upregulate the expression of glucocorticoid receptors, the exact mechanisms by which glucocorticoids ameliorate tumoral edema are not well understood [11]. In practice, dexamethasone should be titrated to the lowest dose that achieves symptom control, thereby decreasing the risk of side effects from long-term steroid use. Management of brain tumor patients on long-term dexamethasone additionally includes low-dose haloperidol for mild psychiatric symptoms, prophylactic antibiotics to prevent *Pneumocystis carinii* pneumonia (PCP) in high-risk patients (lymphopenia, CD4 count <200 cells/mm^3), gastric acid inhibitors for the duration of steroid therapy, as well as calcium/vitamin D supplementation, exercise, and/or bisphosphonate therapy to reduce the risk of osteoporotic fracture.

Anticonvulsants

Seizures occur in 20 percent of MBT patients at presentation [12]. Although the presence of seizures does not significantly affect survival, it has a strong impact on the quality of life in patients with MBT. Metastatic lesions localized to the cortex are more likely to cause seizures [11]; focal seizures comprise the majority of MBT-related seizures, with complex partial and generalized seizures being less common [12]. Interestingly, direct electrical recordings and histological data indicate that epileptic foci are not located within the tumor mass itself but rather localize to the irritated tissue adjacent to the tumor. The mechanism is thought to involve a slightly alkaline environment that favors excitatory neuronal pathways while reducing GABAergic inhibitory pathways. This is consistent with peritumoral biopsies that show increased glutamine- and decreased GABA- and somatostatin-containing neurons [13–15].

Anticonvulsants are typically recommended in all patients with documented seizures, with monotherapy of phenytoin, carbamezepine, or valproate all being reasonable first-line agents. A second traditional agent or a newer agent may be added if seizure activity at high therapeutic levels of monotherapy cannot be controlled. Levetiracetam is a common choice because of its good efficacy and a lack of interaction with other oncologic drug classes. Drug levels should be monitored as needed to ensure appropriate dosing and compliance. Interestingly, a placebo-controlled randomized trial conducted by Glantz et al. to address the issue of prophylactic anticonvulsant therapy in metastatic brain tumor patients demonstrated no significant improvement in subsequent seizure frequency in patients with primary brain tumor or MBT who were given prophylaxis with anticonvulsants [16]. Thus, antiepileptic therapy is typically withheld until seizure activity is documented. Finally, for patients receiving anticonvulsant prophylaxis following surgical removal of metastatic lesions, reducing medication dosage starting one month postsurgery is the typical approach.

DVT Prophylaxis

The incidence of deep venous thrombosis (DVT) following metastatic brain tumor resection is 20 percent, as determined by ^{125}I-labeled fibrinogen scans [17]. Clinical factors that increase the risk of DVT and pulmonary emboli (PE) are arm/leg paresis, history of prior DVT/PE, and longer operative time [11]. Although they did not specifically address brain tumor patients, Danish et al. demonstrated that although subcutaneous heparin administration for DVT prophylaxis decreased the rate of DVT/PE, it also increased the rate of hemorrhage. Given that the benefit of reducing DVT/PE with heparin prophylaxis was only modestly increased compared with simple mechanical prophylactic measures and was far outweighed by poor outcomes secondary to hemorrhage, the recommendation was to withhold prophylactic heparin following surgery [18]. Considering the potential risk of tumor hemorrhage with institution of anticoagulation therapy, an important question to address is the most prudent way to treat a documented thromboembolic event. Most data indicate that the risk of hemorrhage is about 5 percent [11]; thus, most practices advocate conservative anticoagulation therapy: either heparin bridge to warfarin therapy (modest INR goal of 1.5–2.5) or enoxaparin therapy, which is as effective as warfarin and has a lower hemorrhage rate in general, although this has not been specifically evaluated in brain tumor patients [19]. In a subset of patients, including those with tumor bleeding, high fall risk, or documented GI bleeding, placement of an inferior vena cava filter may be considered.

Recursive Partition Analysis	
Class I	age<65, KPS≥70, controlled primary tumor, no extracranial mets
Class II	All other patients
Class III	KPS < 70

Score Index for Radiosurgery			
Score	0	1	2
Age	≥60	51–59	≤50
KPS	≤50	60–70	
Systemic disease	active	stable	none
# lesions	≥3	2	1
Volume of largest lesion (mL)	>13	5–13	<5

Basic Score for Brain Metastases		
Score	0	1
KPS	50–70	80–100
Conrol of primary tumor	>3	2–3
Extracranial mets	Yes	No

Graded Prognostic Assessment			
Score	0	0.5	1
Age	>60	50–59	<50
KPS	<70	70–80	90–100
# CNS mets	>3	2–3	1
Extracranial mets	Yes		No

Figure 27.1. Description of four major prognostic indices for metastatic brain tumors.

Prognostic Factors

A number or variables have been shown to be important predictors of survival in patients with brain metastases. These factors include number of brain metastases, Karnofsky Performance Status (KPS), primary tumor type, age, status of systemic disease control, and treatment scheme. In addition, breast cancer and melanoma studies have revealed that the time interval between primary tumor diagnosis and discovery of brain metastases is important, with an increased duration being associated with a more favorable prognosis [20, 21]. Of the prognostic factors listed above, treatment regimen has the best predictive value, followed by KPS.

In the future, a more robust prognostic index could help clinicians in guiding treatment decisions as well as enabling more efficient design of clinical trials. Three validated prognostic indices have been proposed in recent years: recursive partitioning analysis (RPA), Score Index for Radiosurgery (SIR), and Basic Score for Brain Metastases (BSBM). The RPA scheme, developed from the Radiation Therapy Oncology Group (RTOG) studies, comprises three classes, which incorporate patient age, KPS score, control of primary tumor, and presence of extracranial metastases [22]. SIR sums scores (0–2) from five categories: age, KPS, systemic disease status, number of lesions, and largest lesion volume [23]. BSBM sums scores (0–1) for three categories: KPS, control of primary tumor, and extracranial metastases [24]. Figure 27.1 describes the three proposed scoring schemes in detail. Potential limitations of these indices include required estimation of systemic disease control, which can be difficult, as well as the absence of the number of metastases from the RPA and BSBM schemes. A new grading scheme, termed Graded Prognostic Assessment (GPA), was recently published. This scheme incorporates new RTOG data and attempts to address the shortcomings of the other three indices [25]. The GPA sums scores (0, 0.5, or 1) in four categories (age, KPS, number of intracranial metastases, and presence of extracranial metastases) and is less subjective than the other three measures (Figure 27.1). Using retrospective data to compare performance of all four indices, it was demonstrated that the GPA and RPA have the highest significance among classes. Thus,

it may be reasonable for future trials to stratify patients based on GPA and RPA class to further validate these scales in a prospective manner.

Radiation Therapy

Whole-Brain Radiation Therapy

Traditional therapy for MBT has included symptomatic therapy with steroids and anticonvulsants as well as whole-brain radiation therapy (WBRT). Available data suggest that, compared with no treatment or standard steroid therapy alone, WBRT prolongs survival by three to four months. RTOG data show that partial or complete response, both radiographically and clinically, is seen in approximately 60 percent of patients treated with WBRT [26]. Aggregated data from multiple studies further indicate that WBRT effectiveness is not substantially affected by dosage, timing, or fractionation schedules [27]. Typically, all patients treated for metastatic brain tumors receive WBRT (30 Gy total dose in ten daily fractions), either as monotherapy ($\geq$4 metastases) or in combination with surgical resection, stereotactic radiosurgery, and/or chemotherapy. Recent modifications of WBRT protocols with radiation sensitizers have shown some promise. In particular, subgroup analyses in trials investigating motexafin gadolinium (a redox agent that targets tumor cells and increases radiosensitivity) and efiproxiral (a modifier of hemoglobin that decreases tumor hypoxia, which in turn increases radiation sensitivity) as WBRT enhancers have demonstrated modest improvement in selected outcome measures for lung and breast cancer patients with brain metastases [28, 29].

Stereotactic Radiosurgery

For select patient populations, typically those with one to three metastases, surgical resection has been shown to extend the typical WBRT survival time of three to six months to nine to twelve months [9]. With this proof of principle of local control in patients with MBT, stereotactic radiosurgery (SRS) has been investigated as a potential therapeutic approach. The rationale for the use of SRS is to use convergent radiation to deliver focused therapy while minimizing radiation to surrounding fields. SRS is particularly attractive for treatment of brain metastases because of the discrete nature and uniform geometry of metastases. The safety of SRS for the treatment of metastatic brain tumors and maximum tolerated doses has been determined based on tumor size [30]. For a given tumor size, though, increasing the SRS dose increases toxicity without improvement in the response rate [31].

Two studies have evaluated the role of SRS in addition to WBRT for the initial treatment of brain metastases. Kondziolka et al. evaluated the effect on local tumor control of SRS within one month of WBRT in patients with two to four brain metastases [32]. Given the major improvement in local control seen with the addition of SRS (thirty-six months to recurrence in the SRS arm versus six months in the WBRT-only arm), the study was halted at 60 percent of recruitment goals. As a consequence of the small sample size, although the study proved the superiority of adding SRS for local control, it was unable to demonstrate a significant effect on survival. A second trial, by Andrews et al., randomized 333 patients (RPA class I and II, one to three metastases) to either WBRT or WBRT with SRS to each lesion within one week of WBRT. In this analysis, overall survival was unchanged. However, improvement in survival was achieved in two subsets of patients – those with a single metastasis and those with RPA class I status. Importantly, as intent-to-treat analysis was used and 19 percent of patients in the SRS group did not actually receive SRS treatment, these findings likely underestimate the true benefit of the treatment [33]. Taken together, these trials suggest that SRS as an adjunct to WBRT is a safe and effective therapy for patients with three or fewer metastases.

As the benefit of SRS in achieving local tumor control is now clear, Aoyama et al. conducted a randomized study to examine the efficacy of SRS monotherapy as compared with SRS combined with WBRT for the treatment of patients with one to four metastases less than 3 cm in diameter [34]. Results from this study indicated that, although overall survival was not affected, intracranial relapse occurred more frequently in the SRS-only group, necessitating salvage therapy. Thus, SRS monotherapy is not recommended for treatment of MBT at this time.

Prophylactic Irradiation

The potential benefit of prophylactic cranial irradiation (PCI) in patients with a high likelihood of brain metastases but without known intracranial disease has been investigated. In patients with small-cell lung cancer (SCLC), where the risk of brain metastasis is more than 50 percent at two years, meta-analyses of twelve randomized trials showed that PCI decreased the occurrence of brain metastases by 50 percent and increased survival by 17 percent in the subset of patients who had a complete response to chemotherapy [35]. One of potential adverse effects with PCI is cognitive decline; thus, two randomized trials formally evaluated cognitive status after PCI in SCLC patients. Both trials found no difference in neurocognitive outcomes at one to two years [36, 37]. A Phase I trial is currently investigating the utility of prophylactic irradiation in non–small-cell lung cancer (NSCLC) patients [38].

Surgery

Surgical therapy has been the standard treatment of singe brain metastases since the 1980s. The rationale for surgical resection of MBT includes rapid treatment of mass effect, establishment of diagnosis, and, by definition, local control. Published surgical mortality rates are approximately 2 percent for high-volume centers [39]. In general, the goal of surgery is gross total resection, while minimizing damage to the surrounding tissue. Most centers now use intraoperative MRI-based image guidance systems to assist with planning of appropriate trajectories to minimize damage to important brain structures and facilitate efficient localization of the tumor. For patients with metastatic lesions in or adjacent to eloquent brain regions, preoperative functional MRI and/or intraoperative electrical stimulation techniques aid in sparing language and motor function, an important consideration for patients with a relatively short life expectancy. The pivotal trials that established the importance of surgical resection in the treatment of MBT are discussed in the following sections.

WBRT with or without Surgical Resection for Solitary Brain Metastases

Preliminary studies in the early 1980s suggested that surgical resection of solitary brain metastases may afford a better outcome than WBRT alone [40, 41]. However, concerns persisted regarding the potential confounding of these data owing to the better clinical health of patients being selected for surgery. Three randomized trials examined the benefit of surgical resection versus WBRT alone in the treatment of single metastatic lesions in the brain. In 1990, Patchell et al. published results of a trial evaluating the benefit of surgical resection of a single brain metastasis followed by WBRT (surgical group) versus surgical biopsy followed by WBRT (radiation group) [9]. They found that recurrence at the site of the original metastasis was decreased in the surgical group as compared with the radiation group (20 percent versus 52 percent, respectively). Survival was significantly longer in the surgical group (forty weeks versus fifteen weeks in the radiation group), and patients in the surgical group had a longer duration of functional independence (thirty-eight weeks versus eight weeks for the radiation group). A second trial by Vecht et al. comparing surgery and WBRT to WBRT alone also demonstrated improved survival in the surgery/WBRT arm [42]. In addition, functional independence was achieved faster and lasted longer in the surgery/WBRT group. Importantly, patients with progressive extracranial disease did not benefit from additional surgical therapy in this study.

In contrast, a randomized trial reported by Mintz et al. did not demonstrate any additional benefit of surgery/WBRT versus WBRT alone [43], although this study included more patients with poor prognostic features (low KPS, active extracranial disease), which is in concordance with the lack of benefit seen in patients with progressive systemic disease in the trial by Vecht et al. In summary, surgical excision followed by WBRT is recommended for patients with a single metastatic focus and good prognostic features.

Surgery for Multiple and Recurrent Brain Metastases

Another scenario of interest is the role of surgical resection in patients with multiple brain metastases. In general, in those patients, surgery is limited to cases involving a single dominant symptomatic lesion or for which a tissue diagnosis is required [1]. A retrospective study by Stark et al. of patients receiving surgery for up to three brain metastases suggests that patients with three or fewer lesions may still benefit from surgical resection [44]. In this study, age less than 70, number of metastases fewer than three, resection of all intracranial lesions, and postoperative Glasgow Outcome Score were all associated with improved survival. This study also evaluated the role of surgery for recurrent brain metastases, with multivariate analysis demonstrating a benefit of reoperation.

Surgery with or without WBRT

Given the efficacy of surgical therapy for single/multiple brain metastases, the utility of postoperative WBRT has been questioned. Accordingly, Patchell et al. found that addition of postoperative WBRT improved local, distant, and overall control rates as compared with surgery alone in patients with solitary brain metastases [9]. As with the addition of post-SRS WBRT, post-surgical WBRT did not improve overall survival. However, patients with postoperative WBRT were less likely to die of neurologic causes compared with those treated with surgery only.

Surgery versus SRS

With the established benefit of WBRT combined with either surgical resection or SRS in the treatment of one to four brain metastases, the question arises as to the relative efficacy of surgery versus SRS in this context. Unfortunately, although many patients would likely qualify for either procedure, no randomized trials have directly addressed this question. Retrospective studies aimed at comparing SRS and surgery have largely found no difference in relative efficacy, although one study by Bindal et al. suggested improved survival in the surgery group [45]. In the absence of rigorous

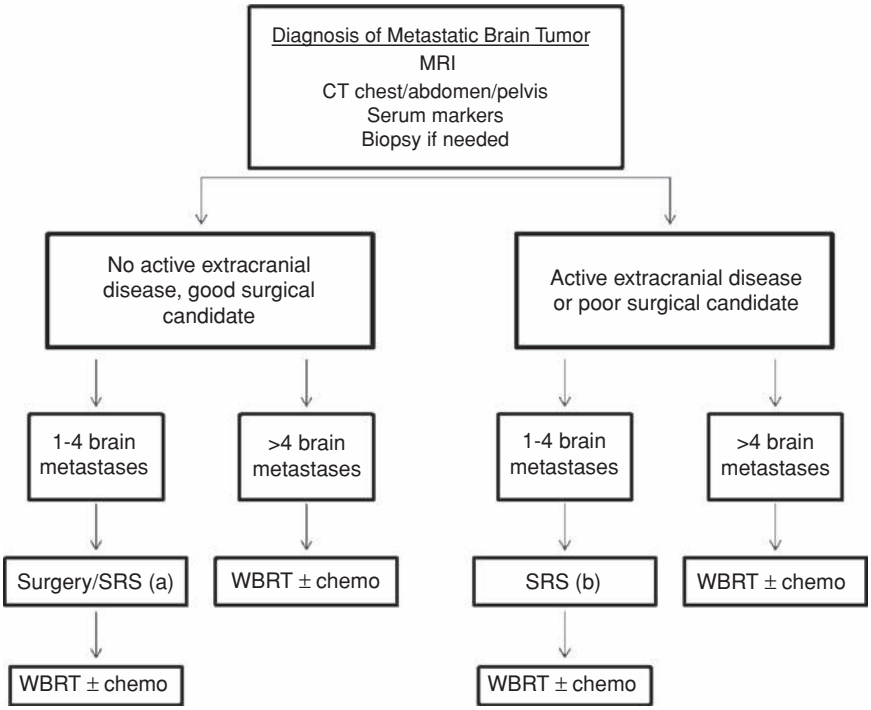

Figure 27.2. Algorithm for treatment of metastatic tumors. (a) Surgical resection of all accessible lesions, SRS for remaining lesions; (b) SRS therapy to all lesions <4 cm in diameter. SRS = stereotactic radiosurgery. Adapted from Kaal et al. [76].

randomized data, factors in choosing surgery over SRS include (1) large lesions with significant mass effect and/or edema that are easily resectable, given the relatively quick resolution of symptoms, and (2) posterior fossa metastases, owing to the potential acute neurological decline from obstructive hydrocephalus that can be seen even with a modest increase in mass effect/edema. SRS may be favored in cases of metastases difficult to address surgically, such as brainstem or eloquent cortex lesions. Combined SRS and surgery (in addition to WBRT) will likely become the standard therapeutic approach in patients with one to four lesions when only a subset of the lesions is surgically resectable. See Figure 27.2 for a proposed management algorithm.

Chemotherapy

Chemotherapy for the treatment of MBT typically has been reserved for salvage or for brain cancers known to be particularly susceptible, such as lymphoma, SCLC, and germ cell tumors. The lack of efficacy in chemotherapeutics has been attributed to inability of these relatively large, hydrophilic compounds to cross the BBB. However, whereas most of the data on BBB permeability has been estimated from animal data of drug concentrations in the serum and the cerebrospinal fluid (CSF), recent evidence indicates that BBB permeability may be significantly increased in metastatic tumor beds. Thus, animal studies may significantly underestimate the activity of chemotherapeutic agents in the brain. Accordingly, studies have shown equivalent intracranial and extracranial activity of chemotherapeutics that theoretically have poor BBB penetration, especially when first-line agents for the primary cancer are used [46]. In this section, we discuss the role of chemotherapy for common sources of brain metastases (lung cancer, breast cancer, and melanoma) as well as leptomeningeal metastatic disease.

Non–Small-Cell Lung Cancer

The activity of chemotherapeutic agents against NSCLC brain metastases is difficult to assess, given that patients have often been treated with primary agents prior to the treatment of brain metastases. If given to naïve patients, cisplatin alone shows an approximately 30 percent response rate [47]; the response rate is 30-45 percent if cisplatin is given in combination with various other chemotherapeutics [1]. In addition, survival rates for intracranial metastatic disease versus extracranial metastatic disease are comparable in these cisplatin-based treatment regimens in naïve patients, suggesting a similar potency for these drugs in the CNS and in other tissues. More recent data suggest that temozolomide (TMZ) has some efficacy in the treatment of recurrent NSCLC brain metastases, with

response rates as high as 20 percent [48]. A trial by Omurio et al. demonstrated a 44 percent response rate in patients treated with TMZ combined with vinorelbine (a lipophilic broad-spectrum agent) for recurrent brain metastases, although the side-effect profile was increased relative to what has been previously published for TMZ monotherapy [49].

In addition to TMZ-based regimens, recent small studies have investigated epidermal growth factor receptor (EGFR) inhibitors. Ceresoli et al. reported a 27 percent disease control rate (10% partial response, 17% stable disease) in forty-one patients with brain metastases following either chemotherapy or WBRT [50]. Interestingly, a recent study demonstrated that MBT patients with EGFR mutations responded to EGFR inhibitor gefitinib, whereas other patients within the same study who lacked EGFR mutations did not respond to the treatment, a finding similar to the experience with these drugs in extracranial disease [51].

Breast Cancer

Breast cancer treatment with first-line regimens such as cyclophosphamide/5-FU/methotrexate [52] and cisplatin/etoposide [53] in chemotherapy-naïve patients is equally effective against intracranial and extracranial disease, with an objective response rate of approximately 55 percent to 60 percent. As with NSCLC, recurrent breast cancer metastases trials have centered on TMZ, because of its low toxicity, BBB permeability, and availability of comparative data in the setting of brain cancer. Although large studies are lacking, preliminary data indicate that TMZ in combination with cisplatin [54] or capecitabine [55], a 5-FU prodrug, has some efficacy in the treatment of recurrent breast cancer metastases to the brain, with response rates of 40 percent and 18 percent, respectively. High-dose methotrexate is another promising therapeutic strategy, given its efficacy against breast cancer and high BBB permeability, although larger studies are needed to assess its efficacy against new and recurrent brain metastases, as well as to monitor potential side effects such as leukoencephalopathy, particularly in the context of post-WBRT treatment [56].

In addition to the more traditional chemotherapy regimens, there is significant interest in targeted therapies aimed at patients with HER (human epidermal growth factor) 2-positive breast cancer. These HER2+ patients are at increased risk for brain metastases because of the increased invasiveness of this subset of tumors, improved control of systemic disease and survival in trastuzumab-treated patients, and poor BBB permeability of trastuzumab, thereby providing a relatively permissive environment for brain metastases. A recent Phase II study by Lin et al. evaluated the efficacy of lapatinib, a dual EGFR and HER2 inhibitor, in women with treated HER2+ breast cancer with at least one recurrent metastatic brain lesion following radiation. This trial demonstrated a 3 percent partial response rate and an 18 percent disease stabilization rate [57]. Further studies evaluating the role of targeted therapies, alone or in combination with additional chemotherapeutics, are warranted.

Melanoma

Cerebral metastases of melanoma are relatively common, seen in nearly 50 percent of melanoma patients at death. Metastatic melanoma is considered a chemoresistant tumor. Given that frequently employed agents such as dacarbazine and interferon have limited BBB penetration, the brain is a frequent site of treatment failure in patients treated with these agents [58]. As with other primary cancers discussed previously, recent clinical trials have evaluated the role of TMZ in treating metastatic melanoma. In a study by Agarwala et al., 151 melanoma patients with MBT were treated monthly with TMZ, with a response rate of 6 percent and stable disease rate of 26 percent [59]. TMZ has also been studied in combination with thalidomide, a known antiangiogenic agent, with a reported 25 percent stabilization rate. However, the toxicity profile of this combination was concerning, with intracranial hemorrhage in 29 percent and thrombosis in 13 percent of the patients [1, 60]. TMZ in combination with WBRT has also been studied, with a response rate of 10 percent [61]. Finally, there are preliminary data to indicate that incorporating TMZ as part of initial therapy may have a preventive effect on future cerebral metastases in melanoma patients [62].

Leptomeningeal Metastasis

Leptomeningeal metastasis, another observed pattern of metastatic spread, refers to the spread of cancer to the pia, subarachnoid space, CSF, and arachnoid membranes. Most cases involve NSCLC, breast cancer, melanoma, or hematologic malignancies [58]. This form of metastasis harbors a poor prognosis, with survival of approximately two months without treatment and six months with best available therapy. Unlike most other MBTs, leptomeningeal metastases are treated primarily with intrathecal chemotherapy (methotrexate or cytosine arabinoside), either by repeated lumbar punctures or via a surgically placed intraventricular Ommaya reservoir. It has been demonstrated that patient response to intrathecal chemotherapy is dependent on normal CSF flow; however, CSF flow is abnormal in 60 percent of patients. Patients with

TABLE 27.2. Distribution of extraneural metastases in children and adults as a function of primary brain tumor type as of 1985.

Primary tumor	Children	Adults	Total
Medulloblastoma	65	22	87
Astrocytoma/glioblastoma	11	68	79
Meningioma	5	49	54
Ependymoma	13	9	22
Germ cell tumor	14	3	17
Pituitary tumor	0	8	8
Oligodendroglioma	0	3	3
PNET	2	1	3
Pineoblastoma	2	0	2

Adapted from Hoffman and Duffner [66].

abnormal CSF flow, as assessed by ventriculography, have decreased survival rates and increased rates of neurologic death compared with patients with normal CSF flow [63]. Therapies currently under investigation include intrathecal administration of rituximab (anti-CD20 antibody) and trastuzumab (anti-HER2 antibody) for the treatment of leptomeningeal spread of lymphoma and breast cancer, respectively [64, 65].

EXTRANEURAL METASTASIS OF PRIMARY BRAIN TUMORS

Although the spread of primary brain tumor cells to other areas of the CNS (dura, ventricles, subarachnoid space, parenchyma) is relatively common, systemic metastasis of primary brain tumors to locations outside the neuroaxis, or extraneural metastasis (ENM), is a relatively rare phenomenon, with 282 cases reported in the literature as of 1985 [66]. The classic reasons given for this lack of metastatic potential, first elaborated by Willis in 1952 [67], include the lack of true lymphatics in the CNS; thin-walled veins collapsing ahead of advancing tumor; veins surrounded by dense dura, thereby preventing vascular invasion; inability of neural tissue to survive at distant sites; and the rapid clinical decline in patients with primary brain tumors that occurs before distant metastases become clinically evident. Another theory, proposed by Pansera, is that the brain environment is not harsh enough to select out metastatic clones, given the lack of extracellular matrix and connective tissue [68]. Several of these ideas have been successfully challenged and disproved, including the demonstration of CNS lymphatic drainage [69], the ability of malignant CNS tumors to invade veins [70], and survival of CNS tumors transplanted to extraneural sites [71]. Thus, the apparent increased occurrence of ENM seen over recent decades is likely a combination of improved surveillance and increased incidence owing to prolonged survival from primary brain tumors.

The most common primary brain tumors exhibiting ENM are glioblastoma in adults and medulloblastoma in children. Meningiomas and ependymomas comprise the majority of the remaining cases, although essentially all known primary brain tumors have been noted to have ENM (Table 27.2). Of note, 40 percent of ENMs occur in children. In addition, the site of metastasis differs based on the primary tumor type, with medulloblastomas primarily seeding the bone, bone marrow, and lymph nodes. In contrast, glioblastomas and ependymomas favor metastasis to the lung and lymph nodes. Meningiomas most often spread to the lung and pleura. Interestingly, ENM has been observed even in the absence of recurrent CNS tumor at autopsy [72].

The method of spread in cases of ENM has been under investigation. One mode of spread, first described in 1954, is via a ventriculoperitoneal shunt (VPS) to the peritoneum and then systemically. VPS-mediated spread is seen in 12 percent of all ENM cases and in 33 percent of children with medulloblastoma-related ENM [66]. One published study showed that insertion of a filter within the shunt system significantly decreased the rate of ENM [73], further establishing a causal relationship. The most common route of metastatic spread is via hematogenous or lymphatic pathways. Given that the vast majority of ENM occurs near the time of craniotomy and is often ipsilateral to the surgical site, it is likely that locally disrupted meningeal vessels and lymphatics during surgery provide a less-restrictive route for tumor cell entry [74]. Finally, continuous tumor expansion with subsequent transdural extension into extraneural tissue has also been described [75]. In summary, ENMs are rare but serious events typically mediated by surgical manipulation or artificial CSF conduits.

CONCLUSIONS

Metastatic brain tumors represent an increasingly important aspect of care in a variety of primary cancers. Recently, the treatment of metastatic tumors has improved, including the establishment of specific roles for stereotactic radiosurgery and surgical resection of metastases, as well as redefining the role of whole-brain radiation. These innovations have led to better outcomes for patients with metastatic brain tumors, in terms of both overall survival and quality of life. Future areas of investigation include optimizing radiation schemes, developing targeted chemotherapeutics based on insights from basic studies of tumorogenesis and metastasis, and establishing reliable prognostic indices to guide tailored multimodal therapy. Finally, as patients with metastatic brain tumors survive longer and radiation becomes a more important part of therapy, it will become increasingly important to consider issues such as cognitive status, psychiatric disorders, and quality of life in providing complete care for these patients.

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28 Head and Neck Cancer Metastasis

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Cancers of the head and neck are a diverse group of diseases arising from different subsites within the region. The incidence varies in different parts of the world, with a cumulative age-standardized incidence of 18.4 in males and 8.7 in females [1]. The majority of these cancers are tobacco-related, and the subsite affected is often determined by the patient's tobacco usage. It is primarily a locoregional disease, in which failure is more often locoregional than systemic. There is additional evidence to suggest that a higher regional burden of disease also predisposes to systemic metastases. However, certain malignancies in this area are more prone for distant dissemination – notably, the undifferentiated cancers of the nasopharynx and adenoid cystic cancers. Although advances have been made in the diagnosis and management of locoregional disease, when systemic failure does occur, there is still a paucity of effective treatment. The ability to predict those patients who will recur or have distant relapse is still not very well established.

INCIDENCE AND SITES OF METASTASIS

Nodal Metastasis in Head and Neck Cancer

The presence of a rich lymphatic network from the base of the skull to the upper mediastinum makes cancers in the head and neck region particularly prone to develop regional nodal metastasis. Nodal metastasis occurs when the lamina propria is breached by infiltrating tumor cells, which then enter the capillary lymphatics. The lymphatic channels have extensive interconnections, which often communicate with channels of the contralateral neck. The relative frequency of regional spread correlates with the density of the capillary network in that subsite. Subsites with the richest lymphatic supply, such as the nasopharynx and hypopharynx, have a far higher incidence of nodal metastasis than those with few or no lymphatic channels, such as the paranasal sinuses, glottis, middle ear, and orbit.

The lymphatic system in the head and neck was first described in detail by Rouviere in 1948 [2]. Even so, the nomenclature of lymph node groups was quite variable and confusing until the 1990s. In 1992, the TNM[1] adopted a system of terminology that divided the nodal regions of the head and the neck into twelve groups [3]. However, it was the Robbins classification, developed under the auspices of the American Academy of Otolaryngology–Head and Neck Surgery, that became more popular for standardizing the terminology pertaining to regions addressed during neck dissection [4]. It described seven nodal regions with clearly defined anatomical boundaries that were candidates for standard neck dissection procedures (Figure 28.1). This classification was adopted quickly and remains the most commonly used system to describe the distribution of nodal metastasis in head and neck cancers.

INCIDENCE AND DISTRIBUTION OF NODAL METASTASIS

The incidence and distribution of clinical lymph node metastasis have enormous implications for planning the surgical and radiotherapeutic treatment of head and neck cancers. Table 28.1 is a compilation of the incidence of clinical nodal involvement in this anatomical region from some of the largest published hospital studies, including our own data from the Tata Memorial Hospital, Mumbai [5–13].

The primary tumor subsite is one of the major determinants of the frequency of lymph node metastasis.

[1] The TNM Classification of Malignant Tumors (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue, N describes regional lymph nodes that are involved, and M describes distant metastasis (spread of cancer from one body part to another).

TABLE 28.1. Incidence of clinically evident lymph node metastasis

Subsite	cN+ (%)	Subsite	cN+ (%)
Oral cavity		Larynx	
Oral tongue	34–65	Supraglottis	31–64
Floor of mouth	30–59	Glottis	0–30
Buccal mucosa	9–31	Subglottis	10
Gingiva	18–52	Hypopharynx	
Hard palate	13–24	Pyriform sinus	52–76
Retromolar trigone	39–56	Postcricoid	14–50
Nasopharynx	86–90	Nasal cavity and paranasal sinuses	
Oropharynx		Ethmoids and nasal cavity	1–2
Base of tongue	50–83	Maxillary sinus	8–9
Tonsillar fossa	50–76	External ear	5–16
Soft palate	37–56	Salivary gland	13–14
Pharyngeal walls	50–71	Thyroid	2–12

cN+, clinically evident nodal metastasis.
Data compiled from Bataini [5], Candela [6, 7], Lindberg [8], Northrop [9], Shah [10], Laskar [11], Rao [12], and Terhaard [13].

Lesions in the pharyngeal subsites, oral tongue, and floor of the mouth are associated with a high incidence of nodal metastasis. The incidence is lower if the tumors are located in the glottic larynx, paranasal sinuses, or the ear. The general propensity for nodal metastasis in tumors of the salivary glands is low but is greatly affected by the histological type of malignancy.

Occult nodal metastasis is found on neck dissection in a significant proportion of patients in whom the neck is clinically and radiologically negative. Some large retrospective studies have reported incidence as high as 33 percent [6, 7, 10, 14]. Based on published evidence,

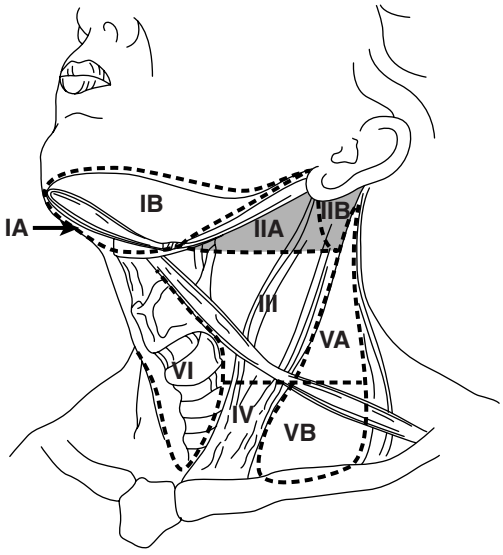

Figure 28.1. Lymph nodal levels of the neck.

Mendenhall et al. have formulated a risk grouping for the likelihood of occult nodal metastasis in different subsites of the head and neck according to the T stage. T1 tumors in the floor of mouth, hard palate, buccal mucosa, retromolar trigone, and gingiva have an incidence of nodal metastasis of less than 20 percent and, in selected cases, can be observed without addressing the neck [15].

The distribution of clinical and occult metastatic lymph nodes is mainly determined by the site of the primary tumor (Table 28.2) [5, 6, 8, 9, 16–19]. Primary tumors in the oral cavity and oropharynx drain mainly into levels I–III, whereas more caudally situated primaries in the hypopharynx and larynx have a higher incidence of metastasis to levels II–V. The nasopharynx, although central and cranially situated, has a relatively high incidence of posterior and lower neck node metastases, approaching 30 percent [20]. The distribution of pathologically positive nodes generally corresponds to that of clinically evident nodes. In patients undergoing neck dissection with clinically evident nodes, occult nodal involvement is often detected at one extra adjacent level, warranting the incorporation of at least one extra level of nodes in the surgical or radiotherapeutic plan of management [10].

The retropharyngeal nodes, extending from the skull base to the caudal border of the C3 vertebra, are a special entity. They are neither a part of the Robbins classification (as they are only rarely dissected) nor are they clinically detectable. Studies incorporating a systematic radiological evaluation reveal that the highest incidence of retropharyngeal lymphadenopathy is found in

TABLE 28.2. Distribution of metastatic lymph nodes

	Distribution of clinically positive lymph nodes										
Subsite	**Level I (%)**		**Level II (%)**		**Level III (%)**		**Level IV (%)**		**Level V (%)**		**CL/BL (%)**
Oral cavity	42		79		18		5		1		15
Nasopharynx	9		71		36		22		32		56
Oropharynx	13		81		23		9		13		25
Hypopharynx	2		80		51		20		24		44
Supraglottic larynx	2		71		48		18		15		29
	Distribution of pathologically positive nodes										
	Level I (%)		**Level II (%)**		**Level III (%)**		**Level IV (%)**		**Level V (%)**		**CL/BL (%)**
	N0	N+	N0	N+	N0	N+	N0	N+	N0	N+	cN0
Oral cavity	20	46	17	44	9	32	3	16	1	3	27
Oropharynx	2	15	25	71	19	42	8	27	2	9	38
Hypopharynx	0	10	12	75	12	72	0	45	0	11	59
Larynx	5	7	19	57	20	59	9	30	3	4	26

CL/BL, contralateral or bilateral nodes; N0, incidence in the node-negative neck; N+, incidence in the node-positive neck.
Data compiled from Bataini [5], Candela [6], Lindberg [8], Northrop [9], Byers [16], Shah [17], Woolgar [18], and Buckley [19].

nasopharyngeal primary tumors (70%–90%), followed by tumors of the pharyngeal walls (20%) and the soft palate (10%–15%). Other subsites have an incidence of 5 percent or less [21–23].

The overall incidence of contralateral lymph node involvement is low, but it is higher for tumors that are centrally located (such as those of the pharynx, larynx, and floor of mouth) or tumors that have a crossover of lymphatic channels (Table 28.2). The nodal levels involved in the contralateral neck generally correspond with ipsilateral nodal levels.

Although lymphatic metastasis follows an overall pattern of orderly progression of nodal involvement, there exists definite evidence of skip metastasis in some head and neck primary tumors, especially oral cancers. The incidence in oral lesions varies between 2 percent and 15 percent and is highest for oral tongue cancers, for which metastasis to lymph nodes in levels III–IV without involvement of level I–II nodes is well recognized [24, 25].

DISTANT METASTASIS IN HEAD AND NECK CANCER

The incidence of distant metastasis in head and neck cancer is relatively low compared with cancers in most other anatomic regions. It is rarely seen at presentation. The reported incidence at diagnosis is below 2 percent in most studies [26, 27]. The detection rate for distant metastasis was 12 percent in one study that extensively investigated cases of locoregionally advanced disease [28].

In large retrospective studies for radically treated patients on long-term follow-up, the overall incidence of clinically detected distant metastasis with squamous cell carcinomas varies between 4.2 percent and 15.1 percent [26, 27, 29–32]. These figures are represented in Table 28.3. In the majority of cases, distant metastasis occurs early – 80 percent develop within two years of diagnosis [31]. The reported prevalence in autopsy studies is higher, with a number of them reporting rates higher than 40 percent [33, 34]. These high rates are a reflection of a more advanced disease profile, as well as the fact that the majority of these patients had persistent or recurrent locoregional disease at the time of autopsy.

In order of frequency, the most common sites of distant metastasis are lung, bone, liver, skin, and nonregional nodes. Various other sites of metastasis have been reported infrequently, including the brain, adrenals, kidneys, heart, spleen, and urinary bladder [35–42].

Distant metastasis occurs most frequently in the lung. The capillary bed in the lungs is the first available location for entrapment of circulating tumor cells. The lungs are affected either alone or in addition to other sites in 55 percent to 85 percent of cases. In approximately 50 percent of the patients with distant spread, the lung is the solitary site of metastasis [30].

The skeletal system is involved in 19 percent to 32 percent of patients with distant spread. Nasopharyngeal undifferentiated cancers have a higher incidence of bone metastasis than differentiated squamous cancers do [31]. The majority of metastatic lesions are found in

TABLE 28.3. Selected large retrospective reports on incidence of clinically evident distant metastasis

Author (Year)	No. of patients	Incidence of distant metastasis (%)	Distribution of metastasis
Probert (1974)	779	9.6	Lung 55%, bone 32%, liver 8%
Merino (1977)	5019	10.8	Lung 52%, bone 23%, liver 6%
Bhatia (1987)	1127	4.2	Lung 69%, bone 19%, liver 6%; distant metastasis at diagnosis 1.1%
Calhoun (1994)	727	11.4	Lung 83%, bone 31%, liver 6%.
Jackel (1999)	1087	11.9	Lung 69%, bone 20%, liver 29%.
Holsinger (2000)	622	15.1	Lung 66%, bone 22%, liver 10%.
Leon (2000)	1880	9.5	Lung 52%, bone 12%, liver 5%.

the axial skeleton, with the vertebrae, pelvis, and skull accounting for 75 percent of lesions [32]. A study using 18-fluorodeoxyglucose (FDG)-PET for the detection of bone metastasis in head and neck cancer identified several occult lesions in the pelvis and femur [43].

The liver is the third most common site, reported in 6 percent to 24 percent of cases. Although it is unusual as the first or solitary site of metastasis, the liver has been found by some authors to be a common site of spread from oropharyngeal primaries and also from some cases of undifferentiated cancers of the nasopharynx [31].

The skin and subcutaneous tissues account for 5 percent to 10 percent of cases with distant spread. The incidence is low (1%–2%) in squamous cell carcinoma (SCC), but considerably higher in certain other histologies, especially the rare atypical carcinoid [44–46]. The dermal lymphatic channels are often responsible for spread to the skin and subcutaneous tissues, and their blockage resulting from tumor infiltration or surgery results in abnormal patterns of lymphatic flow and deposition of metastatic cells [47]. This pattern of direct dermal lymphatic infiltration is more common than hematogenous metastasis.

Nonregional nodal metastasis occurs most commonly to the axillary and mediastinal lymph nodes. In a large retrospective review, the overall incidence of infraclavicular nodal metastasis was 1.5 percent, constituting 8 percent of all distant metastases [48]. In autopsy studies, the incidence of axillary nodal metastasis varies between 2 percent and 9 percent [49, 50], with a higher incidence in individuals who have dermal metastasis [51]. Lymphatic spread to the axilla is probably caused by a retrograde flow of lymph from the jugulosubclavian junction, caused by tumor or fibrosis resulting from postsurgical or radiotherapy treatment. Metastasis to mediastinal nodes is infrequent, but in primary sites of the lower neck, such as the hypopharynx and cervical esophagus, the proportion of patients with occult metastasis may be considerably higher [52, 53].

Brain metastasis is uncommon in head and neck SCC, comprising 2 percent to 6 percent of all patients with metastasis [31, 33, 54, 55]. In general, single metastasis is more common than multiple foci. Solitary brain metastasis is very rare, however, and more than 90 percent of these cases have evidence of metastasis to other sites [56]. Primary cancers commonly associated with brain metastasis include undifferentiated nasopharyngeal cancer and adenoid cystic carcinoma [39, 57–58].

Certain histologies deserve special mention because of their higher propensity for systemic metastasis. The incidence of distant metastases in adenoid cystic carcinomas is often higher than 30 percent [59–61]. The incidence rates at different primary sites vary, with a relatively lower incidence in oral cavity primary tumors [62].

The lung remains the most common site of metastasis. The propensity for perineural spread also predisposes to a relatively high incidence of brain metastasis. Distant metastasis is often a late occurrence, and the median survival ranges between two and three years after metastasis [60, 61].

SYMPTOMS OF METASTASIS

Metastases to cervical lymph nodes initially present as mobile painless lumps in the neck. Their increasing size and extracapsular extension result in fixity to the surrounding neurovascular bundle and muscles. Nerve involvement may cause symptoms of pain and palsy. Skin infiltration may result in external wounds. Rarely, the infiltration and destruction of the walls of blood vessels may result in hemorrhage.

The site of distant metastases is the primary determinant of its symptomatology. Frequently, a patient may remain asymptomatic, and the metastatic disease is detected only by a routine follow-up evaluation. This is more often the case in histologies such as adenoid cystic carcinoma. Symptoms are often confused with posttreatment sequelae and, hence, a high index of

suspicion is the key to timely diagnosis, although it may not have a final bearing on the outcome.

The symptoms for lung metastases include cough, chest pain, breathlessness, and hemoptysis. The diagnostic dilemma then is to differentiate the solitary metastasis from a second primary tumor, especially in the absence of locoregional recurrence. Bone aches and pains or vertebral collapse/compression with neurological deficit or fractures may be seen with disseminated skeletal metastases. Malignant hypercalcemia and related symptoms are a rare presenting feature with extensive bone metastases. Raised intracranial tension, seizures, and cranial nerve palsies may point to the presence of brain metastases or may be due either to intracranial extension of local disease or to metastases at the base of the skull.

The symptoms determine the direction the investigation will take to evaluate the metastatic spread. This inquiry should also be expanded to include the status of the locoregional disease. There is no available evidence to suggest that the early detection and aggressive treatment of systemic metastases alters the outcome of the disease.

PREDICTORS OF METASTASIS

Accurate prediction of metastatic potential is key. A risk stratification of tumors based on their malignant potential is useful not only to establish investigative modalities but also to provide approaches to treatment that will provide the greatest potential benefit while sparing unnecessary cost and morbidity. Several clinical, pathological, and biological factors have been identified as possible predictors of metastasis. Although some have been incorporated into clinical decision making, others are still being investigated and will require further validation.

CLINICAL AND HISTOPATHOLOGICAL PREDICTORS OF METASTASIS

Many of the parameters assessed during clinical and histopathological evaluation are relevant in risk stratification for metastasis.

Prior history of tobacco abuse is an important etiological variable. Tobacco-related primary tumors have been known to harbor a different biological profile from non–tobacco-related cancers, as these primary tumors exhibit a more aggressive profile [63, 64]. It is likely that human papillomavirus (HPV) may be an etiologic agent in a significant proportion of patients who have no history of tobacco abuse. HPV-related cancers are known to have more widespread nodal metastasis at presentation. However, they are more susceptible to the standard nonsurgical treatment approach with radiation and chemotherapy and have an excellent prognosis, without a high incidence of distant spread [65–67].

As discussed in the previous section, the primary subsite is a crucial determinant of the incidence of nodal metastasis. The incidence of nodal metastasis varies widely in different subsites (Table 28.1) and has a strong bearing on the approach to elective nodal treatment.

The size of the primary lesion and infiltration into surrounding soft tissue, bone, and cartilage are factors that determine the T stage. A higher T stage has been shown to correlate with a higher incidence of nodal and distant metastasis [8, 10, 14]. Bone invasion also predicts the propensity for lymphatic spread [68, 69].

The histology of the primary lesion is another determinant of metastatic potential. Undifferentiated nasopharyngeal cancer and adenoid cystic cancer are examples of histologies that have a higher incidence of distant metastasis. Among SCCs, the basaloid variant exhibits aggressive behavior, leading to a higher incidence of distant spread [70, 71].

The grade of the primary tumor is also a marker of biological behavior. Poorly differentiated SCC and higher grades of salivary gland tumors are reported to have a higher incidence of metastasis [61, 72–73].

Following primary surgical excision, some characteristic features in the histopathology are determinants of nodal and distant failure. Lymphovascular emboli and perineural invasion have been associated with both distant and nodal spread [74–76]. The depth of infiltration is an extremely important consideration in oral cancers. The incidence of lymph node metastasis in cancers of the oral tongue with a depth of 5 mm or more is more than double that of superficial tumors [77–80]. A prospective randomized trial of elective versus delayed neck dissection in cancers of the oral tongue demonstrated that a tumor thickness of 4 mm is a clinically useful differentiator to select patients for elective nodal dissection [81].

Several characteristics of nodal metastasis are predictive of regional and distant failure. Two or more metastatic nodes, bilateral nodes, the presence of extracapsular extension, and inferior levels of nodal metastasis (levels IV, V, and VII) are some of these factors [30, 31, 44, 45, 48, 73, 82–86].

BIOLOGICAL PREDICTORS OF METASTASIS

Traditional systems of staging involve mainly anatomical and physical descriptors of the tumor and nodal metastasis. However, the natural progression of a cancer may be better predicted by a more comprehensive understanding of its intrinsic biological behavior. Because the primary tumor may harbor molecular characteristics that can predict its metastatic potential, considerable research has been focused on the

TABLE 28.4. Biological markers of metastasis

Markers of cell cycle, growth and apoptosis
Overexpression or amplification of cyclin D1
Overexpression of EGFR and neu
Mutation of p53
Markers of cell adhesion
Loss of expression of E-cadherin
Loss of expression of Ep-CAM
Overexpression of certain isoforms of CD44
Overexpression of sialyl Lewis protein (21,22)
Syndecan 1
Markers of proteolysis
Overexpression of certain isoforms of matrix metalloproteinases (MMP) and its tissue inhibitors (TIMP)
Markers of vascularization and oxygenation
Increased expression of angiogenic markers (e.g., VEGF)
Increased microvessel density
Overexpression of markers of hypoxia
Chromosomal markers
DNA aneuploidy
Others
Reduced expression of Nm23
Altered expression of plasminogen activators (UPA) and inhibitors (PAI)
Overexpression of COX-2
Reduced expression of prostaglandin E2
Markers of viral etiology
Markers of HPV infection
Serum EBV DNA load

CA-9, carbonic anhydrase 9; COX-2, cyclooxygenase 2; EBV, Epstein-Barr virus; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; HIF, hypoxia-inducible factor; HPV, human papillomavirus; uPA, uroplasminogen; VEGF, vascular endothelial growth factor.

detection of biological markers that can be used in clinical settings.

A summary of potential biological markers of metastasis is presented in Table 28.4. These include the genes and their protein products that are involved in every step of the metastatic cascade, including proliferation, loss of contact with neighboring cells, migration through the interstitial matrix, invasion of blood and lymph vessels, and homing in lymph nodes or distant organ systems. These steps involve several, perhaps simultaneous, changes in cellular characteristics, incurring alterations in genes and their products. Therefore, accurate prediction of metastatic potential requires not only just the identification of individual markers but also a knowledge of their combinations and interactions in a biological environment.

No single biomarker has been found to be unequivocally related to nodal or distant metastasis. Even those with potentially strong correlations to metastatic disease are not generally assessed in the standard evaluation of tumor samples. It is likely that a combination of several markers, rather than any single one, may have a greater predictive value. Comparative genomic hybridization (CGH) and tissue microarrays have the potential to evaluate a combination of markers. Both techniques are in an early stage of development as detection tools for the prognostic biomarkers of metastasis [113–115]. The high throughput of these techniques has the potential to improve on the current predictive value of biomarkers in head and neck cancers by analyzing large sets of markers simultaneously.

Before these markers can be incorporated into the mainstream evaluation of the metastatic potential of head and neck cancers, several hurdles need to be cleared. First, parameters can be evaluated by several different methods, with varying sensitivity and standards. Thus, uniformity and standardization of criteria would be a difficult issue. Second, the biological characterization of small biopsy samples is not necessarily a true reflection of the entire tumor tissue. The ultimate biological behavior could be affected by malignant clones that may not be present in small biopsy samples. However, because of the increasing interest in translational research, further insights on head and neck tumor biology will guide a better informed protocol on the use of biomarkers in the prediction of metastasis. A detailed description of the significance of each marker is beyond the scope of this chapter. Several excellent reviews have been published on this subject [116–118].

DIAGNOSTIC TESTING FOR METASTASIS

Over the last few decades, investigations for the detection of metastasis have evolved from clinical evaluation only to a combination of clinical evaluation with biological imaging and biological assays.

Standard imaging techniques for the evaluation of cervical nodal metastasis are computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US). Fine-needle aspiration cytology with US guidance (US-FNAC) is also performed. The overall sensitivity of CT and MRI for nodal metastasis is above 80 percent. However, their specificity is more variable, at 40 percent to 80 percent [119–121]. The sensitivity and specificity of US is between 70 percent and 96 percent and 75 percent and 95 percent, respectively [120, 122]. Specificity is considerably increased with the addition of FNAC to US, with reported rates as high as 95 percent to 100 percent [119, 122, 123].

A meta-analysis comparing these standard modalities in the assessment of nodal metastasis suggests that that the diagnostic odds ratio is higher with US and US-FNAC than with CT and MRI [124]. However, CT and MRI are used more often in investigations for routine staging. The ability to simultaneously stage the primary lesion is their biggest advantage. US and US-FNAC require additional training and experience for

optimal results. They also have a higher interobserver variation and are not suitable for nodal regions that are deep-seated and surrounded by bony cavities (e.g., retropharyngeal nodes). Unfortunately, the detection of occult metastasis is suboptimal with all these modalities. Because of their primary dependence on size-based criteria, conventional imaging is able to detect fewer than 50 percent of occult positive nodes that could be demonstrated from neck dissection [125, 126].

The difference is negligible between CT and MRI in regard to their overall performance in the detection of nodal metastasis. One study reported a greater likelihood in the detection of metastasis in small lymph nodes using MRI. The use of a special contrast medium containing ultrasmall particles of iron oxide (USPIO) that can potentially discriminate between benign and malignant lymphadenopathy based on functional characteristics has been reported. The sensitivity and specificity for this technique are 84 percent to 88 percent and 77 percent to 97 percent, respectively. It has the potential to reduce the false-positive interpretation of uninvolved but enlarged nodes [127–129]. A metastatic workup is traditionally limited in head and neck cancers because of a very low incidence of metastatic disease at diagnosis. In the majority of cases, the only imaging used is a chest X-ray. Locoregionally advanced disease in sites with a relatively higher incidence of distant metastasis may involve a bone scan, an abdominal ultrasound, or a CT scan to rule out bone and liver metastasis. However, these are rarely employed with an absence of symptoms because of a low yield and a lack of cost-effectiveness.

Recently, biological imaging has made great forays in diagnostic oncologic imaging. The capability of differentiating between tumors and normal tissues on the basis of biological rather than morphological characteristics is attractive. A number of studies have evaluated the use of positron emission tomography (PET) and PET/CT in the primary evaluation of head and neck cancer for the diagnosis of nodal and distant metastasis. 18-FDG is the most commonly used isotope. Several studies have compared PET with standard imaging modalities in the assessment of head and neck cancer and have received mixed results [126, 130–132]. A meta-analysis evaluating the use of 18-FDG-PET in the detection of cervical metastasis has been reported [133]. The overall sensitivity and specificity of PET were 79 percent (95% CI) and 86 percent (95% CI), respectively. In comparison, other standard imaging modalities, such as CT, MRI, or US-FNAC, had a sensitivity and specificity of 75 percent and 79 percent, respectively. In spite of the positive trend, the improvement was not statistically significant. The sensitivity of PET in detecting occult metastasis was only 50 percent (95% CI). These results were not substantially better than conventional imaging techniques (45%), although the specificity remained high, at 87 percent. The limitations in spatial resolution of FDG-PET and the presence of relatively active adjacent salivary glands may be responsible for suboptimal results. These results suggest that even though FDG-PET has some advantage over conventional imaging in nodal staging, the extent of benefit may not be large enough to recommend its routine use in the management of head and neck cancer. The surgical exploration of the node-negative neck to identify and remove microscopic spread remains the optimal procedure.

The routine use of biological imaging for the detection of distant metastasis is also unlikely to be cost-effective. However, the selective use of modalities such as 18-FDG-PET is of some benefit in situations associated with a relatively high incidence of distant metastasis. The most common use of PET in this setting has been in the evaluation of nasopharyngeal cancer, because it has a relatively higher incidence of lower neck and distant metastasis. Some studies have reported an improvement in neck staging with PET in this setting, but its role in the initial staging is still not clear [20, 134, 135]. An FDG-PET–based study has reported a higher detection of occult bone metastasis in head and neck cancer [43]. In this study, bone involvement was detected early in many patients who had no clinical and biochemical evidence of metastasis. Therefore, PET scanning in a selected group of patients who are at high risk of developing distant metastasis may be considered, as it has the potential to change treatment decisions.

In spite of the apparent lack of major benefit with PET scanning in the routine initial diagnostic assessment in head and neck cancer, this modality has significant advantages in the characterization of the primary and metastatic regions. These results can affect the course of treatment, such as the extent of neck dissection or radiation target volumes. Several studies have reported a change in radiation treatment plans based on PET-based target delineation [136–138]. PET is also being used in the noninvasive assessment of tumor hypoxia. Several sites including the head and neck have been investigated using 18-F MISO (misonidazole, a nitroimidazole with selective concentration in hypoxic tissues). Other analogous isotopes, fluoroerythronitroimidazole (F-FETNIM) and fluoroazomycin-arabinofuranoside (F-FAZA), are in development, which could assist not only in prognostication but also in biologically directed treatment plans in radiation therapy (see the section, “Emerging Treatment Options”).

Currently, there are no biochemical tests that are routinely recommended to diagnose nodal or distant metastasis. Liver function tests may be of value in hepatic metastasis but have limited sensitivity in small or early metastatic lesions. The use of biological markers is

Prognostic impact of nodal status on outcomes

Tata Memorial Hospital experience with 1805 patients with head and neck cancer 1990–2000

	n	5 yr DFS	5 yr NC
N0	813	60.7%	89.5%
N1	468	48.5%	68.2%
N2-3	524	34.1%	48.6%
		p < 0.001	p < 0.001

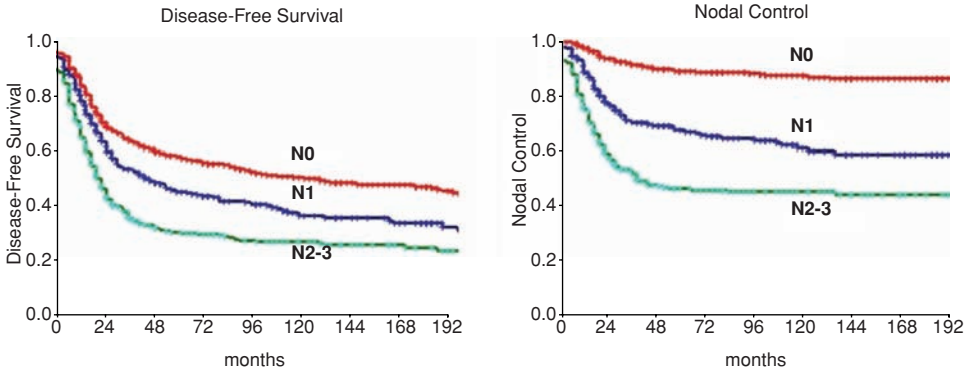

Figure 28.2. The impact of nodal stage on prognosis in head and neck cancer. Analysis of disease-free survival (DFS) and nodal control (NC) in 1805 patients with head and neck cancer (all sites and stages) treated in the Department of Radiation Oncology at Tata Memorial Hospital, 1990–2000.

still being investigated (see the section, "Predictive Testing"). Several molecular prognostic markers are being used more often. Markers of viral etiology are increasingly being employed. These include HPV infection using p16 immunohistochemistry, in situ hybridization, and reverse-transcriptase polymerase chain reaction (RT-PCR) commonly in oropharyngeal primaries because of its prognostic significance; and circulating Epstein-Barr virus DNA (EBV-DNA) load using real-time quantitative PCR, and EBV-encoded early RNAs (EBER1 and EBER2) in tissues in endemic nasopharyngeal cancer. Levels of circulating EBV-DNA have been shown in some studies to predict distant metastasis and survival [112]. Epidermal growth factor receptor (EGFR) overexpression or amplification can be tested using multiple techniques, including immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). Other markers include markers of hypoxia – tissue expression of HIF1a, CA IX, lysyl oxidase with IHC, and plasma osteopontin levels using ELISA. Comparative genomic hybridization and tissue microarrays are being developed to detect multiple genetic and gene product expression to categorize head and neck cancers into risk profiles with potentially different management strategies.

PROGNOSTICATION OF METASTATIC DISEASE

The presence of nodal or distant metastasis has a profound impact on the prognosis for patients with head and neck cancer. In a predominantly locoregional disease, nodal metastasis is arguably the most important prognostic factor affecting survival in all subsites and histologies of this region. Figure 28.2 depicts the impact of nodal stage on overall survival, disease-free survival, and nodal control in patients at the Tata Memorial Hospital who were studied from 1990 to 2000 (previously unpublished). The independent prognostic effect of nodal stage has been demonstrated widely [139–141], and our own experience in head and neck cancer is no different [142, 143].

Multiple aspects of regional nodal metastasis are of prognostic relevance. The number and size of lymph nodes are most important and directly affect the staging of head and neck cancer. Lymph node size and fixity determine resectability, the chances of nodal failure, and the incidence of extracapsular extension (ECE). Pathologic examination shows that the incidence of ECE is higher than 80 percent in nodes larger than 3 cm [144–146]. This factor is of great significance not only in regional control but also in the risk of development of distant metastasis [44, 145, 147–149]. The number of involved nodes may also predict the chances of distant metastasis and long-term survival [44, 149–150].

The location of nodal metastasis is significant. One study reported a progressive worsening of overall survival with an involvement of lower neck levels, from 37 percent with level Ib nodes to 21 percent with level V nodes [151]. Some studies suggest that nodal metastasis may be of prognostic relevance to local control as well, although this has not been unequivocally proven [152]. The risk of nodal recurrence is linked to these prognostic factors as well as to the primary treatment details in terms of modality, operated and radiated

levels, and so forth [153–155]. The results of selective neck dissection at the Tata Memorial Hospital show that 30 percent of all nodal failures occur outside the dissected field [155]. Evidence of immunohistochemical features of micrometastasis in lymph nodes that were negative on routine microscopic examination has been associated with regional recurrence in some studies [156]. Also, a longer disease-free interval (DFI) is predictive of better outcomes and long-term control using salvage therapies [157, 158].

The presence of distant metastasis confers an uniformly poor prognosis with one-year survival rates of 20 percent to 40 percent [29, 159]. Several studies have reported factors that may affect survival in patients with distant metastasis [159–162]. Solitary metastasis has a better prognosis compared with a more disseminated spread. Liver and lung metastasis confer a poorer prognosis. The DFI also predicts survival in patients with metastatic disease who have received treatment.

TREATMENT OF METASTATIC DISEASE

Standard Treatment Options

Principles of Treatment of Nodal Metastasis

The management of the neck in head and neck cancers largely parallels the treatment of the primary tumor. Nodal metastasis can be managed equally well with either surgery or radiotherapy (with or without chemotherapy or biological therapy); therefore, the choice of treatment is usually based on the most appropriate treatment of the primary disease. A single modality of treatment is usually sufficient when the metastatic burden is less or is clinically absent. In more advanced disease, multimodality treatment that frequently uses all three treatments is the most optimal. Elective treatment of the neck is often required even in the presence of a clinicoradiologically negative neck, because of a low sensitivity for diagnosis of occult nodal disease in normal-sized nodes with clinical or available radiological modalities.

Surgical treatment of the neck involves neck dissection. A comprehensive neck dissection involves removal of nodes along the entire longitudinal extent of the neck. In a classic radical neck dissection, the superficial and deep cervical fascia with the enclosed lymph nodes (levels I–V) are removed together with the sternocleidomastoid muscle, the omohyoid muscle, the internal and external jugular veins, the spinal accessory nerve, and the submandibular gland. A modified neck dissection spares certain structures to reduce morbidity and improve functional outcome without compromising disease control. A selective neck dissection is more limited in extent and includes the resection of selected lymph node levels. Common types include the supraomohyoid (levels I–III), lateral (levels II–V), and posterolateral (levels II–V) dissections. These selective neck dissections are usually performed for the clinically negative neck and for the salvage treatment of residual nodal disease after radiotherapy. In other cases, modified neck dissection remains the standard.

Radiation therapy for neck disease can also be used as an elective and therapeutic option. Conventional radiation therapy of the head and neck is performed with a 4–6-MV linear accelerator or a telecobalt unit. Brachytherapy is not commonly employed for nodal metastasis.

The dose and fractionation of radiotherapy depend on treatment indications. Clinicoradiologically positive regions receive 60 to 72 Gy using the standard fractionation of 1.8 to 2 Gy per day. Electively treated regions receive a minimum of 50 Gy with the same fraction sizes. Other schedules using altered fractionation exist. Hyperfractionated or accelerated treatments have shown a small but definite advantage in locoregional control and survival. Hypofractionated schedules are commonly reserved for palliation in the presence of advanced locoregional disease or distant metastasis.

The volumes encompassed by radiotherapy are determined by the nodal levels involved, the site and clinical characteristics of the primary tumor, and the histological type of the primary tumor. Radiation techniques range from simple bilateral or anterolateral portals to more complex three-dimensional conformal radiotherapy (3DCRT) and intensity-modulated radiotherapy (IMRT) techniques that are able to deliver the highest possible dose to the tumor while maximally sparing normal tissues, including the spinal cord, salivary glands, muscles of deglutition, and laryngeal structures.

The combination of radiosensitizing chemotherapeutic agents and radiotherapy has improved on the clinical outcomes. Significant improvements in locoregional disease and overall survival have been demonstrated with the use of concomitant platinum-based chemotherapy, in both the radical and adjuvant settings for high-risk patients (with the risk primarily determined by nodal status) [163, 164].

Treatment of the Node-Negative Neck

Elective neck treatment is based on the estimated incidence of subclinical disease. Either neck dissection or radiation is effective in controlling 90 percent of subclinical disease, and the choice of treatment depends primarily on the optimal strategy for the primary tumor [165]. When surgery is the primary modality chosen, neck dissection is strongly suggested when the incidence of occult metastasis is above 10 percent to 15 percent. If the expected incidence is lower, close observation is usually adequate. A modified neck dissection

is most commonly performed for those who require surgery. Selective nodal dissection is considered adequate for early cancers of the oral cavity, especially when performed in conjunction with frozen-section evaluation of suspected metastatic nodes and the provision to extend the dissection when metastasis is detected.

Primary radiation treatment policies usually include the nodal levels at risk of metastasis within the treatment portals. In general, this is achieved with little additional cost or morbidity. Elective irradiation of the bilateral neck includes several subsites that are at a high risk of occult contralateral spread.

Isolated nodal failure after conservative management of the neck can occur in 20 percent to 30 percent of cases, and salvage treatment is successful in 70 percent to 80 percent of individuals [165].

Treatment of the Node-Positive Neck

As with elective nodal treatment, the choice of initial treatment in the node-positive neck is made according to the most appropriate treatment of the primary. When surgery is the primary treatment, modified neck dissection alone is sufficient for single ipsilateral nodes without extracapsular extension (ECE) [166, 167]. The presence of large or multiple nodes or ECE mandates the use of adjuvant radiation (with chemotherapy) to reduce the incidence of regional recurrence [164, 168].

When a primary nonsurgical approach is selected, radiotherapy with concomitant chemotherapy or biological therapy (with cetuximab) is the current standard of treatment for node-positive head and neck SCC (HNSCC) [163]. At four to eight weeks after radiation or chemoradiation, no further treatment is required for initially small-volume nodal disease that has regressed completely. A suspicion of residual neck disease based on size or morphological criteria that have been determined by imaging should be addressed with a neck dissection. Some studies recommend the routine use of elective neck dissection following radiotherapy for N2b-N3 disease after complete clinicoradiological response [169–171], whereas others advise a more conservative "wait-and-watch" approach [172]. It is our practice to use neck dissection electively after irradiation only for bulky nodal disease with clear evidence of ECE on radiology. Sensitive imaging modalities, such as US-FNAC and PET scanning, may help select patients with the highest risk of nodal relapse [173–176].

Control of nodal disease depends on the initial nodal stage and on the primary management. With only single-modality treatment, the rates of regional failure increase from 10 percent to 15 percent in N1 tumors to 40 percent to 60 percent in N3 disease. A combination of surgery and radiotherapy usually reduces nodal failure by half in advanced nodal disease [177]. The majority of nodal failures are associated with a local failure as well. Isolated neck recurrences are relatively uncommon, occurring in fewer than 10 percent of cases [178, 179]. Salvage treatment for isolated neck recurrences is also based on the previous treatment modality. Surgical salvage rates vary between 25 percent and 60 percent in isolated recurrences with long-term nodal control in about one-third of these patients [180, 181]. The salvage probability depends on the nodal size, fixity, and previous intervention. Radiotherapy also plays an important role, either in combination with surgery or as the sole modality of treatment [157].

Treatment of Distant Metastasis

Metastatic disease in head and neck cancer is a rare primary presentation. The subsequent appearance of metastatic disease is more common and represents a challenge to the oncology community. A diagnosis of distant metastasis has a poor prognosis overall, as the disease has a relatively limited response to systemic treatments. Median survival after the development of metastasis varies between six and nine months with treatment. Therefore, the treatment approach is often a judicious use of systemic therapy, local treatment options including radiotherapy and surgery, and expert supportive care. In the vast majority of cases, the intention is palliation. Several factors help determine the extent of treatment when distant metastases do occur. These factors include the performance status of the patient, the presence of significant comorbidities, the status at the locoregional site, the DFI between primary treatment and occurrence of metastases, and the primary treatment used.

Standard systemic therapy consists of chemotherapeutic agents, commonly platinum-based regimens. The use of platinum-based combination regimens, usually 5-fluorouracil (5-FU) or a taxane, improves response rates over single agent platinum, but this combination regimen has not shown a significant survival benefit in Phase III trials [182, 183]. Response rates rarely exceed 30 percent, which reflects the relative resistance of this epithelial malignancy to standard systemic therapy. Therefore, the decision to offer systemic treatment should take into consideration all the possible risks versus the benefits. Targeted therapies are the main focus of current research in the systemic treatment of metastatic disease. The addition of cetuximab, a monoclonal antibody targeted against EGFR, has resulted in a small but significant survival benefit over the use of platinum alone in recurrent or metastatic head and neck cancer in a randomized Phase III trial [184]. Many other targeted agents are under various stages of development (see the section on current research in metastasis in head and neck cancer).

Local treatment options, such as resection for solitary or oligometastasis in the lung, are useful in several situations [158, 185]. In selected patients, the complete resection rates have been high, between 80 percent and 90 percent. The resultant five-year survival has been reported to be between 30 percent and 50 percent. Thus, in locoregionally controlled patients, this approach may result in an improvement in their survival. In other sites of metastasis, local treatments are directed mainly for symptom palliation. Bone metastasis often requires the use of palliative radiotherapy, with various hypofractionated schedules ranging from a single fraction of 8 Gy to more protracted regimens of 30 Gy in two weeks. Surgical intervention may be required for stabilization of the spine in impending cord compression. Brain metastasis is treated with palliative whole-brain radiation to doses of 20 to 30 Gy. Solitary brain metastasis, although rare, may be approached by using a combination of whole-brain irradiation and surgical resection or stereotactic RT boost [186–189]. Metastasis to nonregional nodes, especially the axilla, can be considered for a nodal dissection if this is the only site of metastasis.

Emerging Options in the Treatment of Head and Neck Cancer Metastasis

Standard treatments for head and neck cancer have evolved into an integrated multimodality approach, leading to a small but definite improvement in outcomes over the past few decades. There is a need for improvement, though, both in terms of disease-free and overall survival in locoregionally advanced and metastatic disease and in the preventable morbidity associated with standard therapies in early stage cancers.

Current research in systemic treatment strategies is focused mainly on targeted treatments. Targeted therapy is a term that can be used to describe treatments that act specifically on cells harboring a certain "target" molecule, without significantly interfering with the functions of other cells. Broadly, two forms of targeted treatments are commonly in use – monoclonal antibodies (mAbs) and small-molecule tyrosine kinase inhibitors (TKIs). In head and neck cancers, the most promising targets include the epidermal growth factor receptor family (erbB), the Src pathway, and the pathway involving angiogenesis. A list of targeted therapies at various stages of clinical development is presented in Table 28.5.

Epidermal growth factor receptors are members of the erbB family of receptor tyrosine kinases that are involved in multiple pathways of growth regulation. There are four members of this family: EGFR, her-2/neu, her-3, and her-4. The first two have an important role to play in HNSCC. EGFR is overexpressed in 90 percent of HNSCC; this has been associated with an increased risk of nodal metastasis and an unfavorable survival [190, 191]. Her-2/neu overexpression is less common, in between 17 percent and 53 percent of HNSCC. Its main role in HNSCC is likely to be as a signaling partner for EGFR, and it has also been associated with poor outcomes in a few series [192, 193]. Among anti-EGFR treatments, cetuximab has already demonstrated a significant benefit in survival both in the concurrent setting (cetuximab with radiation vs. radiation alone) [194] and in the metastatic setting (in combination with platinum-based agents) [184]. Both these treatments are now FDA-approved. Other uses of cetuximab and the novel TKIs, erlotinib, gefitinib, and lapatinib, are currently being investigated in more than 100 clinical trials.

Src kinases are another group of kinases closely linked to signaling by several receptor tyrosine kinases (including EGFR), platelet-derived growth factor receptor (PDGFR), insulinlike growth factor 1 receptor (IGF1R), and G-protein coupled receptors (GPCRs). Of this family, c-Src is overexpressed in several cancers, including head and neck cancer [195]. Inappropriate regulation of these kinases contributes to tumor progression and metastasis [196]. In HNSCC, Src kinases show correlation with EGFR stimulation and inhibition [197]. Drug targeting of Src kinases is a relatively new phenomenon, but some TKIs – in particular, dasatinib – are being investigated in the recurrent and metastatic setting in conjunction with standard treatments.

Angiogenesis plays an extremely important role in tumor growth and is regulated by pro- and antiangiogenic factors. The vascular endothelial growth factor (VEGF) family of proteins is the most important of the proangiogenic factors. The VEGF ligands and their receptors (VEGFR1, 2, and 3) are attractive targets for biological treatments directed against angiogenesis. Bevacizumab, a monoclonal antibody against the VEGF ligand, as well as several TKIs of VEGF receptors, such as sorafenib, sunitinib, and vandetanib, are under evaluation in several trials.

Radiation therapy treatment has changed in the past few decades. The emergence of intensity modulation has allowed for a conformal delivery of dose to the complexly shaped targets in this region, sparing the critical normal structures and enabling dose escalation to involved regions (both primary and nodal). Refinement of image guidance technology has provided an accurate verification of anatomic positioning enabling the radiation oncologist to further reduce the margins of uncertainty. The availability of biologic imaging has added a new dimension to the approach for target volume delineation. PET-based delineation strategies have been tested at various centers. Initial impressions suggest that it may result in changes in the volumes of intended irradiation.

TABLE 28.5. Targeted agents under clinical evaluation in head and neck cancer

Agent	Description	Phase	Trial settings
Anti-EGFR treatments			
Cetuximab	Chimeric mAb	Phase III	Concomitant chemoradiation; recurrent or metastatic
Panitumumab	Human mAb	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation
Zalutumumab	Human mAb	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation
Nimotuzumab	Humanized mouse MoAb	Phase I/II	Concurrent with chemoradiation
Matuzumab	Humanized mouse MoAb	Phase I	Recurrent or metastatic
Erlotinib	TKI of EGFR	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation in definitive and adjuvant settings, chemoprevention
Gefitinib	TKI of EGFR	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation in definitive and adjuvant settings, neoadjuvant
Lapatinib	Dual phase TKI of both EGFR and Her-2/neu	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation in definitive and adjuvant settings, neoadjuvant
Anti- Src treatments			
Dasatinib	TKI of Src, Abl, c-kit and PDGF	Phase II	Recurrent or metastatic
AZD-0530	Inhibitor of Src and Abl	Phase II	Recurrent or metastatic
Anti-angiogenic treatments			
Bevacizumab	Humanized mouse mAb against VEGF	Phase II/III	Recurrent or metastatic, concurrent with chemoradiation
Sorafenib	TKI of VEGFR2, VEGFR3, PGDFRb, Raf1, kit	Phase II	Recurrent or metastatic
Sunitinib	TKI of VEGFR1, VEGFR2, kit	Phase II	Recurrent or metastatic, concurrent with chemoradiation
Vandetanib	TKI of VEGFR2, EGFR, RET	Phase II	Recurrent or metastatic, concurrent with chemoradiation
Semaxanib	TKI of VEGFR2	Phase II	Recurrent or metastatic
XL-880	TKI of VEGFR2, cMET	Phase II	Recurrent or metastatic
Cediranib	Inhibitor of VEGFR1, VEGFR2, VEGFR3	Phase I/II	Recurrent or metastatic
Others			
Bortezomib	Proteasome inhibitor	Phase I/II	Recurrent or metastatic, concurrent with chemoradiation
Celecoxib	COX-2 inhibitor	Phase I/II	Recurrent or metastatic, concurrent with chemoradiation, chemoprevention
TNFerade	Adenovirus vector with radiation-inducible TNF gene	Phase I/II	Concurrent with radiation/chemoradiation
Cilengitide	Anti-integrin	Phase I/II	Recurrent or metastatic
Proxinium	Ep-CAM targeting agent (intratumoral injection)	Phase I/II	Recurrent or persistent
Allovectin 7	Immune inciter with DNA sequences coding HLA B7 and β2 microglobulin (intratumoral injection)	Phase I	Recurrent or persistent

COX, cyclooxygenase; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor; MoAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
Data compiled from the directory of clinical trials at ClinicalTrials.gov (last accessed on September 20, 2008).

The next step is the use of biological imaging to characterize features of different parts of the tumor in order to differentially deliver dose according to biological characteristics, a concept termed "theragnostics" or "dose painting by numbers" [198]. Hypoxic regions within the tumor and nodes are an obvious first target of theragnostic radiotherapy. PET imaging with isotopes concentrated in the hypoxic regions (F-MISO, F-FAZA, Cu-ATSM) has been fused with conventional CT scans in preliminary studies to depict hypoxic regions within the target area that may be treated with different doses and fraction sizes [199–203]. The concurrent use of systemic chemotherapy and biological agents, such as cetuximab, further augments the effects of radiation on locoregionally advanced disease. Nimorazole, a nitroimidazole agent that acts as a hypoxic cell sensitizer, has been used with success in conjunction with radiotherapy [204]. This agent may be a cost-effective addition to radiation-based treatment strategies if used in tumors with known hypoxic characteristics.

One new approach in current surgical research is sentinel node biopsy in the N0 neck in early stage lesions to aid in the eventual surgical approach. Numerous studies, mainly in oral cancers, have been performed to test the feasibility and diagnostic accuracy of this method [205–208]. Lymphoscintigraphy using a radioactive colloid and an intraoperative gamma camera detects sentinel nodes in more than 90 percent of cases. The negative predictive value in most series also exceeds 90 percent. The results in more advanced primary tumors are less consistent. This approach may become an acceptable standard of care in early-stage clinically node-negative HNSCC.

FUTURE DIRECTIONS OF RESEARCH

With the evolution of cancer therapies over the past century, there is a growing realization that despite an immense amount of technological and financial input, the magnitude of improvement in cancer survival is stagnating, and the pace of improvement is slow. This comes with the recognition that cancer cannot be predicted or treated based purely on physical measures of size and number. Standard staging and treatment are often empirical and highly dependent on these physical parameters. The road to further improvement lies in understanding the molecular mechanisms that make similar looking cancers behaviorally different. This process has been gradual and is still far from complete. However, new avenues of research have been found that, if pursued and expanded, may culminate in a scientific breakthrough.

The focus of research in metastatic head and neck cancer has slowly turned toward cancer biology. Further translational research will bring this research closer to the bedside. Molecular markers must be better understood and their role, either singly or in conjunction with others, better defined. This will help not only with the prognostication of the disease but also with the grouping of cancers based on their biological characteristics, and prediction of the chances of metastasis. The use of molecular diagnostic techniques (such as microarrays) should help us categorize each tumor based on multiple characteristics. Radiological techniques should be able to pick up biological characteristics of tumors to predict both the risk of metastasis and also their likely sensitivity to radiation and chemotherapy. Treatments should be based on both physical and biological characteristics, often resulting in a less morbid treatment option for low-risk lesions and in a more aggressive treatment of higher-risk lesions. Radiation doses may be differentially delivered to biologically different regions of the same tumor and metastatic regions. Systemic treatments can be individualized to target the most adverse biological features of each cancer. Multimodality approaches may be redefined in terms of combination and sequencing to best achieve the primary goal of any cancer treatment – maximizing benefit while minimizing morbidity.

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29 Cutaneous Melanoma: Therapeutic Approaches for Metastatic Disease

Ahmad A. Tarhini and John M. Kirkwood

INTRODUCTION TO METASTATIC MELANOMA

Among new cancer cases documented in the United States in 2008, melanoma is estimated to be the sixth and seventh most common in men and women, respectively. This cancer has continued to rise in incidence at a rate exceeding those for all other cancers. In 2010, there were projected to be 68,130 new cases of melanoma, but the vast majority of these were forecast to be early-stage and therefore curable. However, it was estimated that 8,700 patients would die from this disease in 2010 [1]. Annually, about 8,000 patients are found to have metastatic melanoma presenting as a recurrence of an earlier primary melanoma; this number closely approximates the annual number of deaths from this disease. This statistic illustrates the lack of progress that has been made in the treatment of stage IV melanoma over the past several decades.

The American Joint Committee on Cancer (AJCC) divides cutaneous melanoma into four stages. Primary tumors confined to the skin without regional lymph node involvement are assigned stages I and II, depending on the thickness (depth) of the tumor, ulceration of the overlying epithelium, or invasion of the reticular dermis or subcutaneous fat (Clark level IV or V). Stage III is a disease with clinical or pathological evidence of regional lymph node involvement or with the presence of in-transit or satellite metastases. Stage IV disease is defined by the presence of distant metastasis [2]. Patients with stage I melanoma have an excellent prognosis with surgical treatment alone at a cure rate of more than 85 percent. The three- to five-year postsurgical relapse rates in patients with stages IIA and IIB are 20 percent to 30 percent and 40 percent to 55 percent, respectively. Stage III melanoma patients with regional lymph node involvement have a five-year relapse rate of 60 percent to 80 percent, and those with stage IV disease have a dismal prognosis, with a median survival of only six to nine months [3, 4].

Currently, there is no therapeutic agent that is known to prolong survival in patients who have metastatic melanoma. Therapeutic approaches that have been studied in metastatic melanoma include chemotherapy, biochemotherapy, nonspecific immune adjuvants, cancer-specific vaccines, cytokines, monoclonal antibodies, and specific immunostimulants. Chemotherapy with single-agent dacarbazine (DTIC) is the only US FDA-approved chemotherapy agent for metastatic melanoma. Immunological approaches have yielded the only new US FDA-approved agent for metastatic disease in thirty years, high-dose bolus interleukin (IL)-2, based on its durable responses in some patients with metastatic melanoma; however, this agent carries a high toxicity rate and cost. Many novel therapeutic approaches are currently undergoing active clinical investigation (Figure 29.1).

CHEMOTHERAPY FOR METASTATIC MELANOMA

DTIC, an alkylating agent, is the only US FDA-approved chemotherapy agent for metastatic melanoma, and this dates from more than twenty-five years ago. Studies with DTIC since the 1970s have shown response rates ranging from 20 percent in early trials that were assessed by older systems, to a current value of 6.7 percent (in one of the largest recent Phase III trials); its median response durations range from four to six months [4, 6]. However, no randomized controlled trials have ever compared DTIC with a placebo or best supportive care [7]. DTIC is typically given at 200 mg/m^2 intravenously (iv) for five days or 850 mg to 1,000 mg/m^2 iv every two to four weeks, with no apparent difference in terms of response rates and duration between the two schedules. Higher response rates have been achieved by

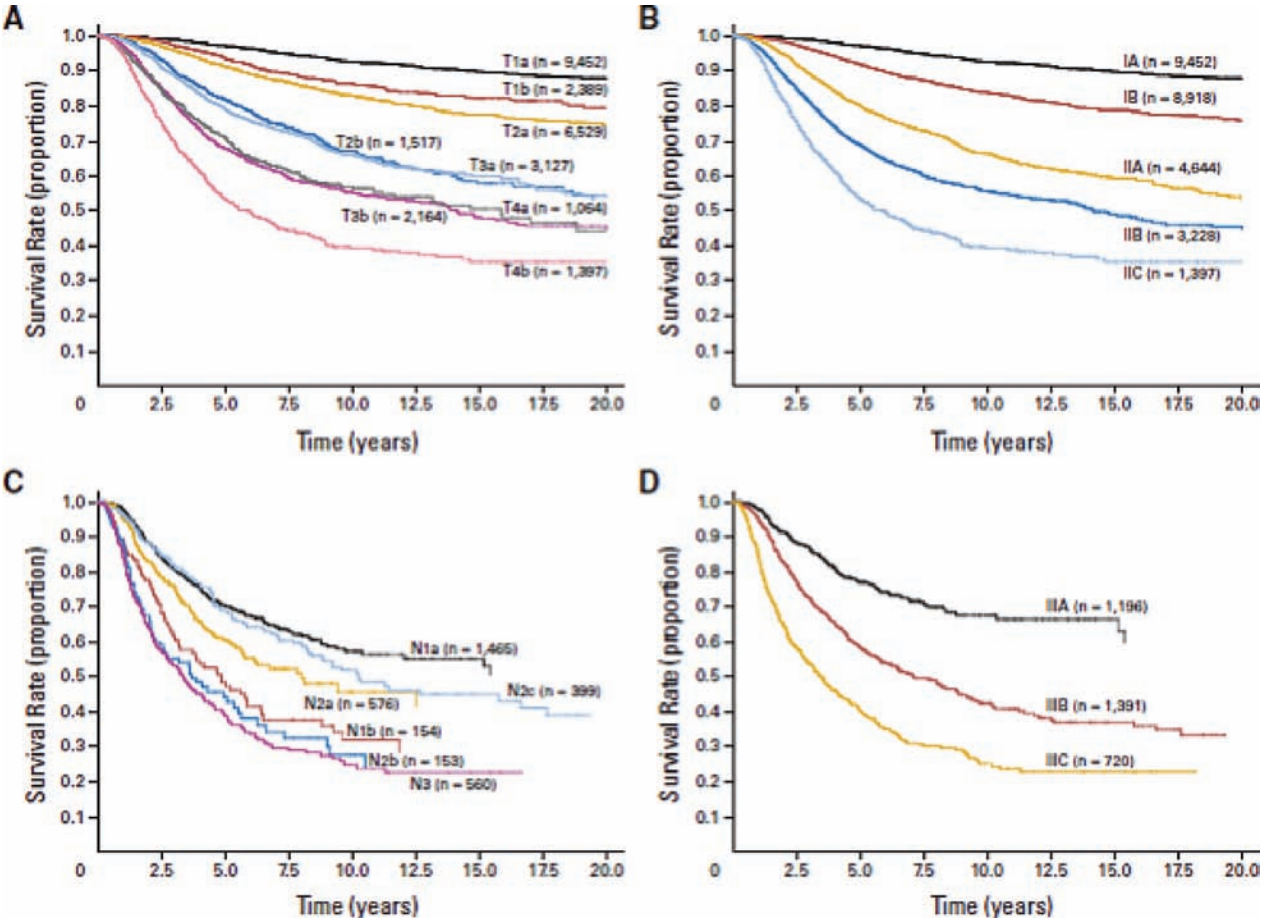

Figure 29.1. Survival in melanoma by stage.

using strategies that involve combination chemotherapy and autologous bone marrow transplant, but these strategies have led to higher toxicities and have no benefit in terms of relapse or survival [8].

Temozolomide (TMZ) is a cytotoxic alkylating agent that transforms in vivo to the same active metabolite as that derived by hepatic metabolism of DTIC, monomethyl triazenoimidazole carboxamide (MTIC). TMZ crosses the blood–brain barrier and does not require metabolic activation; it undergoes spontaneous chemical degradation to MTIC at physiologic pH [9]. A large randomized trial compared oral TMZ administered at a dose of 200 mg/m^2/d × 5 days every 28 days with iv DTIC administered to melanoma patients on their first presentation of metastatic disease. The median overall survival was 7.7 months with TMZ and 6.4 months with DTIC, with a hazard ratio (HR) of 1.18 ($p = 0.2$). The six-month overall survival rate for TMZ compared with DTIC was reported at 61 percent versus 51 percent; $p = 0.063$, HR = 1.36. Even though the difference between the treatment groups for overall survival did not reach statistical significance ($p = 0.20$), the 95 percent confidence interval for the HR (0.92–1.52) indicated that TMZ was at least equivalent to DTIC [10]. A recently reported Phase III trial of 859 patients used TMZ on an extended schedule (150 mg/m^2/day orally on days 1–7, repeated every 14 days) on the hypothesis that an extended schedule may prolong depletion of the DNA repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT) and improve clinical efficacy. There was no significant difference in overall survival (OS; HR = 0.99, median 9.13 [TMZ] vs 9.36 [DTIC] months), progression-free survival (PFS; HR = 0.92, median 2.30 [TMZ] vs 2.17 [DTIC] months) and overall response (complete response/partial response; 14% TMZ vs. 10% DTIC) [11].

Fotemustine, compared with DTIC for untreated metastatic melanoma patients in a Phase III trial, demonstrated improved response rates (15.2% vs. 6.8%), but had no statistically significant improvement in overall survival [12]. Combination chemotherapy regimens including the Dartmouth regimen (DTIC/cisplatin/carmustine/tamoxifen) [8] or the CVD regimen (cisplatin/vinblastine/DTIC) have shown improved response rates but have failed to confer a survival benefit [13]. Further, the addition of tamoxifen and/or interferon alpha (IFN-α) to DTIC offers no discernible benefit [14].

MECHANISMS OF RESISTANCE TO CHEMOTHERAPY IN MELANOMA

MTIC, the active metabolite of DTIC and TMZ, methylates the DNA at the N^7 (70% of base lesions) and O^6 positions (6%) of guanine, and the N^3 position of adenine (9%) [15]. The O^6-methylguanine (O^6-MeG) base lesion, considered to be important for the cytotoxicity of DTIC or TMZ, is repaired by the DNA repair protein MGMT. Depletion of MGMT through approaches that use combinations of two alkylating agents or extended dosing schedules of TMZ have been pursued to reduce resistance and improve clinical outcome [16].

Loss of MGMT expression, as measured by MGMT promoter methylation, has been shown to correlate with an improved response rate and progression-free survival in patients with glioblastoma after treatment with TMZ [17] and with an improved response rate in patients with glioma [18]. In melanoma, studies to date have not shown a correlation with response to DTIC [19] or TMZ [20, 21].

Mechanisms of alkylating agent resistance other than MGMT are known in melanoma, such as activation of base-excision repair or loss of mismatch repair (MMR) [22]. Failure to find a correlation between tumor response to DTIC or TMZ and MGMT expression or promoter methylation suggests that these other resistance mechanisms may be important, either alone or in conjunction with MGMT.

IMMUNITY AND IMMUNOTHERAPY IN MELANOMA

Immunity to melanoma appears to be important for disease control in the adjuvant and advanced disease settings. Spontaneous regression has been reported in melanoma, suggesting a role for host immunity. This role is indirectly supported by the regular presence of lymphoid infiltrates at the site of primary melanoma that are associated frequently with histopathological evidence of tumor regression. Host cellular immune response within melanoma has potential prognostic and predictive significance. T-cell infiltrates in primary melanoma are prognostic of disease outcome [23], and T-cell infiltrates within regional nodal metastasis predict benefit from IFNα2b therapy [24–26].

HIGH-DOSE INTERLEUKIN-2

IL-2 plays a central role in immune regulation, as it affects the survival of key cells of the immune system that are responsible for the antitumor cytotoxicity of T lymphocytes and natural killer (NK) cells, and it has a cofactor role in the activation of B cells and macrophages [33]. The administration of IL-2 at high-bolus iv doses once every eight hours was a regimen developed by the National Cancer Institute (NCI) based on animal models indicating that antitumor activity with this agent was dose-dependent [34]. Initial studies with high-dose bolus (HDB) IL-2 used doses of 600,000 to 720,000 units/kg every eight hours from days 1 through 5 (cycle 1) and days 15 through 19 (cycle 2), with a maximum of fourteen doses per cycle or twenty-eight doses per course (1 course = 2 cycles). Responding or stable patients were offered a second course of therapy eight to twelve weeks later. IL-2 was administered either as a single agent or in combination with immunologically active cells, as so-called adoptive immunotherapy. The latter technique used two types of immune cells: the lymphokine-activated killer (LAK) cells and the tumor-infiltrating lymphocytes (TILs).

Eight clinical trials conducted between 1985 and 1993 and using the HDB IL-2 regimen described above, with or without LAK cells, were reviewed in a retrospective analysis. These trials had an enrollment of 270 patients with advanced metastatic melanoma [38, 39]. In studies that involved the concurrent administration of LAK cells, these cells were obtained using leukapheresis from patients during the rebound lymphocytosis that occurs following treatment and cessation of bolus IL-2 (days 8 to 12). LAK cells were then cultured in IL-2 for three to four days. These generated LAK cells were reinfused with IL-2 during the second cycle of IL-2 administration. The retrospective analysis of these trials with a follow-up through December 1998, along with a more recent update, demonstrated an objective response rate of 16 percent, with durable responses in 4 percent of patients [38, 39]. The median response duration was 8.9 months (range 4–106+ months). Twenty-eight percent of the responding patients, including 59 percent of patients who had achieved a complete response, have remained progression-free at a median follow-up of sixty-two months. Furthermore, no patient who had responses longer than thirty months has relapsed, suggesting the possibility that these patients may be "cured." The frequency of the responses was similar in patients with visceral metastases and/or large tumor burdens, but the responses were lower in patients with poor performance status or those who had received prior systemic therapy. Based on these data, HDB IL-2 received approval by the US FDA for the treatment of metastatic melanoma. However, major toxicities are associated with this regimen, such as a capillary leak syndrome leading to hypotension, renal insufficiency, and hypoxia, precluding its widespread application. The use of high-dose IL-2 is currently limited to specialized programs with experienced personnel, and it is generally offered only to patients with good performance and excellent organ function [40].

In addition to being cumbersome, randomized studies have not shown superiority for IL-2 administered with LAK cells versus therapy with HD IL-2 alone [41].

Furthermore, a randomized Phase III trial of CD8+ TIL given in combination with rIL-2 in metastatic renal cell carcinoma has been negative [42].

BIOCHEMOTHERAPY

The use of combined chemotherapy and immunotherapy has been widely investigated in metastatic melanoma. The two broad approaches to this concept have involved either sequential chemotherapy (cisplatin, vinblastine, and dacarbazine [CVD]) followed by immunotherapy (IL-2 given by continuous infusion at 9 MIU/m^2 and IFN-α) or concurrent chemoimmunotherapy. Both approaches have produced promising results in Phase II trials, with an overall response rate between 40 percent and 60 percent and a long-term remission rate of about 9 percent. The sequential approach was compared to chemotherapy alone in a randomized trial. Although response rate and time to progression were improved for the sequential biochemotherapy (BCT) group, the survival difference was only of borderline significance, and the toxicity was formidable [44]. A concurrent biochemotherapy regimen of CVD together with IL-2 and IFN-α was tested in a Phase II study by Atkins and colleagues [45]. The results appeared to be equivalent to those obtained with the sequential regimen, with greater practicality and lower toxicity. The concurrent CVD/IL-2/IFN-α regimen (BCT) was subsequently adopted by the U.S. Intergroup and compared with CVD in an important randomized Phase III trial (ECOG 3695). This trial was stopped early after an interim analysis that revealed the failure of the BCT arm to produce significantly better response rates, PFS, OS, or durable complete responses relative to chemotherapy alone. In addition, toxicity, particularly grade IV, was greater for BCT [46]. Two other Phase III trials that were conducted in Europe using slightly different BCT regimens failed to demonstrate an improvement in response or relapse rates or OS [47, 48]. A recent meta-analysis from eighteen trials (11 trials of chemotherapy ± IFN and 7 trials of chemotherapy ± IFN+IL-2) showed no benefit for biochemotherapy on OS [49]. Thus, there is no convincing evidence that BCT is superior to chemotherapy alone in metastatic melanoma.

Patients who achieve a remission after intensive BCT regimens have a median time to progression of more than six months. In an attempt to extend the remission period, one strategy has been the use of maintenance biotherapy after "induction" BCT. A regimen of low-dose subcutaneously administered IL-2 and GM-CSF initiated with continuous infusions of decreasing doses of IL-2 has been developed [50]. O'Day et al. reported a series of patients with metastatic melanoma who achieved a partial response or stable disease to induction BCT and were treated with chronic low-dose IL-2 and intermittent pulses of intermediate/high-dose decrescendo IL-2 over a twelve-month period. Median survival was higher than that for historic controls, but this concept has yet to be tested in randomized trials [50].

SYSTEMIC THERAPEUTIC OPTIONS IN CLINICAL TRIALS

Cancer Vaccines in Melanoma (Peptide Vaccines, Genetic HSP, DC-Based Vaccines)

Tumor vaccines are generally designed either to increase immune recognition of tumor cells or to enhance the antitumor effector immune response through lymphocyte activation [51]. By 1990, preclinical studies with vaccines had shown evidence of complete tumor regression and/or prolonged stabilization of tumor growth [52–54]. In early clinical trials, vaccines prepared from whole tumor cells were associated with limited activity, presumably owing to the already adversely biased Th2 nature of the host immune response to melanoma-associated antigens [51]. The discovery and cloning of a number of shared melanoma-associated lineage antigens, such as Gp100, Mart-1, and tyrosinase, as well as more restricted cancer-germline antigens (e.g., MAGE-1 and NY-ESO-1), during the early 1990s spurred interest in the development of peptide vaccines [51, 55]. However, peptide vaccines combined with incomplete Freund's adjuvant were insufficiently immunogenic and did not elicit robust antitumor immune responses in the absence of exogenous cytokines [56]. Complex polyvalent vaccines prepared from whole-cell lysates of cultured tumor cell lines were considered to offer a broader repertoire of antigens. For example, Canvaxin is a polyvalent melanoma cell vaccine containing more than twenty tumor antigens [57]. In general, such vaccines were well tolerated in early-phase clinical trials, with preliminary suggestions of clinical benefit in nonrandomized historically referenced series [58, 59]. However, when rigorously tested in prospectively randomized trials both for resected stage III/IV melanoma, adjuvant Canvaxin therapy failed to improve either relapse-free or overall survival compared with Bacille Calmette-Guérin [60]. In addition, some objective responses were reported from other early vaccine studies [58, 59]. However, when compared with chemotherapy in randomized Phase III studies, melanoma vaccines such as Allovectin-7, Canvaxin, and Melacine have generally failed to meet primary endpoints of improved response or survival [61].

Among the strategies employed in melanoma vaccination is the use of T-cell–defined tumor-associated antigens. Melanoma has been shown to express multiple T-cell–defined epitopes, some of which are melanosomal markers of the tissue lineage, whereas

others are cancer-restricted in adults. MHC class I-restricted epitopes represent short peptides derived from tumor-associated antigens that are presented to CD8+ T-cells. The HLA-A2 class I allele (expressed by ≥45% of melanoma patients) appears to play a major role in presenting melanoma epitopes. The majority (70–80%) of antimelanoma cytotoxic T-lymphocyte clones derived from HLA-A2+ tumor-infiltrating lymphocyte cultures appear to react against MART-1-derived epitopes, with 10 percent to 20 percent of the clones reacting against gp100-derived sequences and 1 percent to 10 percent of the clones reacting against tyrosinase-derived sequences [62–65]. In addition, MHC class II-presented epitopes of each of these peptides have been identified. Vaccination with multiepitope peptide vaccine containing MART-1 (27–35), gp100 (209–217, 210M), and tyrosinase (368–276, 370D) peptides has been employed in several clinical trials with consistent evidence that the vaccination is well tolerated and could be associated with immunological and clinical responses in melanoma [66].

ECOG 1696 is a completed Phase II trial of multiepitope peptide vaccination for metastatic melanoma with or without IFNα2b or GM-CSF as an immune adjuvant, in a 2×2 factorial design. This study accrued 120 patients, and complete immunological data are available for 75 who had undergone three months of immune assessment. Immunity to CD8 epitopes of one or more of three lineage antigens inducible in 35 percent of patients with measurable metastatic melanoma was demonstrated. Ellispot assay responses, defined by the doubling of pretreatment T-cell precursor frequencies, were found to be associated with longer median survival but not with PFS. The influence of GM-CSF and IFNα2b, both given systemically, on the vaccine's immunological and antitumor responses did not reach statistical significance [67]. Studies are ongoing that are aimed at improving immunization against MART-1, gp100, and tyrosinase peptides by employing potent immunological adjuvants such as GM-CSF, given locally in oil-adjuvant, and cytosine-guanine oligodeoxynucleotide.

Heat shock proteins (HSPs) are a family of proteins that provide a variety of housekeeping and cytoprotective functions. They have important immunological features, including the chaperoning of an array of peptides to elicit a polyclonal immune response [68]. Immunization of metastatic melanoma patients with autologous tumor-derived HSPPC-96 induces a significant increase in melanoma-specific T-cell–mediated reactions [69], which are associated with favorable clinical responses [70]. In a Phase II trial, patients with metastatic melanoma were immunized with autologous tumor-derived HSPPC-96 at weekly intervals following surgical resection of metastases. In twenty-eight patients with residual measurable disease, clinical responses were observed in 18 percent. There were two with complete responses (CRs) (24 and ≥48 months) and three with stable disease (SD) (153, 191, 272 days). Immune monitoring revealed that HSPPC-96 vaccination induced tumor-specific T-cell responses (11 of 23 patients) and NK activation (8 of 16 patients). Clinical responses were associated with melanoma-specific T-cell–mediated responses (the two CRs and two of the three SDs were among the patients with detectable immunologic responses) [70]. A subsequent Phase II study of HSPPC-96 in combination with GM-CSF and IFN-α in patients with metastatic melanoma ($n = 28$) has recently been completed, and a Phase III trial randomizing patients with metastatic melanoma to vaccination with HSPPC-96 versus treatment with IL-2/chemotherapy/surgery is ongoing, with a target accrual of 350 patients.

Dendritic cell (DC)–based vaccination is actively being tested in clinical trials. DCs are pulsed with whole tumor cells, tumor-cell lysates, or specific peptides in an effort to improve antigen presentation to T lymphocytes and induce a more potent immune response. A Phase II clinical trial tested a DC-based vaccine (Uvidem [IDD-3]) by loading a patient's DCs with a cell lysate from three allogeneic melanoma cell lines. Among thirty-three patients treated, there was one CR, two partial responses, and six patients with SD.

Molecular Approaches to Inhibiting Deranged Signaling Pathways

BAY 43–9006 is a novel multikinase inhibitor that inhibits intracellular Raf kinases (CRAF, BRAF, and mutant BRAF), and cell surface kinase receptors (VEGFR-2, VEGFR-3, PDGFR-β, cKIT, and FLT-3). The BRAF gene encodes a Ras-regulated kinase that mediates cell growth and activates the malignant transformation kinase pathway. Activating mutations in BRAF have been described in two-thirds of the melanoma tumors in primary culture and in 70 percent of melanoma cell lines [71]. BAY 43-9006 is available orally and has been shown to be well tolerated in Phase I trials [72, 73]. In a randomized Phase II discontinuation trial, thirty-nine patients with metastatic melanoma were treated with single-agent BAY 43-9006 at 400 mg BID orally. At twelve weeks, the drug was generally well tolerated; one patient had a partial response and seven patients had SD [74]. These results suggested antitumor activity with BAY 43-9006 monotherapy. In a Phase I/II trial of BAY 43-9006 in combination with carboplatin and paclitaxel, thirty-five melanoma patients were treated for at least six weeks. Among thirty-two evaluable patients, eleven (31%) had partial responses, including ten ongoing at three to sixteen months. Nineteen patients had SD as their best response. The combination not only demonstrated activity in melanoma,

but also had a favorable safety profile and no apparent pharmacokinetic interactions [75].

Based on these results, a Phase III intergroup trial coordinated by the Eastern Cooperative Oncology Group has recently been completed, in which 800 patients with chemotherapy-naïve metastatic melanoma were randomized to carboplatin and paclitaxel with either BAY 43-9006 or a placebo. Based on these results, two Phase III trials were conducted. The first (PRISM trial) randomized previously treated patients (progressed on previous DTIC- or TMZ-containing chemotherapy) to carboplatin and paclitaxel with either sorafenib or placebo. There was no difference in PFS (the primary endpoint) with or without sorafenib (17.4 vs. 17.9 weeks, respectively; HR = 0.91; 99% CI, 0.63–1.31; two-sided log-rank test P = 0.49) or RR (12% vs. 11%). The percentage of patients on both arms with stable disease was also similar (54% and 51%), as was the median OS (42 weeks on both study arms; HR = 1.01 (95% CI, 0.76–1.36; P = 0.92 [5]. The second trial (trial E2603) had a similar design for the first-line treatment of patients with advanced melanoma. In April 2009, the Eastern Cooperative Oncology Group's Data Monitoring Committee recommended that the trial be stopped, having met the protocol criteria for futility.

V600E *BRAF* is the most common kinase mutation in melanoma (60%). Results of a Phase I study with a selective inhibitor of the oncogenic V600E mutant BRAF kinase, PLX4032, were recently reported. Among fifty-four patients enrolled, forty-nine had metastatic melanoma, and there were three thyroid, one rectal, and one ovarian carcinomas. Thirteen melanoma patients treated at doses of 240 mg BID or higher had a minimum follow-up of eight weeks. Among these, five of the seven BRAF V600E+ patients had tumor regression, with one confirmed partial response and 1 unconfirmed partial response (too early); two of four patients with unknown V600E status had tumor regression, with one confirmed partial response; 2 BRAF wild-type patients had progressive disease. All seven patients with tumor regression remained progression-free for at least four to fourteen months [118]. Updated data in June 2009 (at the 2009 ASCO Annual Meeting) reported that among sixteen BRAF V600E+ melanoma patients, there were nine partial responses (seven confirmed and two unconfirmed). These data have been updated in a recently published report [119] showing evidence of complete or partial tumor regression in the majority of patients. A larger phase II trial has confirmed these initial findings, and a Phase III trial is ongoing.

Recently, mutations and amplifications in the receptor tyrosine kinase c-kit have been reported in acral melanomas (which occur in sun-protected areas, such as the palms, soles, or subungual sites), mucosal melanomas, and cutaneous melanomas that arise in the setting of chronic sun damage. These melanoma categories account for only about a quarter of all melanomas in Western countries, but acral and mucosal melanomas are the most prevalent melanoma types in the rest of the world. In a cohort of 102 primary melanomas, the incidence of KIT genetic alterations, as defined by mutations or copy number increases were 15/38 (39%) of mucosal melanomas, 10/28 (36%) of acral melanomas, 5/18 (28%) of skin with chronic sun-induced damage, and 0/18 of melanomas on skin without sun-induced damage [120]. Because of data suggesting the expression of KIT in melanoma, three Phase II trials were initiated to examine the role of inhibition of KIT/PDGFR in patients with metastatic melanoma regardless of whether their tumors displayed expression of KIT/PDGFR. Among sixty-two patients, only one response was noted in a patient with acral melanoma [121, 122]. Also, a Phase II study with a similar design testing dasatinib showed modest activity [123]. Based on emerging data implicating KIT as an oncogene in the progression of melanomas of the mucosa and cutaneous melanomas arising in acral sites or in sun-damaged skin, a study with imatinib mesylate enrolled only patients with unresectable melanoma arising from acral, mucosal, and chronic sun-damaged sites. Patients were allowed if their tumor harbored amplifications on chromosome 4q12 (which includes KIT) by FISH or a mutation in KIT (exons 9, 11, 13, 17, 18) [124]. Of 146 patient tumors screened, 21 percent (31/146) of tumors were characterized by either mutation or amplification of *KIT*. Among the first twelve patients treated in this ongoing trial, the response rate was 33 percent (4/12) with two CR (18+ and 37+ weeks), two partial responses, and six SD. An Eastern Cooperative Oncology Group study (E2607) is currently testing the role of dasatinib in this selected patient population.

Antiapoptotic Approaches

Bcl-2 antisense therapy has been actively tested in metastatic melanoma. The Bcl-2 gene was the first component of the cell death pathway to be identified. Bcl-2 protein prevents apoptosis by blocking the release of cytochrome c from mitochondria [79]. Oblimersen (Genesense) is a Bcl-2 antisense compound that selectively targets Bcl-2 mRNA, causing its degradation and subsequent decreases in Bcl-2 protein translation. Preclinical studies with oblimersen have shown promising results. A subsequent Phase I/II study investigated the combination of oblimersen and DTIC in fourteen patients with advanced malignant melanoma expressing Bcl-2. The combination regimen was well tolerated, downregulated the target Bcl-2 protein, and increased tumor cell apoptosis, which was enhanced after DTIC treatment. Antitumor responses were seen in six patients (one complete, two partial, three minor), and the estimated median survival of all patients

exceeded twelve months [80]. This was followed by a Phase III multicenter clinical trial that enrolled 771 patients with metastatic melanoma who were randomized to therapy with either DTIC alone, or therapy with oblimersen followed by DTIC. In combination with DTIC, oblimersen was found to significantly increase response rate (13.0 vs 7.0%, $P = 0.006$) and progression-free survival (78 vs 49 days, $P = 0.0003$, HR = 0.73). However, there was no significant benefit on overall survival, with a median survival of 9.1 months for the combination group versus 7.9 months for DTIC alone ($P = 0.184$; intent-to-treat). Exploratory analyses suggested a statistically significant intent-to-treat survival benefit for the combination therapy at the fifteen- and eighteen-month landmarks ($P = 0.03$ for each), but it remains to be seen whether longer follow-up will reveal a survival difference between the two treatment arms [81]. The lack of corollary studies that may have confirmed the role of antisense to Bcl-2 in vivo in the context of this large trial was an issue, and the observation of benefit among patients without elevated LDH, as a marker of poor prognosis, has spurred a smaller study using patients with nonelevated LDH to confirm this finding.

Antibodies and Adoptive Transfer Strategies to Reverse Host Immune Tolerance and Redirect Autoimmunity

The roles of several critical regulatory elements of the immune system have recently been elucidated, providing insight into the disease process and new targets for overcoming tolerance. Enhanced expression of co-stimulatory molecules on the surface of DCs is one approach to enhance presentation of tumor-associated antigens. This can be achieved through stimulation of DC receptors such as CD40 and toll-like receptor 9 (TLR-9) [82–84]. Another avenue is to enhance or prolong T-cell activation by blocking negative signaling receptors such as CTLA4 [85]. New strategies, such as the administration of oligodeoxynucleotides that activate TLR-9 or of monoclonal antibodies (mAbs) that activate CD40 or block CTLA4, may provide more effective immunotherapies that can possibly overcome tumor-induced tolerance.

CYTOTOXIC T-LYMPHOCYTE–ASSOCIATED ANTIGEN 4 BLOCKADE

CTLA4 is a key element in immune tolerance and a critical negative regulator of T-cell–mediated antitumor immune responses. Identification of the amino acid sequence of CTLA4 in 1987 allowed further exploration of its role in T-cell tolerance [86]. Early preclinical studies suggested that this molecule serves as a natural braking mechanism for T-cell activation, which allows a return to homeostasis following an immune response. This was most profoundly demonstrated in murine CTLA4 knockout models. Mice lacking CTLA4 developed a massive lymphoproliferative disorder, leading to lymphocytic infiltration and destruction of major organs [87–89]. CTLA4 is a homolog of CD28 that functions as an inhibitory receptor for B7 co-stimulatory molecules expressed on mature APCs [90, 91]. Following T-cell activation, CTLA4 cell surface receptors are upregulated and successfully compete with CD28 for binding to B7, sending an inhibitory signal that downregulates T-cell activation [85, 91]. This inhibitory signal affects downstream targets of CTLA4 that include cytokine production by Th1 and Th2 cells [92] and key components of the cell cycle machinery (Cdk-4, Cdk-6, and cyclin D3) that are required for cell cycle progression [93–95]. Therefore, it was hypothesized that blocking the interaction of B7 with CTLA4 might enhance T-cell activation, leading to a more robust antitumor immune response.

Anti-CTLA4 mAbs with a much greater affinity for CTLA4 than B7 (competitive inhibition) were cloned and shown to inhibit the interaction of B7 and CTLA4 [85]. The inhibitory signal produced by CTLA4 is therefore blocked, and T-cell activation is enhanced (i.e., releasing the brake). In vitro, anti-CTLA4 mAbs were shown to enhance T-cell function, as measured by increased production of IL-2, γ-interferon, and other cytokines [90, 92]. Multiple animal models have confirmed that the CTLA4 blockade, either alone or when combined with other interventions, enhances antitumor T-cell immune function and T-cell–mediated killing, and inhibits tumor recurrence [96–98]. In a murine sarcoma model, the combination of CTLA4 blockade and a poxvirus vaccine provided a significant survival advantage compared with vaccine alone ($P < 0.001$) [99]. Treatment with an anti-CTLA4 mAb also reduced tumor recurrence in a murine prostate cancer model [97]. Using a human/SCID mouse chimeric model, CTLA4 blockade was also shown to significantly enhance human lymphocyte-mediated tumor suppression in mice co-engrafted with peripheral blood leukocytes and tumor cells [100]. Augmentation of IFN-γ production, upregulation of MHC class I expression on the tumor, enhancement of tumor cell apoptosis, and reduction of angiogenesis have been proposed as mechanisms for the antitumor effects of CTLA4 blockade [101]. Based on the strength of these preclinical data, clinical trials have been initiated with two fully human anti-CTLA4 mAbs, which have different pharmacokinetic and pharmacodynamic properties.

Tremelimumab

Tremelimumab (CP-675,206; Pfizer Inc.) is a fully human IgG2 mAb directed against CTLA4 with a serum half-life of approximately twenty-two days [85]. Tremelimumab was shown to enhance human T-cell activation by inducing IL-2 production in cultures

of staphylococcal enterotoxin A (SEA) superantigen-stimulated peripheral blood mononuclear cells or whole blood [102]. In an open-label, Phase I dose-escalation study, thirty-nine patients with solid malignancies received an intravenous infusion of tremelimumab at one of seven dose levels ranging from 0.01 to 15 mg/kg [85]. Among twenty-nine patients with melanoma and measurable disease, toxicities were generally mild to moderate in severity and were dose related [85]. The most commonly reported treatment-related toxicities were diarrhea, dermatitis, pruritus, and fatigue [85]. Two (7%) patients had complete responses CRs by RECIST criteria, two (7%) patients experienced partial responses, and four (14%) patients had SD [85]. Furthermore, objective responses were durable (ranging from 37 to 51+ months) [103], suggesting a memory T-cell response to tumor-associated antigens.

Subsequently, an open-label Phase II trial was conducted in patients with advanced melanoma who were randomized to receive either 10 mg/kg tremelimumab monthly ($n = 44$) or 15 mg/kg tremelimumab every three months ($n = 45$) [104, 105]. Four (9%) patients treated with 10 mg/kg every month (Q1M) had results of one CR and three partial responses, and three (7%) patients treated with 15 mg/kg every three months (Q3M) had responses of one CR and two partial responses, including responses from the lungs, liver, bone, lymph nodes, skin, and adrenal glands [105]. Although the response rate was not significantly different between the two arms, the 15 mg/kg Q3M regimen was associated with a lower incidence of grade 3/4 adverse events [105]. Consequently, the 15 mg/kg Q3M dosing regimen was selected for further study and was investigated for single-agent antitumor activity in a larger Phase II open-label trial and in a randomized comparative Phase III study against DTIC or TMZ in patients with advanced relapsed or refractory melanoma.

The Phase III study compared the overall survival of patients who were randomized to single-agent tremelimumab ($n = 328$) to that of patients who received standard-of-care chemotherapy ($n = 327$), either DTIC or TMZ at the physician's discretion [106]. Patients received either 15 mg/kg tremelimumab ($n = 324$) every three months for up to four cycles or chemotherapy ($n = 319$) with either DTIC (1000 mg/m^2) every three weeks for up to twelve cycles or TMZ (200 mg/m^2) on days 1 through 5 of every four weeks for up to twelve cycles [106]. Overall survival was the primary endpoint. At the second interim analysis, the trial was halted based on recommendations from the Data Safety Monitoring Board because the log rank test-statistic ($P = 0.729$) crossed the prespecified O'Brien-Fleming boundary for futility ($P > 0.473$). At that time, the observed median survival in the tremelimumab and chemotherapy arms was 11.76 and 10.71 months respectively (HR chemotherapy/tremelimumab = 1.04). The patients who received a clinical benefit with tremelimumab are remaining in the study, and more mature survival and response data are anticipated. The role of excluding patients with higher than two times the upper limit of normal LDH blood values and crossover of patients in the control arm to another anti-CTLA4 mAb are not yet known.

Ipilimumab

Ipilimumab (MDX-010; Medarex, Inc./Bristol-Myers Squibb Co.) is an IgG1κ mAb against CTLA4 with a serum half-life of approximately twelve days [107]. Of HLA-A*0201$^+$ patients with stage IV melanoma ($N = 56$) who were treated with 3 mg/kg ipilimumab every three weeks or 3 mg/kg initially and then 1 mg/kg every three weeks in combination with a gp100 peptide vaccine, the overall objective response rate was 13 percent (2 CRs, 5 partial responses) [108]. Tumor regression was noted in the lungs, liver, brain, lymph nodes, and skin. Fourteen (25%) patients had grade 3/4 immune-mediated side effects, including colitis, dermatitis, uveitis, enterocolitis, hepatitis, and hypophysitis [108]. In a Phase I/II study, metastatic melanoma patients ($N = 36$) received a combination therapy with ipilimumab (0.1–3.0 mg/kg) plus high-dose IL-2 (720,000 IU/kg every 8 hours for a maximum of 15 doses). Objective tumor responses (3 CRs, 5 partial responses) were experienced in eight (22%) of the patients [91]. Although the combination therapy of ipilimumab and IL-2 was tolerable, there was no evidence of synergistic antitumor effect [91]. A Phase III study testing ipilimumab as a single agent or in combination with DTIC for patients with advanced relapsed or refractory melanoma has been completed, and another Phase III study involving ipilimumab administered in conjunction with the gp100 peptide vaccine has also been conducted. Final results of both studies are anticipated. Promising results with this agent in the metastatic setting have lead to the initiation of the adjuvant randomized trials EORTC 18071 (ongoing) and E1609 (anticipated).

CONCLUSION AND FUTURE DIRECTIONS

For metastatic melanoma, current available medical options have limited benefits with no known survival advantage. For this group of patients, participation in clinical trials is currently the best strategy to maximize therapeutic options and to access novel drugs in clinical development. Outside of a clinical trial, HDB IL-2 is associated with durable responses in a minority of carefully selected patients. DTIC, TMZ, and the combination of carboplatin and paclitaxel have modest clinical activity. Future progress will likely come

from the use of combinations of agents that modulate immune response in the host, and target the tumor cell progression pathways that are identified in melanoma. Future advances will also likely come from individualized approaches targeting groups of patients with specific activating mutations that are driving malignant proliferation, such as the V600E BRAF mutation and mutations and amplifications in the receptor tyrosine kinase c-kit.

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30 Gastric Cancer Metastasis

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The treatment outcome of gastric cancer has markedly improved with the advancement of diagnostic technology, widespread use of radical resection combined with lymph node dissection, and advances in chemotherapy.[1,2] However, these results must be improved as about 700,000 people worldwide die from gastric cancer annually.[3] Therefore, elucidation of the metastatic mechanism of gastric cancer, prevention and early detection of metastasis, and development of drugs based on the metastatic mechanism are important for achieving optimal treatment outcome.

CLINICAL FEATURES OF GASTRIC CANCER METASTASIS

There are two major forms of gastric cancer in terms of clinical metastasis. In histologically scirrhous gastric cancer, which is composed primarily of poorly differentiated adenocarcinoma/signet ring carcinoma, a large amount of interstitial component is morphologically observed, and cancer cells single-handedly assume the course of diffuse invasion. This type of gastric cancer spreads by the peritoneal or the lymph node metastatic route. In histologically nonscirrhous gastric cancer, the main presenting histological features are those of well- or moderately differentiated adenocarcinoma. In this form, cancer cells frequently invade vascular channels as tumor cell clusters (or tumor cell clumps) and spread to distant organs (hematogenous metastasis).

Lymph node metastasis is diagnosed by computed tomography, magnetic resonance imaging, endoscopic ultrasound, or positron emission tomography, but the sensitivity and specificity of these techniques are insufficient. The need for and the extent of lymph node dissection, as well as the significance of and treatment protocol for micrometastasis (MM) in lymph node metastasis, remains debatable. In the United States, the standard procedure is to control lymph node metastasis by performing postoperative chemoradiotherapy after D0 or D1 lymph node dissection.[4] In Europe, the recurrence rate of gastric cancer is reduced by a combination of pre- and postoperative chemotherapy.[2] In Japan, D2 lymph node dissection has been demonstrated to contribute to prognostic improvement. Postoperative adjuvant chemotherapy has recently been administered in stage II/III patients.[5]

Peritoneal metastasis occurs most frequently as a recurrent pattern of advanced gastric cancer. The prognosis for patients with peritoneal metastasis of gastric cancer is extremely poor; therefore, an understanding of the mechanism underlying such metastasis and an identification of a therapeutic target are urgently needed.[6] The metastatic sequence commences when primary tumor cells infiltrate the serosa. These cells are then released into the peritoneal cavity and implanted in the peritoneum. This metastatic pattern is mostly disseminative and rarely hematogenous or lymphogenous. Bowel obstruction, ascites, hydronephrosis, jaundice, and other uncontrollable clinical conditions also frequently develop.

Hematogenous metastasis to the liver, lung, bone, and brain also occurs, causing clinical symptoms specific to individual organs. These metastases are treated with palliative chemotherapy prescribed per the systemic disease.

EARLY DIAGNOSIS OF GASTRIC CANCER METASTASIS

Although the histopathological technique has routinely been used for the qualitative and quantitative diagnosis of cancer, attention has recently been paid to the clinical significance of a minuscule quantity of cancer cells in a resected sample that cannot be identified by histopathological analysis. Such cases are generally

categorized as MM and defined, according to the International Union Against Cancer, as follows:

> *TNM Classification*.[7] Metastasized cancer in a lymph node or a distant organ measuring between 0.2 mm and 2 mm is defined as MM, and a cluster of cells or a single tumor cell measuring less than 0.2 mm is defined as an isolated tumor cell (ITC).

In addition to the histopathological technique, molecular biological analyses have recently been carried out for the diagnosis of MM, and their physiological and clinical features have also been studied. However, the significance of MM associated with gastric cancer has not yet been established, and its relevance toward determining a prognosis remains controversial.

One of the reasons that diagnostic techniques for the early detection of metastasis have not yet been established is that sensitivity and quantitative performance vary among different detection methods. The current detection methods include immunostaining targeting protein expression, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) targeting mRNA, and mutant allele specific amplification-PCR targeting DNA mutation. Immunostaining is usually time-consuming, and false-positive results are occasionally obtained owing to immunocompetent cells. RT-PCR can detect micrometastatic cells, but it is difficult to determine whether such cells have any clinical significance. Thus, it is important to overcome the limitations of these different detection techniques to completely understand the significance of MM. Transcription–reverse transcription concerted reaction and one-step nucleic acid amplification have recently been developed for directly amplifying target RNA. Because they are more convenient and rapid than RT-PCR, their clinical application for intraoperative rapid diagnosis is eagerly awaited.[8,9] The following sections provide descriptions of the diagnostic techniques used for early detection of MM using clinical specimens.

Lymph Node Metastasis

In general, lymph node metastasis of gastric cancer is diagnosed after staining the central cut surface of a lymph node, which is divided onto several hematoxylin and eosin (H&E) glass slides, and then assessing any metastatic lesion smaller than the maximum cut surface as negative. Using this diagnostic staining method, a clear difference in prognosis was observed between patients diagnosed with lymph node metastasis and those without lymph node metastasis. Therefore, this method is considered appropriate for the clinical diagnosis of lymph node MM. In a report on the relationship between lymph node MM and prognosis,[10–12] Siewert et al. demonstrated that ITC in a lymph node acquires proliferative activity after Ki-67 expression.[13,14] However, many experts opine that MM and ITC can be controlled by D2 lymph node dissection because the prognosis remained good even in N1 cases after the patients had undergone this procedure.

The relationship between prognosis and the results of immunostaining for lymph node MM remains controversial, with some advocating its existence and others discounting it. According to a report that investigated 300 patients with pN0 early gastric cancer, MM was detected in 10 percent of the samples by cytokeratin (CK) immunostaining.[15] In addition, decreased E-cadherin and MMP-2 expressions were reported.[16,17] It was also shown in one report that when multiple-marker RT-PCR was employed on a resected lymph node sample using carcinoembryonic antigen (CEA), CK20, and melanoma antigenic epitope-3 as genetic markers, the MM detection rate was improved and the classification stage became higher in 38 percent of curative resection cases.[18] Comparative genomic hybridization has recently been used for chromosome abnormality analysis, and DNA microarray analysis has also been performed to comprehensively investigate genetic expression abnormality. Nevertheless, none of these techniques has been established as a standard procedure for lymph node MM detection in gastric cancer.

In gastric cancer, sentinel node (SN) navigation surgery is also occasionally performed because it is believed that metastasis develops initially in SNs. Not only does lymph node metastasis occur first in SNs, but also the MM/ITC levels are high in SN. In this regard, multiple-section SN sampling is advantageous in terms of sensitivity, economy, and the time required for metastasis diagnosis. However, this technique has not yet been standardized, as there is no consistency either in the methods employed or in the basic data obtained. Because of the complexity of the lymphatic route, uncertainty remains regarding the introduction of SN navigation surgery for detecting metastasis.[19]

Peritoneal Metastasis

Peritoneal lavage cytology (CY) is one of the methods employed to determine the classification of staging and to assess the curability of gastric cancer. The significance of MM in the peritoneal cavity has long been recognized. It has also been reported that the prognosis of CY-positive patients is poorer than that of CY-negative patients, and that the number of cancer cells determined by CY testing may affect prognosis.[20] However, the biggest limitation of CY is that many cases are difficult or impossible to assess, and even CY-negative patients may have peritoneal recurrence. Therefore, CY is not yet used as a standardized diagnostic procedure.

Because there are few immunocytes in the peritoneal cavity as compared with the lymph node, bone marrow,

or peripheral blood, even a small number of cancer cells can form a tumor on the peritoneum. RT-PCR is considered useful for the detection of peritoneal metastasis. A large number of studies, including CK-20, E-cadherin, trypsinogen, telomerase, and matrix metalloproteinase (MMP) have reported CEA as a target.[21–23] The CEA test produced satisfactory results – 89 percent sensitivity and 82 percent specificity – when the recurrence of peritoneal metastasis within the postoperative five years was used as an indicator[24] and a prospective validation study was carried out.[25] Patients with high CEA levels in ascitic fluid had a significantly worse prognosis and had a more frequent recurrence than those with low CEA levels.[24]

In the case of the RT-PCR approach, several problems have been reported, such as the absence of a specific marker. The CEA test produced a false-positive result in response to minimal expression of lymph systematic cells or mesothelial cells in the peritoneal cavity. In addition, the genetic expression patterns are diverse, and these expression patterns are often different even among gastric cancer patients. It has also been reported that the CEA expression level is low in undifferentiated gastric cancer.

To address the aforementioned problems, a new method has been developed and used in recent years. This method provides a comprehensive comparison of genetic expression between a gastric cancer peritoneal metastatic cell line and its parental line by DNA array to discover an unknown relevant factor.[26] In addition to cell-adhesion–related genes, including CD44 and integrin, another gene was discovered, but its relevance to the peritoneal metastasis of gastric cancer has not yet been demonstrated. To date, no single diagnostic marker has been shown to be superior to CEA.

Bone Marrow Metastasis and Circulating Tumor Cells

Because the bone marrow performs the role of filtering peripheral blood, it is possibly a more sensitive marker of hematogenous metastasis than peripheral blood itself. It has been reported that MM in the bone marrow occurred in 20 percent of gastric cancer patients, and that bone marrow metastasis may serve as a predictive factor for early postoperative recurrence.[27,28] Another report found that bone marrow metastasis was related to microangiogenesis, but not to prognosis. Additional studies have shown that cytokeratin immunostaining or real-time RT-PCR could be employed for detecting bone marrow MM[27,29]. Nevertheless, few cancer cells cannot continue to survive in the bone marrow, and there are other noncancerous cells positive for cytokeratin. Therefore, the data accumulated thus far on gastric cancer MM in the bone marrow remains insufficient for clinical application.

Circulating tumor cells (CTCs) in the blood have also been examined as a possible marker of hematogenous metastasis of gastric cancer. One of the advantages of the method of identifying CTCs is that it is less invasive than collecting a lymph node or bone marrow sample, but the problem lies in the fact that the number of CTCs that can be collected is very small. Some studies have reported on the relationship between CTCs and the prognosis of gastric cancer – that is, surviving-expressing CTCs are associated with recurrence rate, and the metastasis rate is related to membrane type 1 MMP-expressing CTCs. Clinical application is not in place as yet because of the lack of data on CTCs.[30,31]

MECHANISM OF METASTASIS OF GASTRIC CANCER

The metastatic process of gastric cancer goes through various sequential steps, starting with the detachment of cancer cells from the primary lesion, which then leads to the destruction of surrounding tissue, invasion and transfer into blood vessels and lymph channels, settlement in the target organ, and, finally, ectopic proliferation. Detachment from the primary lesion as the first step of metastasis is induced by the collapse of the mechanism of cell–cell or cell–stroma adhesion. The structures and abnormal expression of E-cadherin and related α-catenin, β-catenin, adenomatous polyposis coli, and dysadherin are known to be associated with the collapse of the adhesion mechanism of gastric cancer cells. The epithelial–mesenchymal transition (EMT) is also being investigated as one of the metastatic mechanisms of cancer.[32,33]

Reduced E-cadherin expression has been reported for gastric cancer.[34] In animal experiments, after the addition of the culture supernatant of gastric fibroblasts, CD44H (an adhesive molecule binding to ligand hyaluronic acid) expression increased with the detachment of peritoneal metastatic cells from the primary lesion, thus accelerating peritoneal metastasis.[35]

The involvement of transforming growth factor (TGF)-β in this process has also been reported. Moreover, integrin, an adhesive molecule binding to peritoneal extracellular matrix as the ligand, was intensely expressed in peritoneal metastastic cells. In clinical specimens, the expressions of $\alpha 2\beta 1$-integrin and $\alpha 3\beta 1$-integrin increased at the peritoneal metastatic site of gastric cancer compared with those found in the primary lesion.[36] The RGD or YIGSR peptide, which has the same amino acid sequence as the $\beta 1$-integrin adhesion domain, not only adheres to the B1-integrin expressed in cancer cells but also blocks its adhesive function. In addition, it has been shown that the administration of both agents prolonged the survival of mice with peritoneal tumor dissemination.[36] Snail, Twist, MMP-3, met, TGF-β, FOXC2, GSK3β, and Smad-3 have

also been identified as EMT regulators, and the development of molecular-targeted therapy is under way, focusing on these regulators.[37]

In gastric cancer invasion, the interactions between cancer cells and interstitial tissue are important. The invasion process begins with MMP expression increasing at the local site of cancer. Next, the proteolytic zinc-dependent MMP degrades the extracellular matrix, resulting in the destruction of interstitial tissue and facilitating the dissemination of cancer cells. MMP-7 is not expressed on the interstitial cells of cancer tissue but is specifically expressed on cancer cells, and it is one of the target genes for Wnt/β-catenin signals. It was found that increased MMP-7 expression can serve as a predictive marker of lymph node metastasis of gastric cancer.[38–40] An agent that can selectively inhibit MMP-7 has recently been discovered and is now in the research stage. It appears that this agent can stop the invasion/metastasis of colon cancer.

In relation to this development, the Ets-related transcription factor E1AF/PEA3 was found to play an important role in the enhancement of MMP-7 transcription.[41] Specifically, E1AF is known to play an important role in increasing MMP-7 expression in gastric cancer and is considered to be a useful predictive marker in the recurrence and prognosis of postoperative gastric cancer.[38,40] MMP-1 expression in resected specimens was reported to be relevant to the development of peritoneal metastasis as well.[42] Different levels of tissue destruction are considered to be induced by differences in the quantitative balance between MMP-1 and the tissue inhibitor of metalloproteinase, which inhibits the activities of MMP-1, MMP-3, and MMP-9; a possible correlation between MMP-1 and metastasis of gastric cancer has been indicated. When cells derived from scirrhous gastric cancer were cultured in a stroma (type I collagen), the cell proliferation rate increased. Although cytokines such as TGF-β, an insulinlike growth factor, are accumulated abundantly in the stroma of cancer tissue, the proliferative inhibitory mechanism of TGF-β is not very effective, owing to the absence of or a decrease in the number of TGF-β I receptors.[43] These findings suggest that scirrhous-forming gastric cancer cells naturally create an environment for accumulating stroma inside cancer tissue. This is a new interpretation, being different from the conventional perception that scirrhous tissue is formed as a result of local fiber growth for tissue reconstruction following tissue destruction.

There are currently many published reports on the process of lymph node metastasis. Lymph node metastasis induced by vascular endothelial growth factor (VEGF)-C is a clever mechanism of cancer cells that accomplishes dissemination through the utilization of biological reactions. Cancer cells secrete VEGF-C, which then acts on vascular endothelial growth factor receptor (VEGFR)-3, specifically expressed on lymphatic endothelial cells, to induce lymph node metastasis. The extensive lymph node system provides more opportunities for cancer cell invasion, thus promoting lymph node metastasis.[44]

In a model of lymph node metastasis established by gastric cancer orthotopic implantation, VEGF-C expression on the hypermetastatic lymph node gastric cancer cell line increased compared with that of the parent cell line, suggesting that tumorous lymph node metastasis was promoted.[45] Because an assay method for assessing lymph channels in tumors of clinical samples has not yet been established, findings regarding the density of lymph channels in the primary lesion and in lymph node metastasis remain controversial.

In one report on the chemokine reaction of a gastric cancer cell line, it was demonstrated that the expression level of the chemokine receptor CCR7 increased, that chemotaxis to CCL21 (SLC), a CCR7 ligand, increased in vitro, and that hyperexpression of CCL21 in lymph nodes and a high degree of malignancy were observed in patients with lymph node metastasis.[46] On the other hand, CCR7, which is not expressed in the normal state, was shown to be expressed in patients with *Helicobacter pylori*-induced gastritis, and CCR7 expression level increased in intestinal metaplasia, dysplasia, and cancer.[47] Moreover, metastasis was shown to be inhibited by a chemokine antibody/inhibitor in an experimental animal model. Thus, the chemokine/chemokine receptor has attracted attention as a candidate target of next-generation molecular-targeted therapy.

Different histopathological types of cancer are characterized by different genetic expressions. Scirrhous gastric cancer is characterized by amplification of the *K-samII* gene, one of the many types of cancer genes.[48] The *K-samII* gene is homologous to fibroblast growth factor receptor 2 (FGFR2); is frequently expressed in poorly differentiated adenocarcinoma, particularly in scirrhous gastric cancer (33%); and is involved in the proliferation of this cancer. Ki23057, a FGFR2 inhibitor, significantly prolongs the survival time in peritoneal dissemination mice.

The c-met gene encodes the receptor of hepatocyte growth factor (HGF), a scatter factor; its amplification, 19 percent in well-differentiated adenocarcinoma but as high as 39 percent in scirrhous gastric cancer in terms of histological type, was observed only in advanced cancer.[49] When a cyclooxygenase-2 inhibitor (JTE522) blocked HGF produced by fibroblasts, scirrhous gastric cancer cell invasion was suppressed in mice with gastric cancer peritoneal metastasis. Concurrent use of TS-1 prolonged the survival time in mice as well.[50] Amplification of the c-erbB2 (HER2) gene was observed in about 20 percent of patients with gastric cancer, for the most part with well-differentiated adenocarcinoma, and these patients with gene amplification or overexpression showed poor prognosis.[51]

Although some types of gastric cancer do not develop frequently, their mechanism of development has already been clarified. Germ-cell inactive mutation of E-cadherin is found in Maori families in New Zealand and is associated with a high risk of juvenile diffuse gastric cancer.[52] Moreover, 13 percent of all gastric cancer patients are positive for microsatellite instability (MSI), which has been caused, in the majority of cases, by the methylation of the MLH1 promoter domain. The intestinal type of MSI-positive gastric cancer that occurs most frequently in the distal region is characterized by less frequent lymph node metastasis or distant metastasis and good prognosis.[53,54] Approximately 10 percent of gastric cancers patients are said to also be infected with Epstein-Barr virus (EBV), and lesions that histologically present substantial lymphocyte infiltration are mostly of the diffuse type that frequently occurs in the proximal region and are characterized by less frequent lymph node metastasis and good prognosis.[55,56] Therefore, EBV infection and a histological finding of lymphoepithelioma-like cancer have evolved as markers for a low risk of lymph node metastasis.

INTERVENTION FOR GASTRIC CANCER METASTASIS

The findings of these clinical investigations and molecular biological studies have been clinically applied to the development of therapeutic strategies and novel drugs against gastric cancer metastasis.

Intervention by Peritoneal Washing Cytology

Peritoneal metastasis appears to be predisposed in gastric cancer of the macroscopic type 3 or 4 because serosal infiltration is frequently observed. A clinical trial was carried out for either type of gastric cancer on patients who had undergone preoperative chemotherapy. The chemotherapy was administered to obtain a higher cure rate if the diagnostic laparoscopy finding was CY0 and also was expected to improve treatment outcome.[57] The Japan Clinical Oncology Group (JCOG) is currently carrying out a randomized control study of both types of gastric cancer, using one group that underwent surgery only compared with another group that had accompanying preoperative chemotherapy. Although several reports on peritoneal thermochemotherapy and intraoperative peritoneal chemotherapy in CY1 cases were previously published, no agreement and standardization have been achieved thus far.[58]

Intervention Using Molecular-Targeted Drugs

In patients with advanced gastric or gastroesophageal junction cancer with high expression of HER2 protein (immunohistochemistry 2+ and FISH positive or immunohistochemistry 3+), addition of trastuzumab to cisplatin plus capecitabine or 5-fluorouracil significantly improved overall survival compared with chemotherapy alone. The HR for patients with high HER2 expression was 0.65 (95% CI 0.51–0.83) and median overall survival was 16.0 months (95% CI 15–19) in trastuzumab plus chemotherapy compared with 11.8 months (10–13) in chemotherapy alone.[55] Although lapatimib, a dual inhibitor of HER1 and HER2, produced only a 5 percent response rate in metastatic gastric cancer, a Phase III study is currently under way for the development of a second-line therapy with paclitaxel.[59] A Phase II study for gastric cancer was previously carried out using gefitinib, a tyrosine kinase inhibitor (TKI) of the endothelial growth factor receptor (EGFR), but the expected therapeutic outcome was not achieved, with a 0 percent response rate and an 18 percent disease stabilization rate.[60] In contrast, a 9 percent response rate was achieved with erlotinib, a similar TKI agent, but only in patients who had esophagogastric junctional cancer.[61] These results suggest that an EGFR-targeted agent alone is not effective against gastric cancer and should be combined with the existing cytotoxic agent. Good results were obtained in a Phase II study with cetuximab + folinic acid, fluorouracil (5FU) and irinotecan (FOLFIRI), and 5FU (FOLFIRI) (5FU), leucovorin, and oxaliplatin (FUFOX [D1]).[62,63] A randomized Phase III study with cisplatin plus capecitabine (XP) with or without cetuximab is currently under way. An interim analysis result of a Phase I gastric cancer study of the poorly differentiated adenocarcinoma type using GSK1363089, a dual c-MET/VEGFR-2 inhibitor, as a third-line or subsequent therapy has been published. However, the results were not satisfactory, with a 0 percent response rate and a 28 percent disease stabilization rate. A central pathology review determined that although c-MET gene amplification was observed in 54 percent of cases, high-level amplification was found in moderately differentiated adenocarcinoma, thus proving that c-MET gene amplification is not observed solely in the poorly differentiated type. Another Phase I study is under way in Asia for MK2461, a TKI of activated c-MET. VEGF-R1 expression was found in the tumor cells of 65 of 86 tumors (76%) and in the stromal vessels of 36 tumors (42%). VEGF-R2 expression was found in tumor cells and stromal vessels of 0 and 46 tumors (0 and 53%), respectively, and VEGF-R3 expression was found in tumor cells and stromal vessels of 0 and 75 tumors (0 and 87%), respectively. Univariate analysis revealed that VEGF-R expression correlated with shorter survival (VEGF-R1 in stromal vessels, $P = 0.001$; VEGF-R2 in stromal vessels, $P = 0.009$; VEGF-R3 in stromal vessels, $P = 0.005$). Multivariate analysis of potential prognostic factors

showed that VEGF-R1 and VEGF-R2 in stromal vessels were independent predictors of poor outcome.[66] However, bevacizumab, which is a humanized anti-VEGF monoclonal antibody, did not show survival benefit in a Phase III trial between XP versus XP plus bevacizumab for patients with advanced gastric cancer.[67]

CONCLUSION

The prevention and early diagnosis of gastric cancer metastasis have unfortunately not been completely successful to date. Comprehensive investigation and identification of the molecules related to the mechanism of gastric cancer metastasis and state-of-the-art bioinformatic technology will undoubtedly contribute to the further elucidation of the molecular mechanism underlying gastric cancer metastasis. Specific inhibitors against these molecules will be developed, and it is hoped that genetic information-based new diagnostic and therapeutic systems will be established for realizing an effective treatment against gastric cancer.

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31 Metastatic Pancreatic Cancer

José Eduardo M. Cunha, Marcos V. Perini, and Daniela Freitas

Pancreatic cancer is one of the most aggressive of human malignancies. In the United States, more than 37,000 people develop pancreatic adenocarcinoma each year, and almost all are expected to die from this disease [1], which is the fifth leading cause of cancer death in the country [2]. In Europe, about 40,000 deaths from pancreatic cancer are observed each year [3]. Median survival is only eight to twelve months for patients with locally advanced disease and three to six months for patients with metastatic disease, regardless of the therapy chosen. The overall survival rate is less than 5% [2]. Surgical resection is the only potentially curative treatment for pancreatic cancer. Unfortunately, the disease typically presents late, and therefore by the time a diagnosis is made, only a limited number of patients are candidates for pancreatectomy [4]. In large series of patients, only 5% to 22% were found to have resectable tumors at diagnosis, owing to the presence of advanced local tumor growth, peritoneal spread, or hepatic metastases [5, 6].

IMPORTANCE OF METASTASES

The treatment of metastatic disease remains the primary challenge in the treatment of pancreatic malignancy. Although distant metastasis can occur, locoregional metastasis is the most common recurrence or spread. Aggressive behavior, neurotrophic growth, and early spread are the main characteristics of this tumor.

Distant spread to the liver, peritoneum, lung, and bones is associated with poor prognosis [7] and with a short median survival of three to six months, depending on the extent of the disease and the performance status [8].

Several recent studies have shown that positive lymph nodes present at the resected specimen of a pancreatectomy for pancreatic cancer are adverse prognostic factors with respect to survival [9, 10], although a positive association between lymph node metastases and survival could not be definitively proved in a previous report [11]. This is certainly a consequence of study design and a small number of retrieved lymph nodes rather than a true finding [10, 12].

PATHOGENESIS OF METASTASIS

The mere dissemination of cancer cells into the vasculature or to a secondary site does not constitute metastasis. Development of clinically significant metastases requires that a cancer cell arriving in a secondary site completes a series of well-defined steps, generally referred to as the *metastatic cascade* [13]. This involves release of cancer cells from the primary focus, vascular invasion, adhesion to vascular endothelial cells, extravasation and invasion, and manipulation of the local environment in a manner that permits survival, proliferation, and development of a secondary tumor growth [14]. If a cell fails to complete any one of these steps, overt metastases will not develop [13–15].

Many aspects of metastatic progression remain unknown. During the growth of the primary pancreatic tumor, newly formed cancer cells migrate out, invade adjacent tissues, and extravasate to the local vasculature, from which they circulate to distant organs and may form new colonies [16]. Studies based on nonmorphologic variables have shown that metastatic cells and cells of the primary tumor are less similar than was previously thought [17].

Although it is known that the movement of neoplastic cells is not an arbitrary process, the mechanisms determining their route of transit, survival in foreign tissue environments, and choice of residence at a final destination have remained uncertain [18]. It has been suggested that organs distant from the original malignancy actively attract cancer cells via expression of adhesion receptors or by secretion of soluble chemotactic factors [19]. Identification of adhesion receptors on endothelial cells in blood vessels of

distant organs that specifically trap circulating cancer cells supports this theory [20]. However, this has not been demonstrated for pancreatic cancer metastasis. It has been reported that inflammatory cytokines enhance the expression of adhesion molecules such as intracellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 [21]. It is also likely that the different microenvironments within distant organs offer variable development conditions for specific circulating cell types [22, 23]. It then appears that the malignant cell migration to an ectopic tissue might be ruled by various mechanisms independently from those regulating its growth and/or survival after its destination has been achieved [18].

Matrix Metalloproteinases in Pancreatic Cancer Dissemination

The detachment of cancer cells from the primary tumor is followed by their migration and invasion of the surrounding tissue. This process depends on the loss of junctional contact between tumor cells and the adjacent epithelial cells and cell–matrix associations [24]. The matrix metalloproteinases (MMPs) compose a family of secreted or transmembrane protein enzymes involved in degradation of extracellular matrix components such as collagen, gelatin, and fibronectin. MMPs from malignant cells are thought to mediate cancer invasion and metastasis [25]. Recent studies of the activity of MMPs and their tissue inhibitors in invasive neoplasms have indicated that these enzymes play a crucial role in the degradation of connective tissue, which is associated with the progress of tumor metastases [26].

The importance of MMPs in the development of tumor metastasis may be related to their proteolytic activity against type V collagen, which is a major constituent of epithelial basement membranes. In an experimental model of human pancreatic carcinoma xenografts in nude mice, expression of the active forms of MMP-2 and MMP-9 was higher in pancreatic carcinomas from patients with liver metastases than those from patients without liver metastases [27]. This finding suggests that tumors with potential for bloodborne metastasis express MMP-2 and MMP-9. Furthermore, inhibition of MMPs reduces the growth of experimental pancreatic cancer metastases as a result of impaired cancer cell attachment, migration, and organ invasion [25].

Angiogenesis and Pancreatic Cancer Metastasis

The formation of new blood vessels (angiogenesis) is an essential step not only in the growth of primary tumors but also in the formation of metastases [28]. It is well known that angiogenesis, which can be induced by the release of multiple factors produced by cancer and stromal cells, facilitates both the local and systemic expansions of the tumor mass [29]. Furthermore, the growth of the invading cancer cells in the target organs after they have detached from the primary tumor and migrated through the circulatory system requires the development of a new vascular supply via angiogenesis and vasculogenesis [30]. This involves more than simply endothelial cell proliferation; endothelial cells must divide, invade the basement membrane, migrate, and finally go through differentiation and capillary tube formation. Furthermore, although the contribution of bone-marrow–derived cells (BMDCs) to tumor neovascularization is controversial, recruitment of circulating bone-marrow–derived endothelial progenitor cells is certainly implicated in the angiogenic process. BMDCs also act on the formation of premetastatic recesses, to which metastatic cells adhere via integrins [31] The breakdown of the basement membrane requires the action of many factors that are overexpressed in pancreatic cancer, including vascular endothelial growth factor (VEGF) and interleukin (IL)-8 [32]. Additionally, overexpression of several proangiogenic cytokines in pancreatic cancer tissues correlates with tumor aggressiveness and a poor prognosis [33].

In conclusion, for cells from the primary tumor to disseminate and grow into overt metastases in secondary organs, they must survive and proliferate in the surrounding tissue after extravasation. They must be able to initiate appropriate, context-dependent signaling cascades, which allow them to survive, enter the cell cycle, and divide. Although disseminated cancer cells may be detectable in numerous organs, only certain environments appear to allow their survival and subsequent development [34].

Inflammation

Chronic pancreatitis (CP) significantly increases the risk of developing pancreatic cancer, which suggests that chronic inflammation within the pancreas may be a predisposing factor to the development of cancer, although relatively few studies have directly assessed this. Nuclear factor-κB (NF-κB) and IL-8 are key mediators of the inflammatory process in CP; both have been implicated in the development of other malignancies. The exact mechanisms and inflammatory mediators that link CP and pancreatic cancer remain undefined.

The dense fibrotic stroma that forms around the remaining acinar cells in CP contains inflammatory cells, proliferating fibroblasts, and cytokines. Similarly, pancreatic cancer induces a strong desmoplastic reaction that may provide a source of inflammatory mediators and growth factors to support tumor growth and metastases. This stroma is composed of the same cell

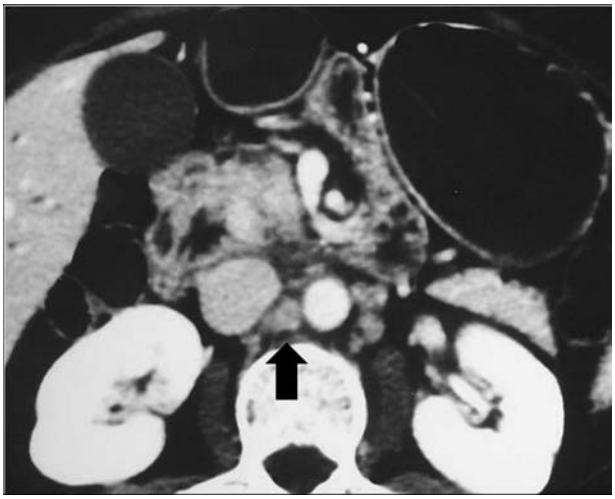

Figure 31.1. Lymph node metastasis (black arrow) in pancreatic cancer.

types in both CP and pancreatic cancer; thus, it may provide a source of cytokine expression and growth factors, which facilitates the development of pancreatic cancer from CP.

Cyclooxygenase-2 (COX-2) plays an important role in both the development and the progression of gastrointestinal malignancies. Its role in carcinogenesis derives from the observed regression of neoplastic lesions in patients receiving nonsteroidal antiinflammatory agents that act via the COX pathway. On the other hand, COX-2 expression has been associated with worse survival after curative resection for pancreatic cancer [35].

DIAGNOSIS OF PANCREATIC METASTASIS

Clinical suspicion of metastatic disease in patients with pancreatic carcinoma must be made in the presence of extreme weight loss and ascites. Very high levels of the serum tumor marker CA19–9 are also usually associated with tumor unresectability owing to metastasis. However, confirmation of pancreatic metastasis is, in most cases, made by diagnostic imaging. Transabdominal ultrasound (US) is usually the initial screening investigation in patients presenting with jaundice. The presence of lymph node or hepatic metastasis can be determined by this method. Although transabdominal US is the first imaging modality used, the current "gold standard" imaging for diagnosis and staging of pancreatic cancer is contrast-enhanced, dual-phase multidetector computed tomography (CT) (Figure 31.1). However, although this provides a better tumor definition than does US, small hepatic or peritoneal metastases may still be missed by CT. Therefore some centers advocate the use of exploratory laparoscopy for preoperative staging of pancreatic cancer [36]. Although its main limitation is in determining critical vascular invasion and/or lymph node involvement, laparoscopy remains an excellent tool for visualizing liver metastases with a sensitivity of 77% in determining unresectability [37]. Some studies have suggested that measurement of serum CA19–9 levels may be useful to aid in the selection of patients with radiographically resectable disease for staging laparoscopy [38–40].

TREATMENT OF SPECIFIC SITES OF PANCREATIC METASTASIS

Lymph Node Metastasis

Lymph nodes (LNs) are the most common metastatic site in pancreatic cancer (Figure 31.1). The reported incidence of LN metastasis in resected specimens varies from 56% to 86% [41]. The most common lymph nodes involved are the peripancreatic nodes. The incidence of paraaortic lymph node metastasis for pancreatic head carcinoma varies from 16% to 26% [42], whereas nodal involvement in pancreatic body and tail carcinoma is somewhat lower, reported at 13% to 17% [41]. These findings led many surgical centers in Japan to propose the performance of extended lymphadenectomy for the treatment of pancreatic cancer.

The lymphatic flow from the pancreatic head tumor to the paraaortic lymph node via the posterior surface of the pancreatic head and around the superior mesenteric artery has been suggested in many reports [41, 43]. Furthermore, the pattern of lymphatic spread of metastatic disease in pancreatic cancer may vary because of the multidirectional lymph drainage of the pancreas to superior, inferior, anterior, posterior, and left lymph nodes [44].

According to some studies, the ventral and the dorsal parts of the pancreas have differing lymphatic drainage as a consequence of their different embryology. Thus, the lymphatic spread for tumors located at the ventral pancreas occurs to the peripancreatic lymph nodes and to the LNs along the superior mesenteric artery, whereas for those tumors located at the dorsal pancreas, LN metastasis occurs in the nodes along the common hepatic artery besides the peripancreatic LNs [43, 45]. However, this distinction has no clinical impact on survival, as randomized trials on extended lymphadenectomy have not shown a difference in survival rates. For this reason, extended lymphadenectomy for the treatment of pancreatic adenocarcinoma should not be recommended [46–48].

Liver Metastases

The liver is the most common site of distant metastasis in pancreatic cancer, the organ being affected

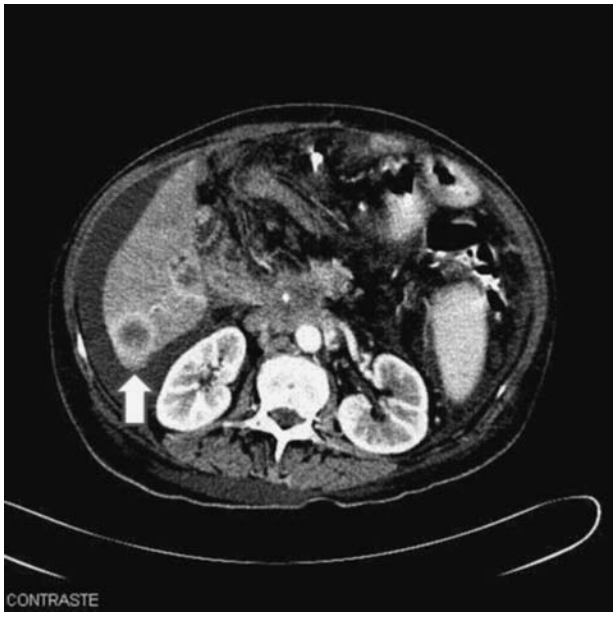

Figure 31.2. CT shows hepatic metastasis (arrow) and ascites after resection for pancreatic adenocarcinoma.

in approximately 60% of the cases [49]. Metastatic liver disease may be diagnosed either at the initial consultation or during the follow-up period after resection for pancreatic adenocarcinoma (Figure 31.2)

Although there are isolated reports of prolonged survival after surgical resection of solitary metachronous liver metastasis in highly selected patients without local recurrence after surgical treatment of pancreatic carcinoma [50], patients presenting with metastatic liver tumors from pancreatic cancer are not usually considered to be candidates for surgical treatment [51]. Previous studies showed that morbidity ranged from 19% to 35% and mortality from 0% to 20% for hepatectomy in this subset of patients [52]. Regardless of treatment, patients with liver metastasis from pancreatic cancer usually die within one year. The six-month median survival of patients undergoing liver resection is not statistically different from the four-month survival after palliative bypass [53].

On the other hand, pancreatic resection in patients with metastatic liver disease has very strict palliative indications, such as recurrent bleeding from duodenal ulceration previously managed by conservative treatment, and should not be routinely recommended.

Peritoneal Metastases

Peritoneal spreading with carcinogenic ascites is a hallmark of tumoral spreading beyond the abdominal compartment (Figure 31.3). This can be demonstrated by the detection of micrometastasis in the bone marrow and/or bloodstream.

Cytology is used for the detection of disseminated tumor cells in peritoneal cavity washings. The detection rate varies from 13.4% to 35.5 %, depending on the concentration of the tumor cells in the peritoneal cavity washings and on the tumor stage [54, 55]. The implications of peritoneal washing cytology in patients with pancreatic cancer are still a matter of debate. According to some studies, positive cytology indicates disseminated disease and precludes tumor resection with curative intent [56], whereas other authors affirm that cytologic status has little predictive value for survival, and patients whose pancreatic cancer is considered otherwise resectable should not be denied curative resection [54, 55]

TREATMENT ENDPOINTS

Evaluating response remains a great challenge for investigators and clinicians and can be highly inaccurate. Traditional two-dimensional or unidimensional tumor measurements to determine objective response to therapy are often inadequate when evaluating the primary site in the pancreas, owing to the characteristic desmoplastic reaction and local inflammatory response. More recent trials have included clinical parameters such as pain, weight loss, performance status, and quality-of-life endpoints as well as survival data. Because so many patients with pancreatic cancer present with pain and depression, exploring these outcomes allows more effective estimation of the treatment benefit.

The surgical treatment of metastatic disease in pancreatic cancer cannot be generally accepted. Pancreaticoduodenectomy for pancreatic carcinoma with simultaneous resection of synchronous liver metastases does

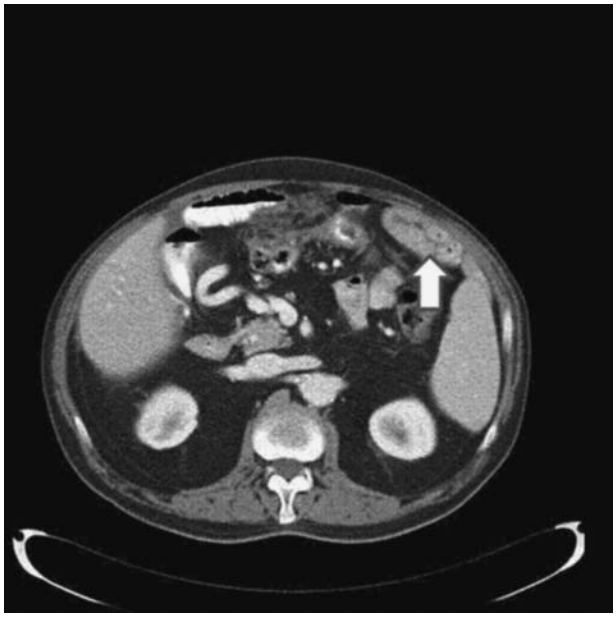

Figure 31.3. Abdominal CT shows peritoneal metastasis in a patient with pancreatic adenocarcinoma.

not result in long-term survival in the overwhelming majority of patients [57]. Therefore, medical treatment still is the most appropriate method for management of these patients.

CHEMOTHERAPY FOR METASTATIC PANCREATIC CANCER

The main goal of systemic therapy for metastatic pancreatic cancer is to minimize disease-related symptoms and to prolong survival. The better survival outcomes achieved with 5-fluorouracil (5-FU)–based combinations compared with control groups (patients to whom only optimal supportive care was offered) demonstrate the benefit of this treatment modality for patients with advanced pancreatic cancer. The median survival times achieved by 5-FU–based regimens were consistently in the range of four to six months, whereas for those receiving only the best support care, the estimated survival is around three months. However, in a meta-analysis, 5-FU combinations did not demonstrate a survival benefit when compared with 5-FU alone. The 5-FU is an inexpensive drug and usually is well tolerated, with main side effects of diarrhea, oral mucositis, and hand–foot syndrome (edema, pain, hyperemia, and desquamation of the skin of the hands and feet). However, at the present time 5-FU is no longer considered the first choice in the treatment of metastatic pancreas cancer; rather, gemcitabine is regarded as the standard of care worldwide after a randomized study showing its superior clinical benefit and a gain in overall survival over 5-FU treatment.

Gemcitabine is a nucleoside analog with structural similarity to cytarabine, with a broad spectrum of preclinical activity in solid tumor models, and responses in patients with pancreas cancer during Phase I evaluation. A Phase III trial comparing gemcitabine with 5-FU demonstrated that clinical benefit, defined as an improvement in pain, performance status, and weight gain, was experienced by 23.8% of gemcitabine-treated patients compared with 4.8% of 5-FU–treated patients ($p = 0.0022$). The median survival durations were 5.65 and 4.41 months for gemcitabine-treated and 5-FU–treated patients, respectively ($p = 0.0025$). The survival rate at twelve months was 18% for gemcitabine patients and 2% for 5-FU patients [58].

Gemcitabine has been studied in combination with many agents. Despite the suggestion of promising results from preliminary Phase II trials [59], the combination results with 5-FU were clearly negative in at least two randomized trials [60, 61]. In a multinational trial comparing gemcitabine with or without capecitabine, there were no significant differences with regard to median survival [62, 63]. The combination of gemcitabine and a platinum agent was evaluated in large Phase III trials. When single-agent gemcitabine was compared with the same agent combined with cisplatin, no survival improvement was achieved [64]. On the other hand, the combination of oxaliplatin and gemcitabine in a regimen known as GEMOX, compared with gemcitabine alone, presented different results in two multicenter trials [65]. The United States Intergroup trial found no survival difference between the groups, whereas, in a second Phase III study, the combination was associated with significantly higher response rates and a nonsignificant trend toward improved median survival (9 vs. 7.1 months, $p = 0.13$) [66].

Besides myelosuppression, which represents the principal toxic effect of gemcitabine, other toxic effects include acute lung, liver, and kidney injury. Gemcitabine administration to patients with baseline hyperbilirubinemia may increase the risk for liver function deterioration.

Target Therapy Combinations

Erlotinib

Human epidermal growth factor receptor (EGFR) type 1 is overexpressed in many pancreatic tumors and is associated with poor prognosis and disease progression. In experimental models, blocking EGFR tyrosine kinase improves the anticancer effects of gemcitabine. Ongoing studies are incorporating small-molecule tyrosine kinase inhibitors of the EGF receptor (e.g., erlotinib) as well as monoclonal antibodies directed against this molecule (e.g., cetuximab).

A Phase III trial from the National Cancer Institute of Canada compared gemcitabine with and without erlotinib in patients with locally advanced or metastatic pancreatic cancer. The addition of erlotinib resulted in a statistically significant benefit in overall survival (HR: 0.82; 95% CI, 0.69–0.99; $p = 0.038$). Improvement of median survival was from 5.9 to 6.4 months, and the one-year survival rate improved from 17% to 23% ($p = 0.023$) on the erlotinib-plus-gemcitabine arm. However, the modest, two-week improvement in median survival; erlotinib toxicity, which includes rash, diarrhea, infection, and stomatitis; and the great financial impact associated with its use may discourage the use of this combination as a palliative treatment for metastatic pancreatic cancer.

Bevacizumab and Cetuximab

VEGF and EGF play an important role in the growth and metastasis development of many tumors, including pancreatic cancer. Bevacizumab and cetuximab are anti-VEGF and anti-EGF monoclonal antibodies that have shown clinical benefit in metastatic colon, lung, and breast cancers. Concomitant administration of the monoclonal antibodies and tyrosine kinase inhibitors together and in combination with chemotherapeutic

agents may both augment their therapeutic activity and offset mechanisms of resistance. However, randomized Phase II and III studies on the efficiency of these targeted agents in pancreatic metastatic cancer have failed to demonstrate a benefit for the addition of cetuximab or bevacizumab to gemcitabine [67, 68]

Although some incremental progress in the treatment of pancreatic cancer has been made, the prognosis of patients with this disease remains extremely poor. The fact that gemcitabine in combination with other drugs (target or not) has demonstrated, in two large randomized Phase III studies, the superiority of a gemcitabine-containing combination over single-agent gemcitabine, capecitabine plus gemcitabine (GemCap) and erlotinib plus gemcitabine seem to direct the new tendency in treatment of advanced disease. Nevertheless, gemcitabine plus/minus erlotinib or capecitabine are considered the standard of care for advanced pancreatic cancer patients with good performance status in North America.

Several therapeutic strategies are being explored for the treatment of pancreatic cancer; certainly, the identification of new pathogenic targets is expected to have a clinical impact. One such target, S100P, can supposedly be overexpressed in pancreatic cancer. Overexpression of S100P may enhance tumor growth and metastasis and decrease patient survival. Pancreatic cancer cells with high endogenous levels of S100P may show resistance to gemcitabine.

Furthermore, an antiallergic drug, cromolyn, may inhibit tumor growth and increase the effectiveness of gemcitabine through binding to S100P. However, further studies are needed before the anticancer activity of cromolyn can be established.

In addition, despite the negative results with the use of bevacizumab, cetuximab, and everolimus, adding a second targeted agent to chemotherapy is definitely where the future is heading. We are gradually moving toward realizing that individualized cancer treatment with molecular data can improve the outcomes of currently available treatments by better patient selection and novel drug administration schemas, as well as intelligent combinations. However, despite promising results of studies exploring the role of targeted therapy in the adjuvant setting of pancreatic cancer, numerous questions remain unanswered, including the rational selection of the patients who are more likely to obtain benefit from targeted therapy.

PALLIATION OF METASTATIC PANCREATIC CANCER

Pain

The initial approach to pancreatic-cancer–associated pain is based on the use of long-acting narcotics such as extended-release oral morphine, oxycodone preparations, or transdermal fentanyl. Intermittent, postprandial epigastric discomfort, suggesting pancreatic enzyme insufficiency, may be successfully palliated with the initiation of pancreatic enzyme replacement therapy (PERT) alone.

Existing studies suggest that neurolytic celiac plexus block (NCPB) is a well-tolerated intervention that may improve analgesia, decrease opioid requirements, and minimize deterioration of quality of life [69–71]. Patients with tumor-associated pain that fails to respond adequately to systemic narcotic analgesics are candidates for NCPB, which is likely to have prompt and long-lasting analgesic efficacy for pancreatic cancer. NCPB has durable (at least three months) partial or complete pain relief in approximately 90% of patients with pancreatic and other intraabdominal cancers [69, 72]. Besides its rescue effect in controlling pain in nonresponder patients on systemic narcotic analgesics, some studies have shown a beneficial effect of NCPB when used at the initiation of opioid therapy [70].

Exocrine Enzyme Deficiency

Pancreatic cancer patients may develop symptoms of exocrine insufficiency owing to pancreatic duct obstruction, which may include abdominal discomfort and/or distension, pain, excessive flatus, belching, diarrhea, steatorrhea, and weight loss. Management of pancreatic exocrine insufficiency can usually be achieved through the administration of 25,000 to 40,000 IU of lipase for a standard meal. The dosage may be increased if steatorrhea or other evidence of malabsorption persists. The efficacy of enteric-coated pancreatic enzyme extracts can be optimized through the inhibition of gastric acid secretion by the additional use of a proton pump inhibitor.

Biliary Obstruction

The management of jaundice secondary to biliary obstruction in patients with metastatic pancreatic cancer who present a short life expectancy is preferably achieved by endoscopic placement of plastic or metal biliary stents [73]. Expandable metal stents are preferably used because they remain patent for a longer period of time compared with plastic stents [74]. In addition to the relief of jaundice and pruritus, biliary decompression has been shown to improve quality of life by increasing appetite and reducing indigestion [75].

Gastric Outlet Obstruction

Duodenal obstruction leading to gastric outlet obstruction occurs in 15% to 20% of patients with advanced

pancreatic cancer. Endoscopically placed metal stents are an acceptable option in patients with metastatic disease when a short survival is expected. They offer palliative benefit with less morbidity [76]. One study evaluating 29 patients and showed satisfactory results for 81% of cases and reobstruction by tumor in less than 7% of patients after an average of 183 days [77].

METASTASIS TO THE PANCREAS

The pancreas is a rare site of metastasis from other malignancies, accounting, in larger series, for fewer than 5% of pancreatic tumors removed [78–80]. In autopsy series, metastasis to the pancreas varies from 3% to 12%. Besides other tumors, such as breast, prostate, colon, or lung cancer, renal cell carcinoma is the most common primary neoplasm known to metastasize to the pancreas, accounting for about 30% of metastatic pancreatic tumors [81], representing 0.25% to 3% of all resected specimens [82, 83]. In autopsy series in patients with renal cell carcinoma, metastases in the pancreas were noted in 1.3% to 1.9% [84, 85].

Metastases to the pancreas occur in two different clinicopathological settings, either as one manifestation in widespread metastatic disease or as an isolated mass of the pancreas. The mode of spread of renal cell carcinoma to the pancreas remains unclear. A possible metastatic route to the pancreas is lymphogenous spread through some lymphatic routes running from the head of the pancreas to the dorsal side of the renal artery. Despite this lymphatic spread of metastatic pancreatic cancer, an interesting observation is that lymph node involvement is less frequently reported in pancreatic metastasis than that usually found in ductal adenocarcinoma. [82, 83]

The distinction between a pancreatic metastasis of a renal cell carcinoma and a primary pancreatic adenocarcinoma is important for prognostic and therapeutic reasons, considering that metastatic renal cell carcinoma has a better prognosis than a primary pancreatic neoplasm. However, clinical symptoms of secondary tumors to the pancreas are similar to those of primary tumors, and include abdominal or back pain, body weight loss, digestive tract bleeding and/or obstruction, and jaundice. Secondary tumors can be asymptomatic and detected only on routine radiological studies. In a series of 18 patients with pancreatic metastasis 15 were asymptomatic and the pancreatic mass was detected on routine follow-up examination. Acute pancreatitis is rare, but hyperamylasemia can be seen. The interval between primary diagnosis of renal cancer and subsequent nephrectomy until occurrence of pancreatic metastases range up to more than 25 years but may be as low as one or three years in some patients.

The diagnosis of metastasis to the pancreas is based on a history of relevant cancer. The interval between the diagnosis of the primary tumor and the metastasis can be as long as 20 years but may also be as short as one to three years. In any case, a careful long-term follow-up in patients with a history of renal cell carcinoma is strongly recommended.

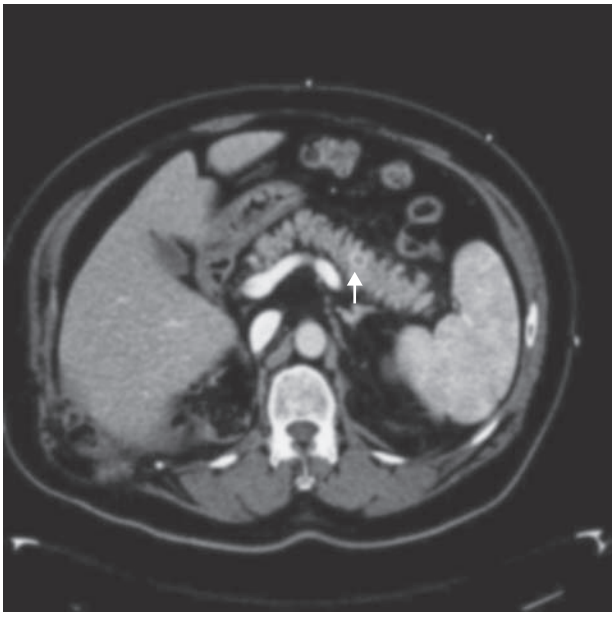

Figure 31.4. Single pancreatic metastasis (arrow) shown on a CT of a patient previously operated on for a renal cell carcinoma.

US is a useful imaging technique for diagnosing both primary and metastatic pancreatic tumors. In cases of pancreatic metastasis, US characteristically shows a rounded, well-delineated mass that is hypoechoic compared with the adjacent pancreas. CT scan is the imaging method of choice, and metastasis to the pancreas can be seen as one or more lesions (Figures 31.4 and 31.5). Radiological characteristics such as vascular hyperdensity or peripheral rim enhancement can be seen in lesions larger than 1.5 cm. Multifocal lesions

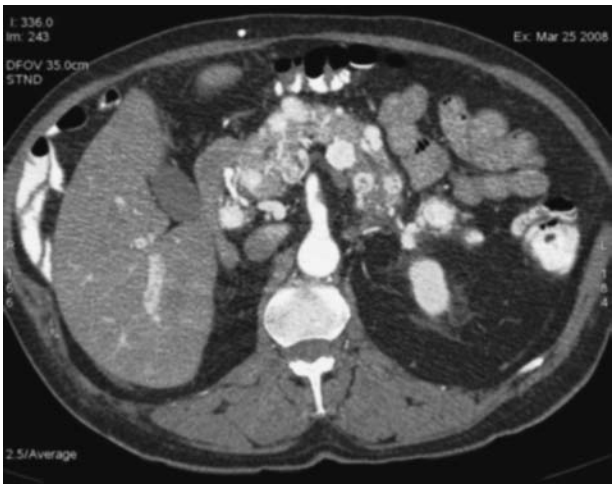

Figure 31.5. Multiple pancreatic metastases shown on a CT of a patient previously operated on for a renal cell carcinoma.

are more common; when an isolated lesion is seen, it could be misdiagnosed as a primary tumor. There are no specific image features, such as acinar cell tumors, focal pancreatitis and, mainly, neuroendocrine tumors, that differentiate metastatic from primary pancreatic lesions. However, in patients with a history of renal cell carcinoma and a CT examination that reveals a solid pancreatic tumor with vascular enhancement, this should be considered as a pancreatic metastasis. Furthermore, the multifocal pattern of involvement makes secondary pancreatic tumors more likely [79]. Wirsung (pancreatic duct) and common bile duct dilation can occur in lesions located in the pancreatic head. In body or tail lesions, focal Wirsung dilation and pancreatic atrophy can be seen. Fine-needle aspiration and pancreatic biopsy guided either by CT or by endoscopic ultrasonography (EUS) constitute the most reliable method of diagnosis, but FNA-guided biopsy may be associated with a high risk of hemorrhage owing to lesion vascularity.

Surgical resection for solitary pancreatic metastases from renal cell carcinoma is the most effective treatment option, leading to five-year survival rates of up to 75%, mainly when there is a long time interval between the primary tumor removal and the appearance of the metastasis in the pancreas [86]. Furthermore, surgical resections of pancreatic metastases from various malignancies may provide a five-year survival rate of 25% and a mean survival of almost two years [87]. Pancreatic resection for patients with metastasis to the pancreas should be tailored to the patient's individual needs, and achieve adequate resection margins and maximal tissue preservation of the pancreas. Therefore, depending on the localization of the tumor burden, the surgeon should consider a pancreatoduodenectomy, a segmental pancreatic resection or a distal pancreatectomy for focal lesions, or a total pancreatectomy for multifocal lesions.

Although more than 80% of the patients undergoing pancreatectomy survive longer than twelve months, an accurate estimate of the average survival is difficult to assess, as the majority of cases are reported within two years of pancreatic resection [88].

The resectability rates of pancreatectomy for pancreatic metastases are higher than those attempted for pancreatic ductal adenocarcinoma. This finding is possibly the result of a better definition of the metastatic lesion boundaries as compared with pancreatic adenocarcinoma and the less frequent vascular involvement by the metastatic lesions.

Pancreatic resections can nowadays be performed with low morbidity and mortality rates, particularly in high-volume centers [89]. Results of surgical resection of isolated metastases to the pancreas not only from renal cell carcinoma but also from many other primary tumors indicate improvement in long-term survival, with a clearly better prognosis than for primary pancreatic ductal adenocarcinoma [87]. The low complication rate and an overall good survival rate justify an aggressive approach for the treatment of metastatic disease to the pancreas, especially when the metastatic tumor is small and amenable to complete resection.

CONCLUSION

Long-term survival after pancreatoduodenectomy for pancreatic carcinoma is far from excellent. Although a five-year survival rate ranging from 10% to 20% could be achieved, in the past decade the number of patients surviving more than five years has been far from our expectations. In fact, the lack of noteworthy clinical progress in the treatment of metastatic pancreatic cancer makes it one of the most frustrating malignancies to investigate and treat. Despite the fact that the multidisciplinary approach is now counting on new fields such as targeted therapy, and target drugs are expected to play an important role in the treatment of metastatic disease, it is unlikely that the wide-ranging testing of new agents proposed to be efficient against most patients' pancreatic cancer will result in considerable benefits. Therefore, new schemes to match an individual's malignancy with the most effective existing drug must be strongly pursued in the future. Considering the extreme limited value of the surgical management of this entity and the poor palliative benefits associated with the various chemotherapeutic regimens, supportive care strategies in helping patients handle this disease must be strongly emphasized.

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32 Metastasis of Primary Liver Cancer

Zhao-You Tang and Lun-Xiu Qin

Primary liver cancer, largely hepatocellular carcinoma (HCC), is the third most common cause of cancer death in the world; the overall five-year survival is only 3 percent to 5 percent [1]. Fifty-five percent of deaths occur in China [1]. Metastasis and primary tumor recurrence are the major causes of death. After curative resection (en bloc removal of tumor mass with margins free of tumor at resection), the five-year recurrence rate has been reported to be 61.5 percent, although it is lower (43.5%) after resection of small HCC tumors, which is mainly the result of intrahepatic metastasis via vascular invasion [2]. Because of the hypervascularity of the tumors, vascular invasion occurs in the majority of cases, and HCC metastases to lung, bone, adrenal gland, and other sites via the bloodstream are commonly encountered. Lymph node metastases, particularly in the hepatic hilar area, also occur with high incidence.

Studies of HCC metastasis during the past decades have included early detection and re-resection for subclinical recurrence after curative resection [3], establishment of a metastatic human HCC model system for screening novel therapeutic approaches [4–6], finding of a molecular signature with 153 genes and an immune response signature in the liver microenvironment that can also predict HCC metastasis [7–8], discovery of the association between chromosome 8p deletion and HCC metastasis [9], translation of several biomarkers for clinical prediction of HCC metastasis/recurrence [10–13], identifying novel markers for prediction and therapeutic target [14, 15], demonstrating the inhibitory effect of interferon-alpha on the metastatic recurrence of HBV-related HCC [16, 17], exploring other interventional agents [18–20], and optimizing radiotherapy for HCC metastasis [21, 22].

CLINICOPATHOLOGICAL FEATURES OF METASTATIC PRIMARY HCC

Extrahepatic Metastasis

Extrahepatic metastases of HCC are not uncommon. However, there are few detailed clinical reports about the pattern of metastatic spread, and its incidence remains unclear. Extrahepatic metastases of HCC have been reported to occur in 13.5 percent to 36.7 percent of patients with HCC [23, 24]. The most common metastatic sites are lung (observed in 34%–58% of autopsy cases) [25], regional lymph node (10%–42%), and bone (4%–28%) [26]. Less commonly, metastases are observed in the adrenal gland (6%–27%) [25, 26], peritoneum [26], skin [27], brain [28], and muscle [24]. There have also been rare reports of oral [29], nasal [30], pituitary [31], thyroid [32], breast [33], esophageal [34], cardiac [35, 36], spleen [37], pancreatic [38], renal [39], and testicular metastases [40] from HCC.

HCC metastases are typically first detected in the lung. Conversely, the less common metastatic sites almost never represent as the initial manifestation of extrahepatic HCC. In most cases, lung metastases present as a nodular shadow (or shadows) seen by chest radiography, computed tomography (CT), or other imaging. Pleural effusion may also be observed.

Regional lymphadenopathy is usually seen in the periceliac and the portohepatic lymph nodes. However, patients with liver cirrhosis may have benign enlarged lymph nodes; therefore, this finding is not specific for metastatic HCC. Helical biphasic CT scanning can be helpful in differentiating malignant from benign lymphadenopathy. Whereas the size of the malignant lymph nodes is not a reliable criterion of malignancy,

the presence of arterial phase enhancement or interval size increase is suggestive, and the finding of malignant cells at biopsy has been accepted as diagnostic of malignant lymph node involvement [26]. Similarly, the presence of an enlarged adrenal mass does not always imply malignancy. Adrenal adenomas statistically are a more common cause. Arterial phase enhancement in an adrenal mass (in 25% of adrenal metastases) suggests metastatic disease.

Postoperative Tumor Recurrence

Intrahepatic recurrence after surgical resection is common, with five-year recurrence rates around 38 percent to 61.5 percent [2]. Recurrence may be a result of either intrahepatic metastasis (IM) or multicentric occurrence (MO), which is a newly developed lesion in the cirrhotic background. IM is a major cause of recurrence of advanced HCCs with varying degrees of vascular invasion. More than 60 percent of multiple HCCs at the time of first presentation resulted from IM [41]. MO is a cause of HCC recurrence in early-stage patients with no obvious vascular invasion, particularly in those with severe liver cirrhosis or HCV-related HCC. The prognosis of patients with HCC recurrence from MO is significantly better than that of patients with recurrence owing to IM [42].

Many parameters – including gross appearance, size, location and histological features of the tumors, time of recurrence, imaging patterns, and genetic markers – have been applied to discriminate these two origins of recurrences [41]. Genetic assessment of DNA alterations is the most accurate method for differentiating between IM and MO. Clonalities analysis of the integration pattern of HBV DNA in HBV-related HCCs, DNA fingerprinting with loss of heterozygosity (LOH) assay and comparative genomic hybridization (CGH), as well as analysis of the p53 mutation patterns, have been used for determining IM or MO in recurrent HCC. Of these, LOH analysis can be used in most patients even before surgical resection, as this assay can be readily applied routinely to either liver biopsies or fine-needle aspirates [41].

PREDICTION AND DIAGNOSIS OF HCC METASTASIS

Lessons from Experimental Studies of HCC Metastasis

Metastasis is a multistep process that involves multiple genes and interactions among cancer cells, their microenvironment, and the host. Exploring the molecular background could be helpful in the prediction and diagnosis of metastatic disease, and may also suggest therapeutic targets for the treatment of HCC metastasis. During the past decade, many molecular factors involved in the process of HCC invasion and metastasis, including adhesion molecules (E-cadherin, catenins, ICAM-1, laminin-5, CD44 variants, and osteopontin [OPN]), proteinases responsible for the degradation of extracellular matrix, angiogenesis regulators, as well as genomic aberrations and expression profiling, have been shown to be potential predictors for HCC metastatic recurrence and clinical outcomes (Table 32.1) [7–9, 12–14, 43–59].

Plasma Level of Osteopontin Can Predict HCC Recurrence and Prognosis of Patients with HCC

Based on the study of metastasis-related molecular signatures, we demonstrated an important role of OPN in HCC metastasis. OPN is overexpressed in metastatic HCC, and an OPN-neutralizing antibody or microRNA against OPN can efficiently block invasion and metastasis of highly metastatic HCC cells, both in vitro and in nude mice models bearing human metastatic HCC [7, 60]. These indicate that OPN can be a potential therapeutic target for metastatic HCC. A higher plasma OPN level was also found to be closely related to a poorer survival among HCC patients [44]. The two-year disease-free survival (DFS) rate of HCC patients with a higher plasma OPN level ($\geq$200 ng/mL) (16.3%) was significantly lower than that of patients with a lower OPN level (<200 ng/mL; 59.0%, $P = 0.0001$). The plasma level of OPN is an independent prognostic factor for both overall survival (OS) and DFS, and can be used as a predictor for HCC recurrence and survival [44] (Figure 32.1).

Chromosome 8p Deletion in HCC Tissue or Plasma DNA of HCC Patients Can Predict HCC Recurrence and Patient's Prognosis

Through complete genome analysis by CGH, chromosome 8p deletion was found to be the most important genetic aberration associated with HCC metastasis [9]. Furthermore, genomewide microsatellite analysis revealed that 8p deletion was confined to 8p23.3 and 8p11.2, two likely regions harboring metastasis-related genes. LOH on 8p could be detected in circulating DNA from patients with HCC (76.0%, 60/79), and more frequently (85.7%) in those with metastatic HCC ($P = 0.023$); LOH on 8p in plasma DNA was significantly correlated with TNM stage, vascular invasion, and a lower DFS and OS [12]. The three-year DFS of patients with 8p deletion detected in circulating DNA (14.3%, $n = 28$) was significantly lower than that of patients without 8p deletion (45.1%, $n = 51$, $P = 0.018$). Recently, LOH at D8S298 detected in HCC tissues was found to be associated with a worse five-year OS and DFS of patients after curative resection, even in those with early-stage

TABLE 32.1. Risk factors for metastasis and recurrence after surgical resection of hepatocellular carcinoma

Items	Risk factors or predictors
Invasion and metastasis-related markers	Osteopontin (OPN) (tissue and serum) [7, 43, 44] Intratumor microvessel density (MVD) level [45, 46] VEGF level (tissue and serum) [47, 48] *p53* gene mutation [49] Reduced expression of p27 [50], E-cadherin [51] Overexpression of laminin-5, MMP-2, MMP-9, MT1-MMP [52, 53]
Genomic aberrations and expression profiling	Genomic aberrations [9, 12, 13, 54, 55] *16q; 8p;* changed restriction landmark genomic scanning (RLGS) spots Gene expression profiling [7, 8, 56, 57] 90 genes associated with intrahepatic metastasis 153 genes predicting signature for metastases and outcome 12 genes predictive system 17 genes related to immune response Proteomics analysis *[14, 58, 59]* CK19, CK10
Coexisting liver disease	Inflammation activity: ALT, GGT, viral load, serum HbeAg [61] Genotype C HBV [62] Liver functional reserve [63]
Pathological features of tumor	pTNM stage [64, 65] Size, number, capsule, differentiation Venous invasion; intrahepatic metastasis (IM) Inflammatory cell infiltration (favorable factor) [66]
Tumor-associated antigens and detection of circulating cancer cells	Serum AFP level (protein, mRNA); AFP-L3 [67] Serum *MAGE, hTERT* mRNA [68, 69]

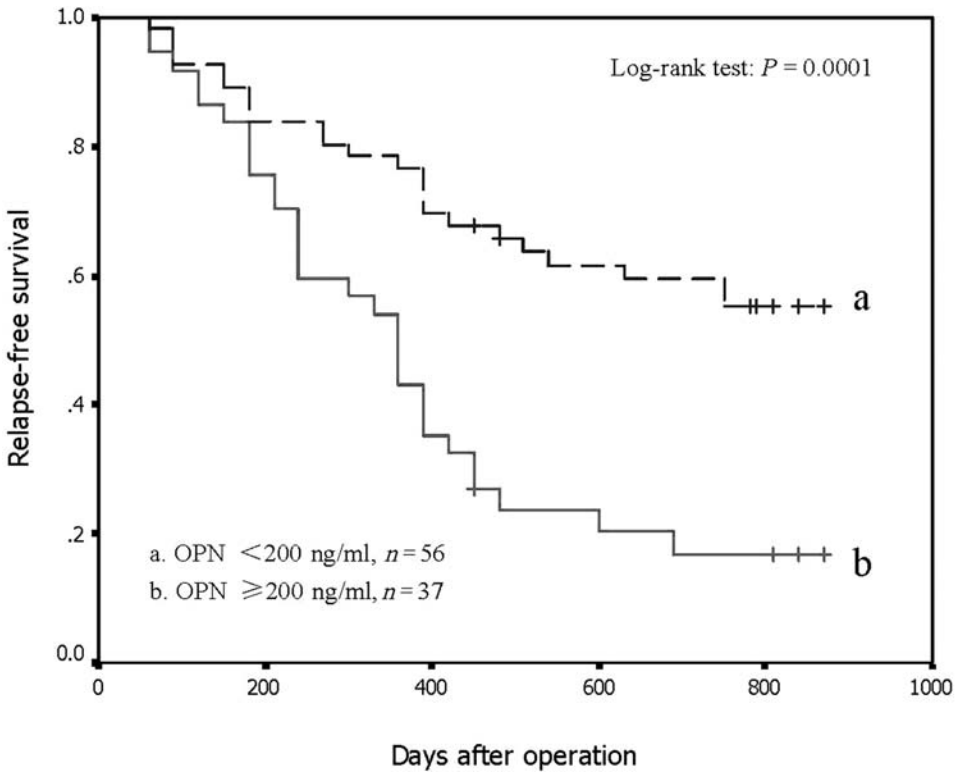

Figure 32.1. Association of plasma osteopontin (OPN) levels with the disease-free survival (DFS) of patients with HCC after resection. The two-year DFS of HCC patients with a higher plasma OPN level (≥200 ng/mL) (n = 37, 16.3%) was significantly lower than that of patients with a lower OPN level (<200 ng/mL) (n = 56, 59.0%, P = 0.0001).

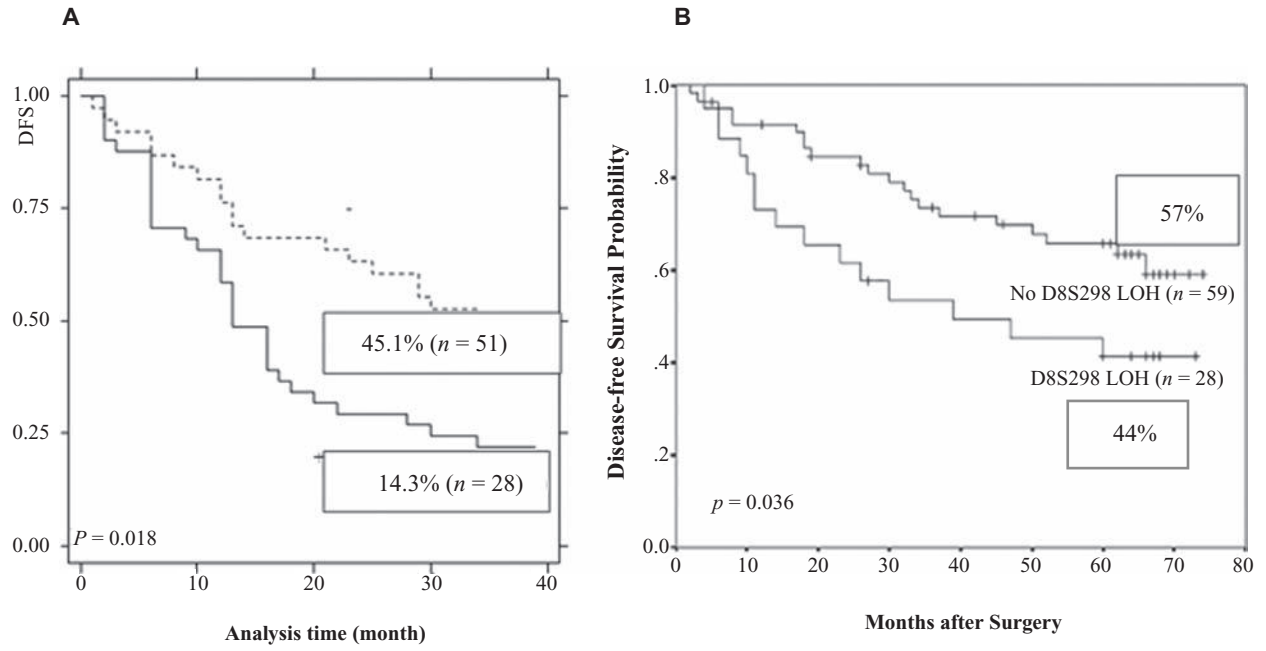

Figure 32.2. 8p deletion detected in HCC tissues and circulating DNA of HCC patients can be used to predict HCC recurrence and patient's prognosis. (A) The association of 8p deletion detected in circulating DNA of HCC patients with the DFS curves of patients. (B) The association of 8p deletion detected in HCC tissues with DFS of patients with TNM stage I of HCC.

HCC (44% vs 57%, $P = 0.036$); it was an independent predictor of decreased DFS [13]. Therefore, 8p deletion can serve as a novel prognostic factor for HCC patients. (Figure 32.2)

Molecular Signature Generated Using 153 Differently Expressed Genes Can Predict HCC Metastasis

Using cDNA microarrays containing 9180 genes, we analyzed the expression profiles of forty HCC samples with or without metastases in a genomewide scale, and found that the metastatic tumors had a similar gene expression signature to their parent HCCs, whereas metastasis-free HCCs were distinct from metastatic primary HCCs (153 genes with significance, $p < 0.001$). Therefore, we proposed that genetic aberrations favoring HCC metastastic progression are initiated early in primary tumors, and that the metastatic potential is even predetermined in primary HCCs. This is in contrast to traditional concepts of cancer metastasis and suggests that prediction and prevention of HCC metastasis may be possible earlier in the course of the disease [7]. Using the 153 genes that were significantly different between metastatic and nonmetastatic HCC, we generated a molecular signature that can be used to predict whether a tumor has the potential to metastasize [7]. Similar molecular predictive signatures have also been constructed to predict early intrahepatic metastasis and recurrence in other centers [57]. These studies provide a new way to predict HCC metastasis.

Inflammatory/Immune Responses in Surrounding Liver Parenchyma Can Predict HCC Metastasis

In addition to examination of the cancer cells, using cDNA microarrays, we also analyzed the gene expression profiles of the noncancerous hepatic tissues around HCCs from patients with and without intrahepatic metastasis. We found that there were 454 genes with significant differences ($p < 0.001$) between these two groups, most of which are associated with either inflammation and/or immune response. The hepatic tissues of metastatic HCC patients have a global decrease in proinflammatory Th1-like cytokines and a more pronounced increase in antiinflammatory Th2-like cytokines. This unique Th1- to Th2-like profile switch is accompanied by an overexpression of macrophage colony stimulating factor (CSF) 1 and a decreased expression of nitric oxide synthase 2. These findings suggest that a shift of inflammatory/immune responses in the microenvironment also plays an important role in metastasis. Using seventeen genes refined from those associated with immune response, we generated a molecular signature that could discriminate liver tissues around HCCs with metastasis from those around HCCs without metastasis. Moreover, this signature was validated as a superior predictor of HCC metastases in another independent cohort containing ninety-five samples with a prediction accuracy of 92 percent, indicating that the immune response molecular signature in the noncancerous

hepatic tissues can accurately predict HCC metastasis and prognosis [8].

Through Proteomics Analysis, CK19 and CK10 Are Identified as Predictors for HCC Metastasis

Differential proteomic analysis was conducted to analyze the protein expression profiles of cell lines and clinical specimens of HCC with different metastatic potentials. Cytokeratin 10 (CK10), CK19, and HSP27 were identified as potential predictive markers for HCC metastasis. Overexpression of CK10 in HCC tissues, as well as serum CK19 levels, might reflect progression in HCC; these proteins may constitute useful prognostic markers and therapeutic targets for metastatic HCC [14, 58, 59].

State-of-the-Art Diagnostic/Prognostic Testing

None of the biomarkers discussed previously has yet been widely accepted in the clinical arena. A combination of these novel parameters with clinicopathological features may be helpful.

Many factors, including other clinical parameters (such as age, sex, coexisting hepatitis, liver function, AFP level), tumor morphology (tumor size, number, capsule status, intra- or extrahepatic spreading, vessel invasion), and tumor histological features, as well as treatment-related factors (surgical techniques, blood transfusion), have been reported as risk factors for metastasis, and as significant predictors of HCC recurrence (Table 32.1).

Coexisting Liver Disease

HCC recurrence is strictly linked to the status of the underlying liver disease. The inflammatory activity, viral load, serum hepatitis B virus e antigen (HBeAg) positivity, and the functional reserve of the remnant cirrhotic liver have been confirmed as independent risk factors for HCC recurrence [61–63].

Pathological Features of Tumor

Many pathologic features, such as tumor size, number, capsule status, cell differentiation, vascular invasion, intrahepatic spreading, and advanced pTNM stage, have been established as risk factors for recurrence and metastasis of HCC. Vascular lake and angiographic condensed pooling are also indicators for early recurrence [64, 65]. Marked inflammatory cell infiltration in the tumor and intratumoral balance of regulatory and cytotoxic T cells are promising independent predictors for recurrence and survival in HCC [66].

Serum AFP and Detection of Circulating HCC cells

Serum AFP is useful not only for diagnosis but also for predicting metastasis and recurrence of HCC. The lens culinaris agglutinin A-reactive fraction of AFP (AFP-L3) is a more useful indicator for distant metastasis; it has shown nine to twelve months of lead time in early recognition of recurrent HCC as compared with imaging techniques, with a specificity of more than 95 percent [67–69]. AFP mRNA in the peripheral blood of HCC patients is proposed as a marker of HCC cells disseminated into the circulation, and might be predictive of early intrahepatic recurrence and distant metastasis after HCC resection [70].

Clinical Staging

A clinical staging system for HCC that predicts the likelihood of tumor recurrence may also be useful to guide patient assessment and therapeutic decisions. Several systems are available for classification of HCC. The most common is the TNM staging system of the International Union Against Cancer (UICC), but the Barcelona Clinic Liver Cancer (BCLC) staging classification and the Cancer of the Liver Italian Program (CLIP) scoring systems are considered more effective in predicting the prognosis of patients with HCC. Although these systems have successfully graded the patients on their prognosis according to each parameter, they still have limitations for accurately predicting the outcome of patients with HCC, especially for those with early-stage disease and for those without vascular invasion [73, 74].

PREVENTION AND TREATMENT

Experimental Intervention on HCC Metastasis

Antiangiogenesis treatment has been investigated in the nude mice model using endostatin, cytostatic calcium influx inhibitor carboxyamido-triazole (CAI), TNP-470, VEGF trapping using decoy circulating Flk, and interferon (IFN)-alpha [75]. Although all of them showed some efficacy in terms of reducing tumor angiogenesis, IFN-alpha has been most intensively studied. In the nude mouse model, IFN-alpha treatment delayed tumor growth and inhibited metastasis and recurrence after resection of the primary tumor by suppressing angiogenesis through downregulation of VEGF and direct inhibitory effects on proliferation and motility of endothelial cells, and by direct inhibition of tumor cell growth only when p48 positive [16, 76, 77].

Antisense H-ras oligodeoxynucleotides (ODN; an inducer of apoptotic cell death), heparin (functionally similar to heparan sulfate, metabolite to suramin), and BB94 (a metalloproteinase inhibitor), have also been

found to inhibit HCC tumor growth and metastasis to lung in nude mice bearing human HCC [75]. Synthetic beta-peptide (an ICAM-1 blocker), and cell differentiation agent-II were also shown to suppress HCC lung metastasis [18].

Current Approaches to Prevention of HCC Metastasis and Recurrence

A number of treatment modalities have been explored for the prevention of HCC metastasis and recurrence after surgical resection. These include preoperative transcatheter arterial chemoembolization (TACE), postoperative TACE, systemic or locoregional chemotherapy, immunotherapy, interferon, and acyclic retinoic acid. However, only a few of these treatments have been shown to be effective by randomized control trial (RCT). As yet, there is no evidence demonstrating benefit in terms of survival and prognosis from these various neoadjuvant and adjuvant therapies [78].

Based on the results of RCTs, preTACE is not helpful in terms of decreasing recurrence after resection of resectable HCC [79]. Indeed, for huge resectable HCC, preTACE increased the likelihood of extrahepatic metastasis and tumors invading to adjacent organs. For small HCC, preTACE was not shown to inhibit the intrahepatic micrometastatic lesions and tumor thrombus in microvessels. Therefore, preTACE should be avoided for resectable HCC, particularly in patients with advanced liver cirrhosis.

There is only one RCT reporting a positive result for postTACE, in which a single 1850 MBq dose of intraarterial 131I-lipiodol given after curative resection of HCC was shown to significantly decrease the rate of HCC recurrence and increased the three-year OS rate [80]. In one very recent update report, it was confirmed that it could increase both the five-year DFS and OS [81]. However, in the two earlier RCTs, postTACE is harmful to patients after curative resection of HCC, as it cannot eliminate the recurrence [82], and possibly even increased the rates of recurrence and extrahepatic metastasis [83].

Systemic chemotherapy is generally not effective in most cases of HCC. No RCT evidence indicates that adjuvant chemotherapy is useful, and it may even enhance cancer recurrence and lead to deterioration of the long-term outcome in patients with cirrhosis [84]. However, capecitabine was shown to be effective in inhibiting tumor growth and decreasing the incidence of metastatic recurrence after resection of HCC, which is one potential new approach for the control of the recurrence and metastasis of HCC [19].

Biotherapy has been proposed as the most hopeful strategy to prevent the recurrence and metastasis of HCC after surgery. Many RCTs have indicated that postoperative IFN-alpha therapy can decrease recurrence after resection of HCV-related HCC [85–87]. Based on the experimental finding that IFN-alpha could inhibit the growth and metastasis of HCC [16], a RCT was conducted to evaluate the effect of IFN-alpha treatment (50 micrograms IM tiw for 18 months) on tumor recurrence and survival in 236 patients with HBV-related HCC. The median OS was 63.8 months in the treatment group and 38.8 months in the control group ($P = 0.0003$); the median DFS period was 31.2 versus 17.7 months ($P = 0.142$). Thus, IFN-alpha treatment could improve the OS of patients with HBV-related HCC after curative resection, probably by postponing recurrence [17] (Figure 32.3)

The positive effects of adoptive immunotherapy on recurrence of HCC have been confirmed in many clinical trials. In the study published in the *Lancet*, adoptive immunotherapy with infusion of autologous lymphocytes activated in vitro with recombinant interleukin-2 and CD3 antibody during the first six months after HCC resection could decrease the frequency of recurrence by 18 percent and improve significantly the recurrence-free survival and disease-specific survival, but not OS [88]. Autologous formalin-fixed tumor vaccine (AFTV) could also reduce the risk of HCC recurrence by 81 percent, significantly prolong the time to first recurrence, and improve both the DFS and OS of patients with HCC [89].

Management of Metastasis and Recurrence of HCC

Many treatment strategies, including surgical re-resection, TACE, regional cancer therapies such as radiofrequency ablation (RFA), and chemotherapy have been tried and tested for the control of HCC recurrence and metastasis. Any treatment at recurrence has been regarded as a significant good prognostic factor for patients with recurrent HCC. Therefore, to improve prognosis after recurrence, we should actively treat the metastatic and recurrent lesions whenever possible. However, there are few RCTs to evaluate the effect of these modalities. (Table 32.2)

Surgical Treatment

Many studies have demonstrated that repeat hepatectomy is effective for treating intrahepatic recurrent HCC in selected patients and prolong the survival [90, 91]. The five-year OS rate after re-resection for recurrent HCC is similar to the curative resection for the primary HCC. Re-resection has been performed in 636 patients with subclinical recurrent HCC since the late 1970s; the five-year survival of patients can be as high as 63.9 percent, which is much better than that of the locoregional therapies (51.6% after RFA, and 28.5%

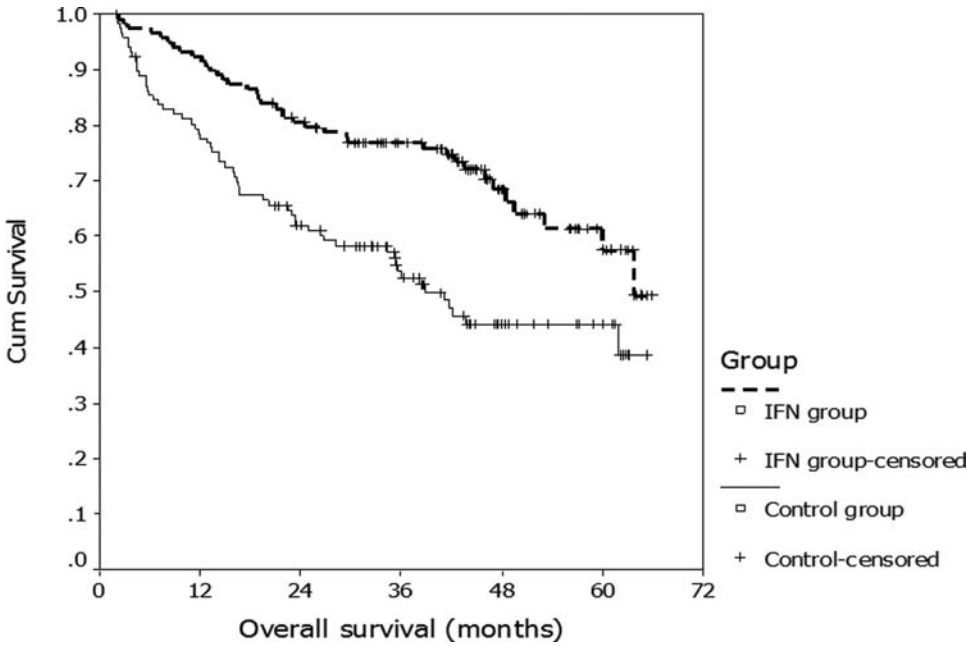

Figure 32.3. IFN-alpha treatment could improve the OS of patients with HBV-related HCC after curative resection. The median OS was 63.8 months in the treatment group and 38.8 months in the control group ($P = 0.0003$).

after TACE); and the ten-year survival is up to 39.2 percent (data not published). Of these patients, 250 have survived more than five years, and 76 patients have survived more than ten years. Thus, repeat hepatectomy is the preferred treatment for patients with recurrent HCC with fewer than three tumor nodules and sufficient liver function. It can also provide a favorable quality of life in patients with recurrent HCC (Figure 32.4).

Liver transplantation has also been suggested as a strategy for patients with recurrent HCC after HCC ("salvage" transplantation), and tumor control by

TABLE 32.2. Management of metastatic and recurrent hepatocellular carcinoma

Strategies	Efficiency	Patient selection
Surgical treatment *Repeat resection*	Most effective, preferred treatment Long-term survival Favorable quality of life	Patients with recurrent HCC ≤3 nodules and sufficient liver function Particularly, patients with recurrence from MO Solitary extrahepatic metastasis (solitary lung, adrenal)
"Salvage" transplantation	Effective in selected patients	Recurrent tumor number: ≤3 Recurrent tumor size: ≤5 cm Liver function: Child-Pugh A or B or even C
Regional cancer therapies *Regional ablation*	Effective in selected patients	Patients with number ≤3 and size ≤3 cm of recurrent nodules Liver function: Child-Pugh A or B Unsuitable for surgery or not willing to undergo surgery
TACE	Effective in selected patients	Patients with multiple intrahepatic recurrence
Radiation therapy	Effective in selected patients	Lymph node metastasis from HCC Adrenal, bone, and spinal metastasis Tumor thrombi in portal vein, bile duct, and IVC
Chemotherapy	Generally not effective TAC + systemic IFN-alpha: new trend	Multiple intra- and/or extrahepatic metastasis with good liver function

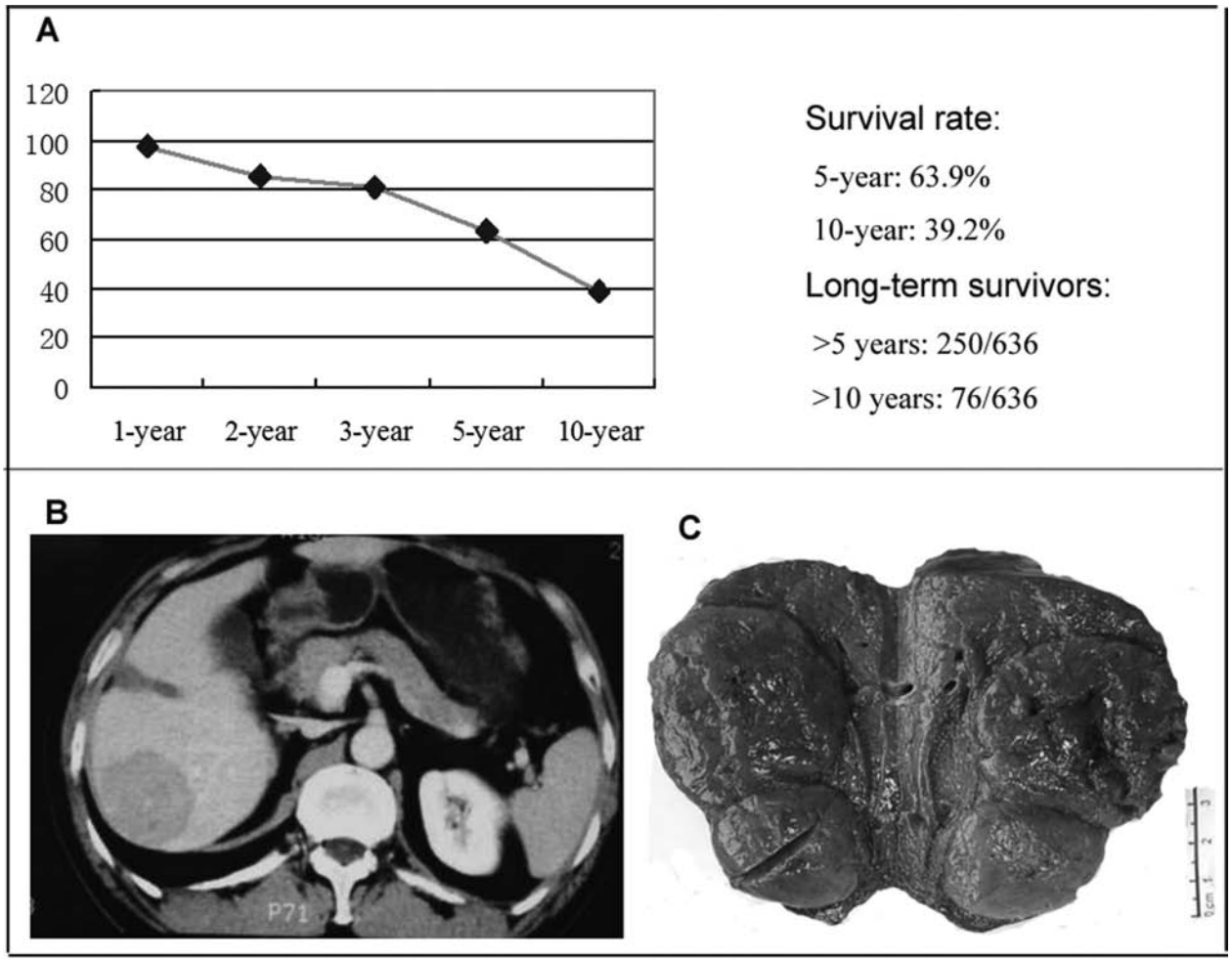

Figure 32.4. Repeat hepatectomy is effective for treating intrahepatic recurrent HCC in selected patients and prolonging their survival. In the authors' institute, re-resection has been performed in 636 patients with subclinical recurrent HCC; the five- and ten-year survival of patients can be 63.9 percent and 39.2 percent, respectively, and 250 patients have survived for more than 5 years (**A**). One recurrent HCC was shown in the right lobe of liver by CT scan during following-up (**B**) and was surgically removed by repeat hepatectomy (**C**).

transplantation is possible in selected patients. However, certain criteria regarding the number (up to three) and size (up to 5 cm) of recurrent tumor nodules have to be observed in order to ensure a low risk of both intra- and extrahepatic spread after operation. Incidence of tumor recurrence after liver transplantation for HCC with Milan criteria was reported to be less than 10 percent, mainly extrahepatic (lung) [92].

The role of resection in extrahepatic HCC recurrences is not well established. The literature supports an aggressive approach in selected cases of extrahepatic HCC recurrence: resectable metastases, preserved liver function, absence of intracranial metastasis, and control of the primary tumor. Surgical resection for pulmonary metastasis from HCC has been shown to prolong survival in selected patients. The average survival after pulmonary resection was 29 months [93]; the one- and three-year survival rates after metastasectomy were 45.3 percent and 23.8 percent, respectively, and the one- and three-year recurrence-free survival rates were 32.4 percent and 21.6 percent, respectively [94]. Furthermore, repeated pulmonary resections via thoracoscopy could result in the long-term survival of patients with pulmonary recurrence of HCC. Adrenalectomy may also be employed to ensure long-term survival in patients with adrenal metastases from HCC, as long as the primary tumor is well controlled therapeutically, there is no additional metastatic disease, and the patient has a good performance status. Adrenalectomy enables survival periods longer than two years [95]. In patients with bone metastases from HCC, surgery should be used to prevent and treat complications such as nerve compression and pathologic fracture. Operative treatment for spinal metastatic HCC lesions may improve quality of life, regardless of whether the survival time is prolonged.

Regional Cancer Therapies

RFA is regarded as the first choice in the management of intrahepatic recurrence that is not suitable for surgery. For multifocal recurrence, TACE is needed. RFA can be useful as a complementary technique for lesions not completely treated by TACE.

Radiation Therapy

Radiation therapy could be beneficial when other therapies present some difficulty regarding application or are not fully effective. It may also provide clinical benefit for patients with lymph node, adrenal, bone, and spinal metastasis, and tumor thrombi in the portal vein, bile duct, and/or inferior vena cava [21, 22, 96]. It provides effective palliation for patients with painful bone metastases from HCC. The major concern is that radiation therapy could suppress the patients' immunity and thereby induce distance metastasis and multiple intrahepatic spreading after radiation therapy.

Chemotherapy

Chemotherapy is not very effective for recurrent and metastatic HCC. Combined therapy consisting of intraarterial chemotherapy (such as cisplatin) and systemic IFN-alpha is a new trend in this area, which may be useful as a palliative treatment for HCC patients with extrahepatic metastasis.

KEY UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

Despite tremendous effort in the research of metastatic HCC, several key issues remain unresolved. Many biomarkers have been identified that are able to predict the likelihood of metastasis and tumor recurrence, but unfortunately none has yet been universally accepted, primarily because of their unsatisfactory sensitivity or specificity. A molecular signature including up to hundred genes has also been found, but its predictive value has not yet been confirmed based on the complicated subsets of HCC with different phenotypes. IFN-alpha is the only treatment that is of proven benefit for the prevention of metastasis. Surgery and regional cancer therapies are accepted to be standard of care for metastasis.

In the future, the following issues deserve to be noted: (1) HCC metastasis is not a regional event; it is a systemic disease. Metastasis results from interactions between host microenvironment (including nervous, endocrine, and immunologic systems as well as metabolism) and cancer. Therefore, the prediction of metastasis and any intervention should focus not only on cancer itself but also on the microenvironment and the host. (2) During the past century, clinical studies for HCC were mainly based on the pathological background. With advancements in molecular biology, modulation of residual cancer using biotherapy will be an important approach to further improve prognosis of conventional therapies. (3) The toxicity profile of the major therapies for HCC is well recognized. However, the "opposite effects," in that these therapies may *enhance* the metastatic potential of the treated cancer, must also be considered. Recently, cyclophosphamide pretreatment was found to induce cancer metastasis [97]. We also have demonstrated that radiotherapy may enhance the long-term metastatic potential of residual cancer. Therefore, the biological concept of major therapies will be another important issue to be studied.

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33 Advances in Management of Metastatic Colorectal Cancer

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Colorectal cancer (CRC) is the third most common type of cancer and has the second highest cancer-related mortality among men and women in the United States. In 2009, approximately 150,000 Americans were expected to be diagnosed with colorectal cancer, and about 50,000 people were expected to die from the disease [1]. Despite the improvement in early detection through tests including fecal occult blood testing, double-contrast barium enema, flexible sigmoidoscopy, and screening colonoscopy, about 20 percent of patients with CRC are found to have metastases at the time of presentation [2]. Liver metastases are seen in about 20 percent to 70 percent of patients with metastatic CRC (mCRC) [3]. When patients present with isolated and limited liver metastases, surgical resection of the liver metastases can provide long-term survival; however, only 10 percent to 20 percent of patients with mCRC are eligible for curative liver resection [4]. The majority of patients with mCRC will receive systemic chemotherapy and palliative liver-directed therapy.

During the past decade, the development of effective chemotherapies such as irinotecan and oxaliplatin and the development of agents targeting the vascular endothelial growth receptor (VEGFR) and the epidermal growth factor receptor (EGFR) have made a tremendous impact on prolonging survival in patients with mCRC. Since the early 1990s, the median survival for a patient with unresectable mCRC has improved from six months with best supportive care to more than two years with current treatments (Figure 33.1). In this chapter, we review the standard approaches to managing mCRC and examine the major advances in the areas of pharmacogenomics, molecular predictors, novel drugs on the horizon, and future directions for mCRC.

DIAGNOSIS AND STAGING

Colonoscopy is the most effective way to detect malignancies in the bowel and to obtain a biopsy of the mass to provide a histological diagnosis. Routine computed tomography (CT) is the most widely used imaging tool for adequate staging. As positron emission tomography (PET) is more sensitive in identifying occult extrahepatic tumors, it has been increasingly used as a screening tool for patients with potentially resectable liver metastases. PET can detect unsuspected tumors in 25 percent of patients who otherwise would be eligible for hepatic resection and, by using this procedure, a better selection of surgical candidate patients has directly translated into higher overall survival [5].

PROGNOSTIC FACTORS IN MCRC

Age is commonly regarded as an adverse prognostic factor in cancer patients; however, it is not a prognostic factor in patients with mCRC. A retrospective analysis was performed on 3742 patients who received FOLFOX, a combination chemotherapy regimen composed of 5-fluorouracil (5-FU), leucovorin (LV), and oxaliplatin. When compared with their younger counterparts, patients older than 70 years of age had a similar response rate, progression-free survival, and overall survival [6].

Performance status (PS) is an important prognostic factor in general. Poor PS patients are unable to proceed to second-line treatment, and their median survival is only 1.7 months after progression of first-line therapy [7].

Circulating tumor cells (CTCs) are clearly associated with the outcome of mCRC patients. First, CTCs can be isolated from the peripheral blood of

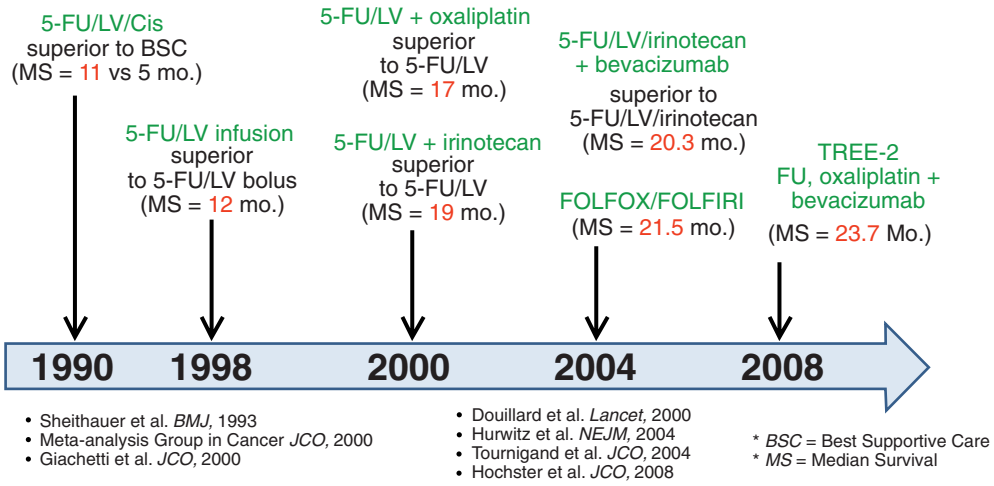

Figure 33.1. Historical perspective.

patients with all major metastatic carcinomas [8]. Recently developed immunomagnetic separation technology enables the easy enumeration of circulating tumor cells by their expression of surface epithelial cell adhesion molecule (EPCAM). Second, and more specifically for mCRC patients, CTCs can serve as a prognostic marker for the disease. In a prospective study, patients with a baseline of ≥3 CTCs/7.5 mL compared with those with <3 CTCs/7.5 mL had a disease-free survival of 4.5 versus 7.9 months ($p = 0.0002$), and the overall survival was 9.4 versus 18.5 months ($p < 0.0001$). Furthermore, a decline of CTCs with treatment was associated with a better prognosis.

The technology measuring CTCs (Cellsearch system) was approved by the Food and Drug Administration (FDA) in November 2007; however, as it is not clear whether CTC levels are a better assessment tool for tumor response than traditional imaging studies, it remains to be determined how physicians should incorporate this technology into their daily management of mCRC patients. Much work remains to be done on characterizing CTCs, and a thorough understanding of the biology of the CTCs will likely shed much-needed light on the identification of drug targets and the development of therapeutic interventions.

MANAGEMENT OF ADVANCED METASTATIC COLORECTAL CANCER

Resectable Liver-Only Metastases

Metastatic cancers are generally considered to be incurable. However, liver-only metastases from CRC present a unique situation, in that long-term survival can be achieved in patients who receive curative hepatic resection [9, 10]. The five-year overall survival rate for mCRC patients receiving hepatic resection ranges from 12 percent to 41 percent, with a median of 30 percent [5, 10]. With the assistance of PET in perioperative screening, the five-year survival rate after liver resection in mCRC has further improved to 58 percent [5]. The benefit of hepatic resection extends beyond five years, in fact, a ten-year overall survival has been seen in 15.7 percent to 28 percent of patients [10–12].

The ***selection criteria for hepatic resection*** have evolved over the years. The principle of curative hepatic resection is to achieve a negative surgical margin while maintaining adequate liver reserve. In the past, the number, size, and locations of the metastases in the liver have been the main determinants for successful resection, and the involvement of more than 70 percent of the liver or more than six segments was thought to be the contraindication for curative resection [13]. However, the current expert consensus statement recommends that the following three criteria be met to achieve a successful resection: 1) the ability to preserve two contiguous hepatic segments; 2) the preservation of adequate vascular inflow, outflow, and biliary drainage; and 3) the ability to preserve more than 20 percent of healthy liver tissue [14]. With effective chemotherapy on board, the presence of extrahepatic disease is no longer an absolute contraindication.

Neoadjuvant chemotherapy is an attractive and rational approach for patients with resectable liver metastases from CRC for several reasons. First, delivery of chemotherapy prior to surgery can potentially decrease micrometastasis at the front end, resulting in a reduced rate of tumor recurrence. Second, the high tumor response rate seen with current chemotherapy regimens can potentially decrease tumor size and increase the likelihood of curative resection. Third, tumors that progress on neoadjuvant chemotherapy

likely would not have been curable, as the disease progression reflects the aggressive and chemoresistant nature of the tumor. These patients should then be spared a morbid and noncurative operation.

The benefit of neoadjuvant chemotherapy was demonstrated in a large randomized European Organization for Research and Treatment Center (EORTC) 40983 trial [15]. In this study, 364 patients with resectable liver metastases (up to four lesions in the liver) were randomly assigned to receive either surgery alone or six cycles of FOLFOX before and after surgery. With regard to the primary endpoint of the study, which was a three-year progression-free survival rate, the absolute benefit of neoadjuvant chemotherapy was 8.1 percent (from 28.1% to 36.2%; HR 0.77; $p = 0.041$) in eligible patients. Not surprisingly, postoperative complications were higher in patients treated with neoadjuvant chemotherapy as compared with those simply receiving surgery (25% to 16%, $p = 0.04$). Although this trial established the role for neoadjuvant chemotherapy in patients with resectable liver metastases, it also raised the concern of increasing postoperative complications resulting from neoadjuvant therapy.

The disadvantages associated with neoadjuvant chemotherapy are not insignificant, and chemotherapy-associated hepatosteatosis (CASH) has gained increased attention recently. The pathological features of CASH include hepatocyte inflammation, fibrosis, and sinusoid dilation. The ninety-day mortality rate after hepatic resection in patients with CASH is 14.7 percent, compared with 1.6 percent for those without CASH [16].

Another concern is the potential curative opportunity that will be missed for those patients who can undergo surgical resection up front, but who choose to be treated with chemotherapy and later are unable to proceed with surgery because of either disease progression or chemotherapy-related toxicity. Even in the setting of effective treatment, the dramatic tumor shrinkage in response to neoadjuvant chemotherapy can pose a challenge for localizing the tumors intraoperatively; therefore, complete resection can be compromised by the fact that the preexisting tumor is no longer visible [17]. At this time, the neoadjuvant approach is gaining favor among surgeons, but it is still not widely used because of the concerns mentioned above. Ultimately, the plan for liver resection should be discussed in multidisciplinary conferences.

Adjuvant chemotherapy has been used routinely with limited supporting evidence. In a multicenter study, 173 patients with complete resection of liver metastases of CRC were randomly assigned to receive either 5-FU and folinic acid or no chemotherapy (observation). The five-year disease-free survival was 33.5 percent for patients in the chemotherapy arm and 26.7 percent in the observation arm; a trend toward overall survival was observed as well (51.1% vs 41.1%, $P = 0.13$) [18]. As the regimen used in this study is now considered suboptimal, the magnitude of benefit may be underestimated in view of the combination chemotherapy that is now available. However, in contrast, a meta-analysis performed in two Phase III trials using 5-FU and LV for the adjuvant regimen showed only a trend toward progression-free survival [19]. Currently, optimal chemotherapy with targeted agents for mCRC patients who underwent hepatic resection is being evaluated in clinical trials. Off protocol, based on the extrapolation of the survival benefit of adjuvant trials in patients with stage III CRC, four to six months of either 5-FU or FOLFOX treatment following liver resection is recommended for mCRC patients.

Hepatic arterial infusion (HAI) as an adjuvant modality has never been widely accepted. Several randomized studies have been done using HAI in the adjuvant setting, but most of these suffer from small sample size, slow accrual, and early termination. Kemeny et al. reported an overall survival rate of 86 percent at two years posthepatic resection in patients treated with HAI floxuridine (FUDR) and systemic 5-FU with or without LV. This is compared with an overall survival rate of 72 percent for patients simply receiving similar systemic therapy without HAI ($p = 0.03$) [20]. A ten-year follow-up of the data showed that only the high-risk patients benefited from Kemeny's approach [21]. At this time, HAI as an adjuvant treatment is limited to research in clinical trials.

Newer techniques have been developed to maximize the efficacy of surgical resection, as it is the only potential cure for patients with liver metastases from CRC. Radiofrequency ablation (RFA) is performed either percutaneously or intraoperatively by placing an RFA needle electrode directly into the tumor and achieving tumor destruction through thermal energy. When RFA is combined with resection, patients who ineligible for hepatic resection because of multiple liver lesions can undergo successful curative surgery. In a retrospective study of 53 mCRC patients with five or more bilobar liver lesions, the thirteen patients who underwent resection with ablation had a similar survival rate to the patients receiving surgery alone [22]. Addition of RFA to hepatic resection does not increase morbidity or mortality [22, 23]. RFA has yet to be compared with surgical resection directly in a randomized trial; until that is done, RFA will simply be used in conjunction with surgery to augment surgical resection rather than to replace surgical resection [24].

Portal vein embolization (PVE) is an effective strategy to increase hepatic reserve prior to resection. This

technique is performed percutaneously or at the time of resection of the primary tumor to occlude the vein to the affected lobe. This procedure results in atrophy, and the lobe not containing metastatic lesions becomes hypertrophic by compensation. By employing this technique, patients who are initially considered unresectable because of poor liver reserve become potential resection candidates. Additionally, PVE can improve postoperative complications. In a prospective study, PVE prior to the right hepatectomy decreased the rate of postoperative complications and shortened hospital stays in patients with chronic liver disease (13 ± 4 vs 30 ± 15 days, $p < 0.001$) [25].

Prognostic factors for mCRC patients receiving liver resection have been under intense investigation. A prognostic scoring system has been used for more than a decade to estimate the chance of cure after liver resection [26]. From a review of 1001 patients who underwent liver resection for CRC metastases, Fong et al. reported seven independent factors that predict a poor long-term outcome. These include a positive margin, extrahepatic disease, node-positive primary disease, a disease-free interval from primary disease to metastases of less than twelve months, more than one hepatic tumor, a largest tumor size of >5 cm, and a CEA >200 ng/mL. A scoring system was then developed based on these independent factors for prediction of clinical outcome [27].

This clinical scoring system continues to be refined [28] and, most recently, male sex, synchronous metastases, more than three metastases, metastatic infiltration of nearby structures, and postoperative morbidity were demonstrated to be the five negative prognostic factors in patients who underwent liver resection [10]. Although various clinical scoring systems provide some insight into prognostic prediction, the ultimate answer will be found by understanding the mechanisms that drive the metastasis process.

Unresectable Liver-Only Disease

Despite the potential cure with liver resection, only 10 percent to 20 percent of mCRC patients have metastases that are eligible for resection at the time of presentation [4]. The majority of mCRC patients are not candidates for liver resection.

Neoadjuvant chemotherapy has been used to downsize unresectable liver metastases. A retrospective study reported that 77 patients of 151 (51%) with initially unresectable liver metastases were able to proceed with curative resection after FOLFOX chemotherapy [29]. From a prospective study of patients with unresectable mCRC, liver resection was possible in 36 percent of patients following a highly effective regimen of 5-FU, LV, and oxaliplatin with irinotecan (FOLFOXIRI) [30]. Studies using chemotherapy in combination with targeted agents are ongoing.

Hepatic artery infusion: Because liver metastases derive most of their blood supply from the hepatic artery, whereas liver parenchyma are supplied predominantly by the portal vein [31], one therapeutic strategy is to deliver chemotherapy through HAI so the concentration of chemotherapy delivered to the tumor sites will be higher [32]. In patients with unresectable liver-only metastases, higher tumor response rates with HAI were seen in multiple clinical trials, but the survival benefits were inconsistent. A meta-analysis was performed on ten randomized controlled trials comparing HAI with systemic chemotherapy for unresectable hepatic metastases [33]. The tumor response rate was higher in the HAI arm (42.9% vs. 18.45%), but the median overall survival was not statistically significant (15.9 vs. 12.4 months). Additionally, the complications associated with HAI, including elevated liver functions, gastrointestinal bleeding, infections of the pump pocket, and hepatic artery thrombosis, can be quite problematic [20, 34. Currently, it is commonly regarded that oxaliplatin- or irinotecan-based systemic chemotherapy with targeted agents should be considered the first-line therapy for nonresectable liver metastases. HAI is then deferred to patients with predominant liver disease who have failed the standard systemic treatments, and its use should be restricted to treatment in an experienced medical center only.

The future of HAI is directly related to the delivery of new chemotherapy agents, such as oxaliplatin and irinotecan, and to the ability to combine this approach with systemic treatments [35, 36]. In patients who failed systemic chemotherapy, oxaliplatin in the form of HAI along with systemic 5-FU and LV resulted in a 62 percent partial response and an overall survival of sixteen months; however, more confirmatory trials are needed before this can be incorporated into regular clinical practice [36].

Selective internal radiation therapy (SIRT) is a relative new locoregional treatment modality. The concept is to infuse resin microspheres impregnated with 90-yttrium through hepatic arterial infusion to deliver a high dose of beta radiation to liver metastases. The device, SIR-Sphere (Sirtex Medical Inc.), gained FDA approval in 2002 based on a prospective randomized trial in which seventy-four patients with liver metastases from mCRC were randomized to either a SIR-Sphere with HAI (FUDR) arm or a HAI monotherapy arm. The tumor response rate was greater in patients receiving SIR-Sphere (44% vs. 17.6%, $p = 0.01$) and the median time to disease progression was longer (19.2 vs. 10.1 months, $p = 0.001$) [37]. This study did not show excessive toxicity with the SIR-Sphere treatment. Currently, clinical trials using SIR-Sphere combined with

systemic chemotherapy in mCRC patients are under way. For example, a FOLFOX plus SIR-Sphere study in combination with the targeted agent bevacizumab is ongoing as first-line treatment to mCRC patients with unresectable liver metastases.

Systemic Treatments in mCRC

Development of current cytotoxic chemotherapies and targeted agents ended the long-lasting era of 5-FU as the only beneficial therapeutic agent in the management of CRC. The median survival for mCRC patients is approximately six months for those who receive best support care, eleven to twelve months with 5-FU and LV [38], and approximately two years with irinotecan or oxaliplatin combination therapy [39, 40]. When agents targeting EGFR and VEGFR inhibition are added to chemotherapy, the median survival in patients with mCRC is extended beyond two years [40, 41].

Chemotherapy Agents in mCRC

5-Fluorouracil and Capecitabine

5-FU, a fluoropyrimidine analog, has been the only agent used to treat CRC in the past four decades, and it has now become the backbone of combination chemotherapy in mCRC. 5-FU undergoes the following metabolic processes after administration: 5-FU is converted to FUrd by thymidine phosphorylase (TP), and then to FdUMP by thymidylate kinase. FdUMP, the cytotoxic metabolite of 5-FU, works as an inhibitor of DNA synthesis by forming a complex with reduced folate and thymidylate synthase (TS) that inhibits DNA synthesis [42]. The schedule of administration of 5-FU has evolved from bolus to continuous infusion. A meta-analysis determined that bolus administration of 5-FU yielded a 14 percent response rate; a higher tumor response rate of 22 percent was obtained by the continuous infusion of 5-FU [43]. LV, a biomodulator for 5-FU, increases the efficacy of 5-FU by increasing the level of reduced folate and further stabilizing TS. The optimal administration schedule for these in combination is a bolus of 5-FU and LV infusion, followed by a prolonged infusion of 5-FU twice a month. This schedule further improves the tumor response rate to 32.6 percent [44].

A few orally active 5-FU analogs have been developed throughout the world; thus far, capecitabine is the only one that has received FDA approval in the United States. Capecitabine is absorbed from the gastrointestinal tract and undergoes a series of metabolic processes converting it to 5-FU, with the last step of conversion being regulated by TP. As TP is highly expressed in tumor tissue, when mCRC patients were given capecitabine for five to seven days prior to surgery, the concentration of 5-FU in resected primary tumors was 3.2 times higher than in adjuvant healthy tissue [45]. As a result, capecitabine was initially considered to possess higher antitumor activity than 5-FU. An analysis performed on 1207 patients with mCRC from two identically designed Phase III studies revealed that capecitabine demonstrates a higher tumor response rate compared with 5-FU/LV (26% vs. 17%; p <0.0002), but the time to tumor progression (4.6 vs. 4.7 months) and overall survival (12.9 vs. 12.8 months) were essentially identical [46]. Capecitabine is now commonly used interchangeably with continuous infusion of 5-FU and LV combination therapy in clinical practice and in most mCRC clinical trials.

Irinotecan, a camptothecin derivative, functions as a DNA topoisomerase I inhibitor. Irinotecan is converted to the active metabolite SN-38 by the carboxylesterase enzyme and is then formed into a complex with topoisomerase I and the DNA complex. This complex can induce DNA breaks during DNA replication and result in tumor cell death [47]. In a study of patients with mCRC who failed 5-FU treatment, irinotecan showed a significant one-year survival benefit when compared with best support care (36.2% vs. 13.8%). In addition, the palliation was better in the irinotecan arm, as indicated by higher quality-of-life scores [48]. In the metastatic setting, irinotecan combined with 5-FU and LV has been compared with 5-FU and LV alone as first-line treatment through a large randomized trial. The response rate was higher in patients in the irinotecan group (49% vs. 31%) and the median overall survival was higher (17.4 vs. 14.1 months, p = 0.031) [49]. This trial proved conclusively the benefits of using irinotecan-based therapy as a first-line treatment for mCRC.

Oxaliplatin, a third-generation water-soluble platinum compound, was approved by the FDA in 2002 for treatment of CRC. It binds to DNA to form interstrand and intrastrand DNA adducts, resulting in cell apoptosis. In a large randomized Phase III study, FOLFOX was compared with 5-FU/LV as the first-line treatment. Median progression-free survival was significantly better in the FOLFOX arm than in the 5-FU/LV arm (9.0 vs. 6.2 months, P = 0.003). The median survival was 16.2 months for the FOLFOX arm and 14.7 months for the 5-FU/LV arm (p = 0.12) [50]. The dose-limiting toxicity of oxaliplatin is peripheral sensory neuropathy, which increases with accumulated doses of oxaliplatin.

Targeted Agents for mCRC

Cetuximab is a chimeric monoclonal IgG1 antibody directed against EGFR, which is highly expressed on the cell surface of many tumors and other epithelial cells in the body. By binding to EGFR, cetuximab can inhibit EGFR signaling by preventing the homodimerization or heterodimerization of EGFR, the necessary step for

activation of EGFR by phosphorylation and subsequent downstream signaling [51]. When cetuximab is combined with irinotecan in patients who have failed on irinotecan treatment alone, the tumor response rate is 22.9 percent, compared with 10.8 percent with cetuximab monotherapy ($p = 0.007$) [52]. As a single agent, compared with the best supportive care, cetuximab improved the overall survival of mCRC patients with a hazard ratio (HR) to death of 0.77, $P = 0.005$ [53]. The response rate of cetuximab as a monotherapy in this study was approximately 9 percent in EGFR-expressing tumors [54]. Trials investigating the combination of FOLFOX with cetuximab as a first-line therapy are ongoing.

Panitumumab is the second EGFR blockade approved by the FDA to treat mCRC. It is a fully human monoclonal IgG2 antibody, so the infusion-related reaction (grade 3 allergic reaction) is less compared to the chimeric monoclonal antibody cetuximab [54]. Compared with best supportive care, panitumumab significantly prolonged progression-free survival (PFS) with a HR of 0.54, $P < 0.001$, but no overall survival benefit was observed [55]. Panitumumab is currently being evaluated with FOLFOX in the first-line setting [56]. Although cetuximab and panitumumab share a similar mechanism of action, treatment comprised of the addition of panitumumab to FOLFOX plus bevacizumab resulted in an inferior PFS and increased toxicity compared with treatment with FOLFOX plus bevacizumab without panitumumab [57, 58]. Thus, panitumumab should be used only as a single agent outside clinical trials.

In the past, the development of a rash in patients receiving EGFR inhibitors has been associated with better tumor response and longer survival [53]. Recently, strong evidence has emerged to support K-ras as a predictive factor for tumor response to EGFR inhibition. We review this exciting molecular predictor in detail later under the section of molecular markers.

Bevacizumab is the only FDA-approved antiangiogenesis agent used to treat mCRC. Angiogenesis is one of the hallmarks of cancer [59], and strategies targeting angiogenesis have translated well into the clinical setting. Bevacizumab is a humanized monoclonal antibody that binds to all isoforms of circulating VEGF, preventing VEGF from binding to VEGF receptors, and thereby blocking the activation of downstream signaling for angiogenesis [60]. In a Phase III clinical trial, patients with mCRC were randomly assigned to receive bolus 5-FU, LV, and irinotecan (IFL) or IFL plus bevacizumab. Bevacizumab was found to improve PFS from 6.2 months to 10.2 months ($P = 0.0014$), and the median survival also increased, from 15.6 months to 20.3 months (HR 0.66, $p < 0.001$) in the bevacizumab arm [41]. Based on the survival benefit derived from bevacizumab, this targeted therapy agent was approved by the FDA for the treatment of mCRC in February 2004.

The side effects of bevacizumab are worth mentioning. Hypertension and proteinuria are the common and easily manageable side effects [41, 61], but arterial thromboemoblic events, gastrointestinal perforation, and significant bleeding are daunting complications. From a pool of five randomized trials including a total of 1745 patients with metastatic cancer, the absolute risk of developing an arterial thromboembolism with the addition of bevacizumab was about 1.4 events per 100 person–years. A personal history of thrombotic events and an age of 65 years or older were found to be risk factors for developing an arterial thromboembolism [41, 61, 62]. The risk of GI perforation with bevacizumab is generally about 1.5 percent [41, 63]. As bevacizumab can delay wound healing [64], it is recommended that bevacizumab therapy should be stopped six to eight weeks before elective surgery.

First-Line Treatment for mCRC

As irinontecan and oxaliplatin combined with 5-FU and LV emerged as effective regimens in mCRC, studies began to address the optimal first-line therapy for mCRC. ***FOLFOX*** and ***FOLFIRI*** were tested in sequence and then reversed to see if there were any differences in clinical benefits. FOLFOX followed by FOLFIRI or vice versa at the time of disease progression appear to have a similar overall survival of 21.5 months versus 20.6 months ($p = 0.99$) [39]. Thus, both regimes are considered equivalent and interchangeable for first-line chemotherapy for mCRC.

Capecitabine, combined with oxaliplatin or irinotecan, is also being investigated in the first-line setting, and using capecitabine rather than 5-FU/LV in the oxaliplatin–combination regimens has shown essentially the same survival [65]. When capecitabine combined with irinotecan (CapeIRI) was compared with FOLFIRI, FOLFIRI demonstrated a longer PFS with less toxicity [40].

Targeted agents have become important constituents of the chemotherapy regimen for first-line treatment of patients with mCRC. The benefit of ***bevacizumab*** was clearly demonstrated in the IFL study, which showed a five-month survival benefit with the addition of bevacizumab to IFL versus IFL therapy alone [41]. The benefit of bevacizumab was also evaluated with an oxaliplatin-based regimen. In this large Phase III study, 1401 patients were randomized to oxaliplatin combined with either 5-FU/LV (FOLFOX) or capecitabine (XELOX), with bevacizumab or without bevacizumab (placebo groups) based on a 2×2 factorial design. The median PFS was 9.4 months in the bevacizumab arm compared with 5.5 months in the placebo group ($p = 0.0023$) [61]. The median overall

survival was 21.3 months in the bevacizumab arm and 19.9 months in the placebo arm. Surprisingly, the benefit of bevacizumab in terms of PFS did not translate into overall survival, which raised the question of bevacizumab being cost-effective. It was also speculated that administering bevacizumab for only a short period of time may compromise the survival benefit. Bevacizumab combined with various irinotecan-based regimens was evaluated in a Phase III clinical trial (BICC-C study); the median survival of bevacizumab with FOLFIRI was 28.0 months compared with 19.2 months in patients treated with bevacizumab with modified IFL [66].

If there are no contraindications, patients with mCRC should receive systemic chemotherapy consisting of bevacizumab with either FOLFOX or FOLFIRI as their first-line therapy. Although both regimens can be used interchangeably, FOLFOX with bevacizumab has been the preferred choice for physicians in the United States.

EGFR inhibitors have also been studied as first-line therapy options. In the CRYSTAL trial, ***cetuximab*** plus FOLFIRI as the first-line treatment was evaluated in patients with EGFR-expressing mCRC. The PFS improved ($p = 0.036$) with the addition of cetuximab, but the overall survival rates were not available for this ongoing study [67]. Inhibition of the EGFR and VEGFR pathways has been evaluated as an addition to the standard chemotherapy regimen. CALBG 80405 is investigating whether the targeted agents, ***cetuximab and bevacizumab in combination,*** provide an additional benefit to patients over administration of either targeted agent alone with either FOLFOX or FOLFIRI. This study was recently amended to exclude patients with the K-ras mutation. The results of this complicated ongoing study may ultimately determine the optimal combination of targeted agents and chemotherapy as first-line therapy for mCRC.

In summary, because of the amount of research being conducted now to determine new and better systemic treatment strategies for first-line mCRC therapy, patients with mCRC should be enrolled in a clinical trial. However, if patients with mCRC cannot be enrolled in a clinical trial, they should be treated with either FOLFOX or FOLFIRI with bevacizumab as the first-line treatment.

Second-Line Treatment for mCRC

The selection of second-line therapy for patients with mCRC is largely based on the unsuccessful first-line therapy that they had received. As the study mentioned earlier showed that the sequence of administering FOLFOX and FOLFIRI did not affect overall survival, it is quite reasonable to administer FOLFOX first followed by FOLFIRI, as the most common regimen for first-line therapy is to use FOLFOX, and then to use FOLFIRI as the second-line therapy. With regard to targeted therapies, bevacizumab, cetuximab, and panitumumab have been approved in the second-line setting. The method of combining the targeted agents with second-line therapy, however, remains to be determined.

One major controversy is whether to continue with bevacizumab in patients who have progressed on bevacizumab with either FOLFOX or FOLFIRI. Based on the Bevacizumab Regimen: Investigation of Treatment Effects and Safety (BRiTE) registry trial, an overall survival of 31.8 months was seen in patients receiving bevacizumab after disease progression compared with 19.9 months in patients who did not continue receiving bevacizumab after first-line therapy [68]. Again, multivariate analysis showed that the use of bevacizumab is associated with longer survival; however, these data were derived from a registry trial rather than a randomized trial. Currently, the use of bevacizumab after disease progression is largely based on individual oncologists' opinions.

Cetuximab and irinotecan in combination can be used as second-line treatment, and panitumumab is approved only for use as a single agent.

Controversial Strategies for mCRC Management

An ***intermittent chemotherapy dosing schedule*** has been evaluated because of the accumulation of oxaliplatin-induced neurotoxicity. A prospective clinical trial was designed to address the very practical question of whether an oxaliplatin-free interval compromises the survival of mCRC patients. The OPTIMOX1 trial randomly assigned 620 patients to either a FOLFOX arm with treatment given until disease progression or a FOLFOX arm in which no oxaliplatin was used as maintenance therapy [69]. The median survival (19.3 vs. 21.2 months) and PFS (9 vs. 8.7 months) were equivalent between the two arms. Overall toxicity in patients who had the oxaliplatin-free interval appeared to be slighter better in the beginning of the study but the benefit had tapered off by the end of the study.

Chemotherapy holiday is a frequent request from patients with mCRC. The OPTIMIOX2 study was designed to address whether a chemotherapy holiday would compromise a survival benefit. Patients in this study were to receive FOLFOX for six cycles first, followed by either 5-FU/LV or no chemotherapy, and then FOLFOX would be reintroduced at the time of disease progression. The overall survival was significantly better in the 5FU/LV maintenance arm (26 vs. 19 months, $p = 0.05$). This study proved that a chemotherapy holiday is not advisable for patients [70]; however, this trial does not address the current standard first-line therapy for mCRC, which includes the use of targeted therapy in combination with chemotherapy.

Pharmacogenomics of mCRC

Major advances have been made in pharmocogenetic testing for mCRC. It is understood that the response and toxicity toward chemotherapy varies individually, and some of the genetic alterations involved in drug metabolism have been identified through pharmacogenomic studies. Excessive toxicity can be avoided by screening patients prior to receiving certain chemotherapies such as 5-FU and irinotecan. This approach is rapidly being introduced into the clinical setting, especially for patients with mCRC, as commercial testing becomes available.

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme responsible for the breakdown of 5-FU. Patients with partial or total DPD deficiency can suffer from severe toxicity, including mucositis, diarrhea, and neutropenia, after treatment with 5-FU [71, 72]. The degree of DPD enzymatic activity is largely associated with either mutation or genetic polymorphism in the dihydropyrimidine dehydrogenase gene (DPYD), with the splice site mutation DPYD*2A being the most common debilitating single-nucleotide polymorphism (SNP) [73]. In a prospective study of 487 patients receiving 5-FU–based chemotherapy, five different types of SNPs were identified in 187 patients, and the two SNPs DPYD*2A and 2846A>T were associated with higher 5-FU related toxicity. For example, 60 percent of patients with the above-referenced two genotypes experienced early toxicity, compared with 6.6 percent in patients with no DPYD SNPs. Despite severe toxicity encountered in patients with certain DPYD genotypes, 5-FU–based treatment can be safely resumed at a lower dose [74]. Therefore, although tests for DPYD gene variants are commercially available, their direct clinical use remains to be determined.

Thymidine synthase is an enzyme responsible for conversion of dUMP to dTMP, and it is encoded by the TYM gene. The 5-FU metabolite, 5-FdUMP, binds to TS and folate and blocks DNA replication. The polymorphism of the TYM gene is a double or triple repeat of a specific 28-bp sequence in the promoter region [75]. Patients with a homozygous TS (2/2) express lower levels of TS, and they have a higher risk of grade 3 and 4 toxicities following 5-FU treatment as compared with patients with TS (3/3) (43% and 3% respectively, $p > 0.01$) [75]. The usefulness of TS testing is currently under investigation.

Uridine diphosphoglucuronosyltransferases (UGTs) are responsible for the glucuronidation of the irinotecan metabolite SN-38, a process enabling the excretion of SN-38 in the bile and urine. Patients with the homozygous polymorphism UGT1A1*28 allele (7/7) have an increased risk for severe neutropenia after receiving irinotecan, especially with the 300–350 mg/m^2 dose regimen [76]. About 10 percent of the North American population has this genotype, which can be assayed in the commercially available kit, Invader UGT1A1 Molecular Assay (Genzyme Corp.). A prospective study involved sixty-six cancer patients who received irinotecan every three weeks; the rate of grade 4 neutropenia was 9.5 percent overall; 50 percent in patients carrying the 7/7 genotype, 12.5 percent in patients carrying the 6/7 genotype, and zero percent in patients with the 6/6 genotype [77]. In 2005, the FDA approved the Invader Assay for use in identifying patients who may be at increased risk of adverse reactions to irinotecan; the FDA also recommended at least one level dose reduction in the starting dose of irinotecan for patients with UGT1A1*28.

Predictive Factors for EGFR Inhibition

Although the monoclonal antibodies cetuximab and panitumumab target EGFR, the EGFR expression level in immunohistochemistry (IHC) detected tumors has not been able to predict tumor response to EGFR inhibitors [52]. Higher EGFR gene copy numbers, as identified by fluorescent in situ hybridization (FISH), may be associated with a better tumor response; however, the data have not been consistent [78–81].

A mutation in the ***K-ras*** oncogene is a negative predictor for tumor response toward EGFR inhibition. It has long been proposed that tumors containing a K-ras mutation activate EGFR downstream of the mitogen-activated protein kinase (MAPK) through the Ras protein directly, so blocking the EGFR pathway in these tumors will have no benefit [82]. A retrospective analysis of eighty-nine patients with mCRC treated with cetuximab showed a 0 percent response rate in patients with a K-ras mutation, compared with a 40 percent response rate in patients with wild-type K-ras ($p < 0.001$) [83]. A similar finding was observed in a panitumumab study. In a large Phase III mCRC trial comparing panitumumab monotherapy with best support care, the K-ras mutation was found in 43 percent of patients. The response rate to panitumumab was 0 percent in the K-ras mutant group and 17 percent in the K-ras wild-type group [84].

The finding that a K-ras mutation predicts anti-EGFR inhibitor resistance holds true in the setting of EGFR inhibitors given in combination with chemotherapy. Based on a retrospective analysis of 540 patients enrolled in the Phase III randomized CRYSTAL trial, in which first-line treatment with FOLFIRI was given with or without cetuximab, patients with tumors harboring a K-ras mutation derived no benefit from the addition of cetuximab to chemotherapy alone (HR 1.07, $p = 0.75$). The benefit of cetuximab, however, is clearly seen in patients without a K-ras mutation (HR 0.68, $p = 0.017$) [67. A similar finding was also reported in the phase II FOLFOX with or without cetuximab (OPUS) trial [85].

Although all these studies are retrospective in nature, the evidence is overwhelmingly convincing that a K-ras mutation confers a high risk for resistance to EGFR inhibitors. Therefore, all protocols sponsored by the National Cancer Institute (NCI) using cetuximab or panitumumab for the treatment of mCRC have been amended to exclude patients with a K-ras mutation.

New Drugs in mCRC Clinical Trials

The ***EGF pathway*** has been investigated intensively for its critical role in cell proliferation, angiogenesis, metastasis, and apoptosis in a variety of tumor types. Two general approaches to targeting the EGF pathway are being studied: (1) blocking the pathway with monoclonal antibodies (mAbs) to EGF receptors, and (2) using small molecules to bind the cytosolic ATP binding site of the EGFR. Interestingly, yet not well explained, mAbs against EGFR have superior clinical efficacy to tyrosine kinase inhibitors (TKIs) for mCRC patients, and the antibody-dependent cellular cytotoxicity (ADCC) was postulated to play an important role in antitumor activity [86]. ***Muthzumab***, a humanized IgG1 EGFR monoclonal antibody, is now in clinical development. ***Nimotuzumab*** is another humanized IgG1 EGFR monoclonal antibody that has been approved in India.

An effective strategy for cancer treatment in general has been to target ***angiogenesis***. The benefit of bevacizumab extends into multiple tumor types, such as lung cancer, breast cancer, and glioblastoma. Understanding of the angiogenesis blockade is also evolving. It was thought that antiangiogenesis therapy blocks the new blood vessel formation that is needed by tumors, but research suggests that instead it normalizes leaky tumor blood vessels, decreasing tumor interstitial pressure and thereby enhancing the delivery of drugs into the tumor [87, 88]. Currently, the soluble decoy receptor for VEGF and placenta growth factor (PIGF) ***VEGF-Trap***, the anti-VEGF antibody ***IMC-1121***,and small molecules including ***sunitinib***, ***sorafinib***, and ***AMG 706*** are being evaluated in clinical trials for mCRC patients.

PI3K/mTOR is a major signaling pathway that regulates cell proliferation, survival, and angiogenesis in cancer. The mammalian target of rapamycin (mTOR) is a downstream effector of the activated PI3K and an attractive therapeutic target [89]. The first oncologic drug among the mTOR inhibitors, temsirolimus, was recently approved by the FDA for metastatic renal cell carcinoma [90]. Currently, temsirolimus and another oral mTOR inhibitor, RAD-001, combined with chemotherapy are being tested in CRC clinical trials.

Interest in the ***insulinlike growth factor*** (***IGF***) pathway has resurfaced over last few years. In general, IGF and insulin bind to the IGF receptor, triggering activation of downstream targets. These targets include the PI3K and MAPK pathways, both of which are involved in cell proliferation and survival [91, 92]. IGF-1 receptors (IGFR), which are frequently expressed in CRC, with their ligands are important for cell proliferation, angiogenesis, and metastasis [93]. Blocking IGF1 inhibits CRC growth and angiogenesis and increases tumor cell apoptosis [94]. It also potentially enhances tumor response to chemoradiation therapy [95]. Small molecules and monoclonal antibodies have been designed to target IGFR, and some of these, such as the human monoclonal IgF1 antibody, IMC-A12, have advanced into early phase CRC clinical trials.

First identified in *Drosophila*, the ***hedgehog*** (Hh) pathway is essential for mammalian gastrointestinal tract development [96] by providing essential signals for gastric gland formation and gastric epithelial differentiation [97]. One of three Hh proteins, sonic Hh, was found to be highly expressed in hyperplastic polyps, adenoma, and adenocarcinoma of the colon [98]; a known Hh inhibitor, ***cyclopamine***, can induce apoptosis in CRC cells [99]. Various approaches have been investigated [100], and ***GDC-0449***, an Hh antagonist, is currently under clinical evaluation with FOLFOX and bevacizumab.

The discovery of ***cancer stem cells*** isolated from acute myelogenous leukemia (AML) in 1994 was a milestone in the field of cancer medicine [101], providing a new paradigm in cancer therapy. A subpopulation of cancer cells are now considered to be stem cells because in xenograft studies only a very small number of cells were required to generate xenograft tumors. The CRC stem cells have been identified as those that express the cell surface markers CD 133 [102, 103], EPCAM(high)/CD44 [104], or Lgr5 [105]. Stem cells are additionally radioresistant and chemoresistant, but further characterization of CRC stem cells is needed to provide insight on the optimal approach for eradication. Targeting interleukin (IL)-4 has been shown to sensitize CRC stem cells to chemotherapy [106]. Another approach, such as targeting Noggin or bone morphogenic protein 4 (BMP4), also yielded an impressive antitumor activity in cell culture and xenograft tumor models. An in-depth understanding of cancer stem cells will be the first step toward the design of curative therapeutic inventions for mCRC patients.

In summary, development of the newer chemotherapies, oxaliplatin and irinotecan, as well as therapies targeting VEGF and EGFR, has provided a significant survival benefit to mCRC patients. Pharmacogenetic testing and further identification of molecular markers that can predict tumor response and treatment-related toxicity will establish the foundation for personalized cancer treatment. With a collaborative effort between basic research scientists and clinicians, advances in the

understanding of tumor biology will be quickly translated into the clinical setting.

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34 Lung Cancer Metastasis

Rafael Rosell, Miquel Taron, and David Jablons

Lung cancer ranks among the most common and most lethal malignancies worldwide. In 2006, approximately 174,470 of an estimated 1,399,790 (12%) new cancer cases, and 162,460 of an estimated 564,830 (28%) total cancer deaths, in the United States were attributable to lung cancer. Similarly, in the 38 countries in Europe, lung cancer accounted for 12 percent of approximately 3.2 million new cancer cases and 19.7 percent of cancer-related deaths.[1] Lung cancer is classified into two major groups: non–small-cell lung cancer (NSCLC), which accounts for 75 percent of all cases, and small-cell lung cancer (SCLC), which accounts for approximately 25 percent. NSCLC is further divided into three histologic subtypes: adenocarcinoma (30%–40%), squamous cell carcinoma (SCC; 20%–25%), and large cell carcinoma (15%–20%).[1] Approximately half of patients with NSCLC already have metastatic (stage IV) disease at the time of diagnosis, and survival times are short regardless of the type of chemotherapy administered.[2]

Several platinum-based chemotherapy regimens are available; they generally yield similar outcomes, with median time to progression of four months and median survival of eight months.[3] Compared with other common primary tumors, such as colorectal and breast cancer, where the median survival is more than twenty months, the prognosis of metastatic NSCLC remains extremely poor.[4] Similar to breast cancer, bone and lung metastases are particularly frequent in NSCLC. However, the different distribution patterns of metastases in lung carcinoma are poorly understood, probably because studies are difficult, given the extremely short survival times and high proportion of patients who have widespread metastases at the time of diagnosis.

PATTERNS OF METASTASIS IN METASTATIC (STAGE IV) NSCLC

Few clinical trials of chemotherapy in stage IV NSCLC provide details of the distribution of metastases. Crawford et al. reported that metastases were seen in the contralateral lung in 26 percent to 28 percent, in bone in 35 percent to 43 percent, in liver in 18 percent to 20 percent, and in the adrenals in 21 percent to 27 percent of patients.[5] In this study, bone metastases were found to be an independent prognostic variable, together with performance status.[5] The most comprehensive analysis of the pattern of metastases is based on the pooled data of 1436 patients with metastatic NSCLC who were treated in two clinical trials.[3,6] Ipsilateral lung metastases were recorded in 67 percent, contralateral lung metastases in 35 percent, bone metastases in 35 percent, liver metastases in 22 percent, pleural involvement in 32 percent, brain metastases in 10 percent, supraclavical nodal involvement in 14 percent, subcutaneous metastases in 4 percent, mediastinum metastases in 53 percent, and metastases in other organs in 32 percent of patients.[7] Subcutaneous and liver metastases and more than four metastatic sites were identified as independent markers of poor prognosis.[7]

Recently, the International Staging Project on Lung Cancer of the International Association for the Study of Lung Cancer (IASLC) evaluated 6596 metastatic NSCLC patients for survival according to the distribution of metastases. Median survival was thirteen months for patients with ipsilateral lung metastases, ten months for those with contralateral lung metastases, eight months for those with pleural dissemination, and six months for those with other distant metastases.[8]

MOLECULAR PATHWAYS IN METASTATIC LUNG CANCER AND IMPLICATIONS FOR THERAPY

Despite the dismal prognosis of NSCLC, recent findings indicate that novel mutations could be present in small subgroups of patients and in some cases may cluster in specific lung adenocarcinoma subtypes.[9,10] These mutations can be targeted; recent findings indicate dramatic responses to specific oral inhibitors in a novel

chromosomal translocation, the EML4-ALK, identified in a subgroup of lung adenocarcinomas.[11,12] For lung cancer patients without targetable mutations, the expression levels of DNA repair genes can be useful to personalize chemotherapy. Recent evidence indicates that the inhibition of poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) is a novel treatment for tumors with DNA repair defects, including BRCA1 or BRCA2 mutations.[13] A clinical trial of customized chemotherapy based on tumor BRCA1 mRNA levels is ongoing (clinicaltrials.gov NCT00617656).

Epidermal Growth Factor Receptor Mutations

Mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR) have been identified as a cause of NSCLC, particularly in adenocarcinoma and bronchoalveolar carcinoma (BAC).[14–18] The most common oncogenic mutations are small in-frame deletions in exon 19 and a point mutation that substitutes leucine 858 with arginine (L858R) in exon 21. These mutations likely cause constitutive activation of the kinase by destabilizing the autoinhibited conformation that is normally maintained in the absence of ligand stimulation.[19] The activating mutations confer dramatic sensitivity to the small-molecule TK inhibitors (TKIs) gefitinib and erlotinib.[14–16] In 165 NSCLC patients with EGFR mutations treated prospectively with erlotinib, overall time to progression was twelve months; however, the time to progression was seven, eleven, and sixteen months in patients with brain, bone, and other metastases, respectively ($P = 0.02$). In the multivariate analysis, the presence of brain metastases, poor performance status, and the L858R mutation emerged as independent markers of poor prognosis.[20]

Interestingly, the distribution of metastases among patients with EGFR mutations is slightly different in BAC than in other histologies. Lung metastases were observed in the majority of BACs, but no brain metastases were seen.[20] In a pilot study of metastatic NSCLC in which patients with EGFR mutations received erlotinib and those without mutations received customized chemotherapy with or without cisplatin based on BRCA1 mRNA levels, a higher incidence of bone, adrenal, lung, and brain metastases was observed in patients with EGFR mutations.[21]

ERCC1 mRNA Expression as a Determinant of Cisplatin Sensitivity

Platinum-based chemotherapy is commonly used in the treatment of metastatic NSCLC. A wealth of data indicates that nucleotide excision repair (NER), a highly versatile pathway for DNA damage removal, is often dysfunctional in NSCLC. NER removes numerous types of DNA helix-distorting lesions, including those induced by platinum compounds.[22,23] NER functions by a so-called cut-and-paste mechanism in which cisplatin damage recognition, local opening of the DNA helix around the lesion, damage excision, and gap filling occur in successive steps through the concerted action of various NER factors.[22] The structure-specific endonuclease excision repair cross-complementing 1 (ERCC1), together with its xeroderma pigmentosum group F (XPF) partner, performs an essential late step in the NER process, where it nicks the damaged DNA strand at the 5′ site of the helix-distorting cisplatin lesion. In addition, the ERCC1/XPF structure-specific nuclease also plays a role in the homologous recombination repair of interstrand crosslinks.[23]

We examined ERCC1 mRNA expression in paraffin-embedded pretreatment tumor specimens from stage IV NSCLC patients treated with cisplatin plus gemcitabine. There were striking differences in survival (15 months for patients with low levels of ERCC1 vs. 5 months for those with high levels) and response rate. Tumor response was higher for tumors with low levels of ERCC1 mRNA than those with high levels (52% vs. 36%), although this difference was not significant.[24] A Phase III trial of customized chemotherapy showed that assessment of ERCC1 mRNA expression in patient tumor tissue was feasible and predicted response to cisplatin-based treatment.[25] The let-7 microRNA (miRNA), a master regulator of gene expression, is highly expressed in normal lung tissue and downregulated in NSCLC. Reduced expression of let-7 in early NSCLC was associated with poor survival.[26,27] In cancer cell lines, the overexpression of let-7 leads to the downregulation of 170 genes, including ERCC1.[28]

Clinical Studies Testing Outcome to Platinum-Based Therapy According to ERCC1 and RRM1 Expression

Ribonucleotide reductase consists of two subunits (RRM1 and RRM2), which are encoded by different genes on separate chromosomes and whose mRNAs are differentially expressed throughout the cell cycle. Increased levels of RRM1 and RRM2 have been observed in tumors and cancer cell lines.[29] Transgenic mice developed lung adenocarcinoma – but not other tumors – in the presence of RRM2 overexpression.[30] In a study of metastatic lung adenocarcinoma patients treated with docetaxel plus gemcitabine, patients with low levels of both RRM1 and RRM2 had a significantly higher response rate (60% vs. 14.2%), time to progression (9.9 vs. 2.3 months), and overall survival (15.4 vs. 3.6 months) than patients with high levels of both genes.[31] Interestingly, downregulation of let-7 miRNA leads to overexpression of RRM1 and RRM2,[28] and RRM1 and RRM2 could be prognostic markers and predictors of gemcitabine response in NSCLC.

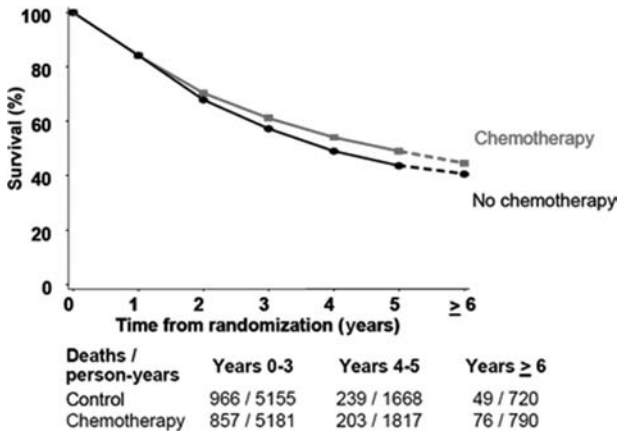

Figure 34.1. Overall survival curves of a meta-analysis of adjuvant chemotherapy in NSCLC.[35]

PATTERNS OF METASTASES IN LOCALLY ADVANCED NSCLC

Locally advanced NSCLC includes patients classified as stage IIIA, mainly because of the presence of metastases in regional mediastinal lymph nodes (N2). For complete details on the definition of tumor size (T), regional lymph node metastases (N), and distant organ metastases (M), see the TNM classification (Mountain[32]). The IASLC Lung Cancer Staging Project has recently proposed a revised TNM classification.[33]

A seminal study of preoperative (neoadjuvant) chemotherapy in locally advanced NSCLC reported a 65 percent complete resection rate among 136 patients, with median survival of twenty-seven months and three- and five-year survival rates of 41 percent and 26 percent, respectively, whereas for patients with incompletely resected or nonresectable cancers, median survival was twelve months, and three- and five-year survival rates were both 5 percent.[34] Of the eighty-nine patients who underwent complete resection, fifty-three relapsed, including eight patients who had attained a complete pathological response without residual tumor. The most frequent sites of first recurrence were the brain (5 patients) and bone (3 patients). Among the remaining forty-five patients with completely resected residual tumors, the most frequent sites of first recurrence were brain only or brain with lung or bone metastases, followed by lung, and – more infrequently – liver or adrenal metastases.[34]

PATTERNS OF METASTASES IN EARLY NSCLC

It is commonly understood that early NSCLCs are relatively small tumors without regional lymph node metastases (T1–2N0M0). In the proposed new TNM stage grouping, stage IB includes tumors under 5 cm in diameter (T2aN0M0). This is the most frequent situation at diagnosis of early NSCLC. (See TNM and stage grouping of subsets in Mountain[32] and Goldstraw et al.[33]) When patients were evaluated according to the proposed new TNM staging, median survival for clinical stage IB was forty-three months, and the five-year survival was 43 percent; median survival for pathological stage IB was eighty-one months, and the five-year survival was 58 percent.[33] These differences between clinical and pathological staging highlight the difficulty of identifying potential micrometastases, as a proportion of patients clinically staged as IB are upstaged after surgery, mainly because of the discovery of mediastinal lymph node metastases at the time of lymph node dissection.

The Lung Adjuvant Cisplatin Evaluation (LACE)[35] performed a pooled analysis of five randomized trials with a total of 4584 patients, including pathological stage I, II (with involvement of hilar lymph nodes – N1) and IIIA (with involvement of mediastinal lymph nodes – N2). The overall hazard ratio (HR) of death was 0.89, which translated to a five-year benefit for adjuvant chemotherapy of 5.4 percent (Figure 34.1). The benefit varied with stage and was more evident for stage II (HR, 0.83) and stage III (HR, 0.83), whereas for patients with early NSCLC, mainly stage IB, the benefit for adjuvant chemotherapy was not clearly demonstrated (HR, 0.93). The benefit – or lack thereof – of adjuvant chemotherapy was clearly shown in one of the randomized trials included in the LACE meta-analysis, in which adjuvant chemotherapy with cisplatin plus vinorelbine conferred improved survival in stage II–IIIA but not in stage IB.[36]

Gene Expression Signatures for Predicting Metastasis and Survival in Early NSCLC

Among completely resected NSCLC patients, 40 percent of stage I, 66 percent of stage II, and 75 percent of stage IIIA patients die within five years of resection,[37] mainly because of the development of distant metastases. The benefit of adjuvant chemotherapy is negligible in stage IB.[35,36] Although at present there are no reliable clinical predictors of relapse after surgery in early-stage NSCLC, transcriptional analysis of primary tumors has identified gene expression profiles strongly related to disease recurrence in adenocarcinoma[38–44] and, to a lesser extent, in SCC.[41–44]

The lung metagene model is a gene expression profile that predicts recurrence in early NSCLC (including stage IA) with an overall accuracy of 72 percent.[44] A meta-analysis[43] of data sets from seven microarray studies[38,40,45,46] identified a 64-gene expression signature that predicted survival with 85 percent accuracy. A study including fifty-one stage I–III lung SCCs identified a 111-gene signature with a 72 percent predictive accuracy for disease recurrence.[47] However, despite the

lack of commonality of many genes identified between the published prognostic signatures, numerous gene expression signatures occupy overlapping prognostic space[48] and may be able to predict outcome in early NSCLC.[49]

The RT-QPCR assay is convenient in terms of laboratory workload and is applicable for large-scale routine use, making it a viable alternative to more complex microarrays. RT-QPCR also allows for accurate and reproducible RNA quantification. The expression pattern of eight genes determined by RT-QPCR correlated with survival in lung adenocarcinoma.[50] Similarly, RT-QPCR–based three-,[51, 52] four-,[53] and five-[54]gene signatures and a five-miRNA signature[55] correlated with metastasis-free survival and overall survival in early NSCLC. A three-gene prognostic model[51] includes a key gene, hypoxia-inducible factor (HIF) 1α. The construction of the small gene signatures developed with RT-QPCR is based on the prognostic value of each gene as determined in a multivariate analysis. Each gene that is significant according to the multivariate analysis is then included in a risk score model, generated by adding the z-scores of the expression levels of each of the genes multiplied by its corresponding coefficient. The risk score is used to classify patients into high or low risk of metastasis and death.[54] The five-gene signature[54] is composed of dual-specificity phosphatase 6 (DUSP6), monocyte-to-macrophage differentiation-associated protein (MMD), signal transducer and activator of transcription (STAT) 1, HER3/neu receptor tyrosine kinase (ERBB3), and lymphocyte-specific protein tyrosine kinase (LCK).

Intriguingly, special AT-rich binding protein 1 (SATB1), originally identified as a protein that recognized double-stranded DNA with a high degree of base-unpairing,[56] is a genome organizer that upregulates metastasis-associated genes, including genes involved in epidermal growth factor (EGF) signaling, such as ERBB1, ERBB2, ERBB3, and ERBB4.[57] In addition, SATB1 upregulates multiple other genes that stimulate invasion and mediate angiogenesis and bone metastasis, such as connective tissue growth factor (CTGF). SATB1 nuclear staining significantly correlated with survival in 985 patients with ductal breast carcinoma stratified by SATB1 expression level.[57] Importantly, because SATB1 tethers multiple genomic loci and regulates chromatin structure and gene expression,[58] the analysis of SATB1 mRNA or protein expression could provide important prognostic information that merits testing in NSCLC in the clinical setting.

Fibronectin is also upregulated by SATB1[57] and has been identified in a six-gene expression signature that predicted survival in diffuse large–B-cell lymphoma.[59] Fibronectin, an extracellular matrix glycoprotein, is highly expressed in tobacco-related lung disease and stimulates lung cancer growth.[60] Median survival of resected SCC patients with low levels of fibronectin mRNA was not reached, whereas it was thirty-one months for those with high levels ($P = 0.002$)[61].

miRNAs are attractive candidates as upstream regulators of metastatic progression because miRNAs can posttranscriptionally regulate entire sets of genes. Quantitative stem-loop PCR of five miRNAs showed that patients with high risk scores in their miRNA signatures had poor overall and disease-free survival, compared with patients with low risk scores.[55] The five-miRNA signature includes two protective miRNAs (let-7a and miR-221) and three miRNAs indicative of poor survival (miR-137, miR-372 and miR-182).[55] Interestingly, miR-335 regulates a set of metastasis genes and has been shown to predict bone and lung metastases in breast cancer.[62]

The wound response signature is composed of 512 genes that define the transcriptional response of fibroblasts to serum, the soluble fraction of clotted blood. In early breast cancer and lung adenocarcinoma, the wound response signature provides prognostic risk stratification of metastasis development and predicts survival in several tumors, including NSCLC.[63] For the sake of practicality, it is important to know that the coordinate amplification of CSN5 (also known as JAB1 or COPS5, residing on 8q13) and MYC (8q24) regulate wound response signature activation in breast cancer. Coexpression of CSN5 with MYC is sufficient to induce the wound response signature.[64] A high expression level of both CSN5 and MYC was a significant predictor of poor patient survival in breast tumors, with efficacy equivalent to that observed for the wound response signature.[64]

The induction of a proteasome signature was associated with an activated wound response signature. The MCF10A breast cancer cell line with the activated wound response signature was more susceptible to death by drugs that inhibit the ubiquitin–proteasome pathway. MCF10A cells expressing MYC or MYC plus CSN5 induced the proteasome signature.[65] CSN5 encodes the catalytic subunit of the COP9 signalosome, a protein complex that regulates cell proliferation, response to extracellular stimuli, cell migration, and DNA damage checkpoints.

The main function of COP9 is to maintain the activity of the multisubunit ubiquitin ligase SCF. CNS5 enhances the cotranscriptional ubiquitination of MYC that activates the transcriptional activity of MYC on a set of target genes promoting cell proliferation, invasion, and angiogenesis. Monomeric CSN5 protein can bind to and modulate the activity of multiple transcription factors and signaling proteins, including interactions with HIF-1α, leading to HIF-1α protein stabilization and increased angiogenic activity.[66]

F-box and WD repeat domain-containing 7 (FBW7) is a component of SCF ubiquitin ligases. FBW7

mediates the ubiquitin-dependent proteolysis of several oncoproteins, including cyclin E, MYC, and Notch.[67] Importantly from the clinical perspective, the SV40 large T antigen oncoprotein binds to and functionally inactivates two major tumor suppressor genes, p53 and retinoblastoma (Rb), that are often inactivated in NSCLC. Large T contains a decoy phospho-degron that inhibits FBW7 function, thus repressing its role in cyclin E, MYC, and Notch.[68]

An integrated gene signature, composed of approximately 150 genes, from multiple transgenic models of epithelial cancers intrinsic to the functions of the Simian virus 40 T/t antigens, has been associated with biological behavior and prognosis. This genetic signature is activated primarily in tumors with aberrant p53, Rb, or BRCA1 expression. Human breast, lung, and prostate tumors expressing this set of genes represent subsets of tumors with the most aggressive phenotype and with poor prognosis.[69] It was observed that SCLC, SCC, and a subset of lung adenocarcinoma harbor the intrinsic T/t-antigen signature.[69] Analysis of the SV40 T/t-antigen signature revealed that BRCA1 is overexpressed in conjunction with a network of genes related to BRCA1 function in breast, lung, and prostate cancers. Furthermore, the T/t-antigen proliferation cluster includes RRM1, which is also a potential target for customizing chemotherapy[31,70,71] and for developing drug therapies targeting RRM1.[69] In addition to repressing the expression of RRM1 and RRM2, let-7 also inhibits BRCA1 expression.[28]

PROGNOSTIC AND PREDICTIVE ROLES OF BRCA1

We performed RT-QPCR in frozen lung cancer tissue specimens from 126 early NSCLC patients who had undergone surgical resection and evaluated the association between survival and expression levels of nine genes involved in DNA repair pathways and in invasion and metastasis. For validation, we used paraffin-embedded specimens from fifty-eight other NSCLC patients. A strong inter-gene correlation was observed between expression levels of all nine genes except nuclear factor of activated T cells (NFAT) – for example, among ERCC1, RRM1, and BRCA1. Along with disease stage (stage I vs II vs III), BRCA1 mRNA expression significantly correlated with overall survival (HR, 1.98; $P = 0.02$). In the independent cohort of fifty-eight patients, BRCA1 mRNA expression also significantly correlated with survival (HR, 2.4; $P = 0.04$).[72] When only stage I patients were examined, median survival was significantly different according to expression levels of ERCC1, MZF1, Twist, and BRCA1.[72] Our findings indicate that although BRCA1 is closely related to ERCC1, RRM1, and other genes, such as MZF1, it stands out as the most significant prognostic marker of relapse. Patients whose tumors had high BRCA1 expression had significantly worse survival and should be candidates for adjuvant chemotherapy.

Intriguingly, in vitro studies have shown that BRCA1 can regulate differential sensitivity to different classes of chemotherapy agents.[73,74] The absence of BRCA1 results in high sensitivity to cisplatin, whereas its presence increases sensitivity to antimicrotubule agents.[73,74] Therefore, it is plausible that patients with the highest expression levels would receive more benefit from antimicrotubule, non–platinum-based chemotherapy.

Along these lines, we have carried out a pilot study of customized adjuvant chemotherapy based on BRCA1 mRNA levels in eighty-eight stage II–IIIA NSCLC patients who underwent complete resection, in which those with the highest expression levels received adjuvant docetaxel and those with the lowest levels received cisplatin-based chemotherapy. The interim analysis shows that recurrence-free survival is similar in both groups. These data support previous findings in stage II–IIIA patients who received neoadjuvant gemcitabine plus cisplatin, when those with the highest BRCA1 levels had a dismal survival of twelve months.[75] The fact that high levels of ERCC1 or RRM1 transcripts conferred a higher risk of relapse[72] provides further evidence for the role of the loss of let-7 in upregulation of ERCC1 and RRM1, as well as BRCA1[28] and for the upregulation of BRCA1 and RRM1 in the SV40 T/t-antigen signature.[69]

Paradoxically, contradictory findings,[76,77] leading to opposed strategies of customizing adjuvant chemotherapy, have reported that the lack of ERCC1 protein implies a higher risk of relapse and a greater sensitivity to cisplatin-based chemotherapy.[76] Nevertheless, the clinical evidence that overexpression of ERCC1, RRM1, and especially BRCA1 confers poor survival in early NSCLC patients indicates the high risk involved in adjuvant chemotherapy. Against the current standard of cisplatin-based chemotherapy, non–cisplatin-based chemotherapy, including antimicrotubule drugs, may be the proper treatment for the majority of patients with a high risk of relapse (Figure 34.2).[72]

BRCA1 Assembly Line in DNA Repair

Experimental findings suggest that both DNA double-strand breaks (DSBs) and a DNA damage response (DDR) can be induced by ionizing radiation,[78] hypoxia,[79] DNA-damaging agents, and activated oncogenes.[80] In precancerous lesions, p53 binding protein 1 (53BP1) localized at foci, and histone H2AX, ataxia telangiectasia (ATM), and checkpoint kinase 2 (Chk2) were phosphorylated, suggesting the presence of DNA DSBs.[81,82] In lung cancer, there is evidence for the presence of DSBs when phosphorylated histone H2AX and 53BP1 foci are present with a high proliferation index

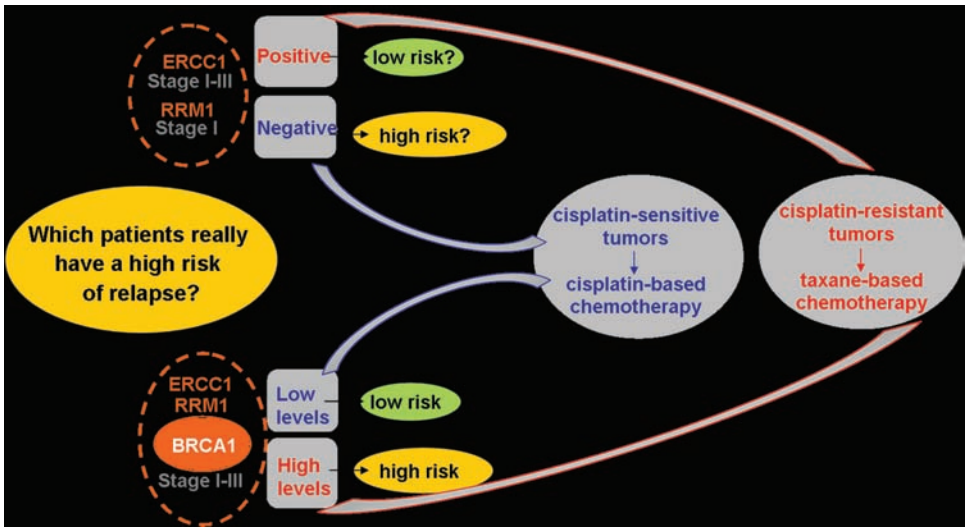

Figure 34.2. A higher risk of relapse in early NSCLC is related to high levels of several transcripts, including ERCC1, RRM1 and, above all, BRCA1 (red circle). The high-risk group could be resistant to cisplatin and sensitive to taxanes or other antimicrotubule drugs.[72]

but low levels of apoptosis.[80,81] More than 50 percent of surgically resected lung cancers show phosphorylation of Chk2.[82] Many proteins, including ATM, γ-H2AX, mediator of DNA damage checkpoint protein (MDC1), BRCA1, Chk1, and Chk2, are involved in the ionizing radiation-induced DDR pathway.[78] Under nonirradiated normoxic conditions, γ-H2AX and 53BP1 are not activated; however, under nonirradiated anoxic conditions, γ-H2AX can be induced through the chromatin.[79] At the core of DDR signaling, ATM is central, activating γ-H2AX. A large-scale proteomic analysis of proteins phosphorylated in DDR identified multiple supercomplexes, including BRCA1, the COP9 signalosome, and the AKT-insulin pathway.[83]

A proposed model for DDR to irradiation involves the formation of a BRCA1 complex.[84,85] In DDR, ATM, and ATR, phosphorylate H2AX on Ser-139,[86] which serves to recruit the MDC1 protein to chromatin, where it is also phosphorylated. Rnf8/Ubc13 complexes go to sites of DNA damage through their forkhead domain and initiate the synthesis of K63 polyubiquitin chains on chromatin that recruit the BRCA1 complex through the ubiquitin-interacting motif domains (UIM) of Rap80[87,88] (Figure 34.3). Rap80 targets a complex containing Abraxas, BRCA1–BARD1 (BRCA1-associated ring domain protein 1), and BRCC36.[84,85,88] BRCC36 is frequently overexpressed in breast cancer, and its depletion disrupts irradiation-induced phosphorylation of BRCA1, thereby sensitizing breast cancer cells to irradiation-induced apoptosis.[89] Cells lacking MDC1 are also sensitive to ionizing irradiation.[90]

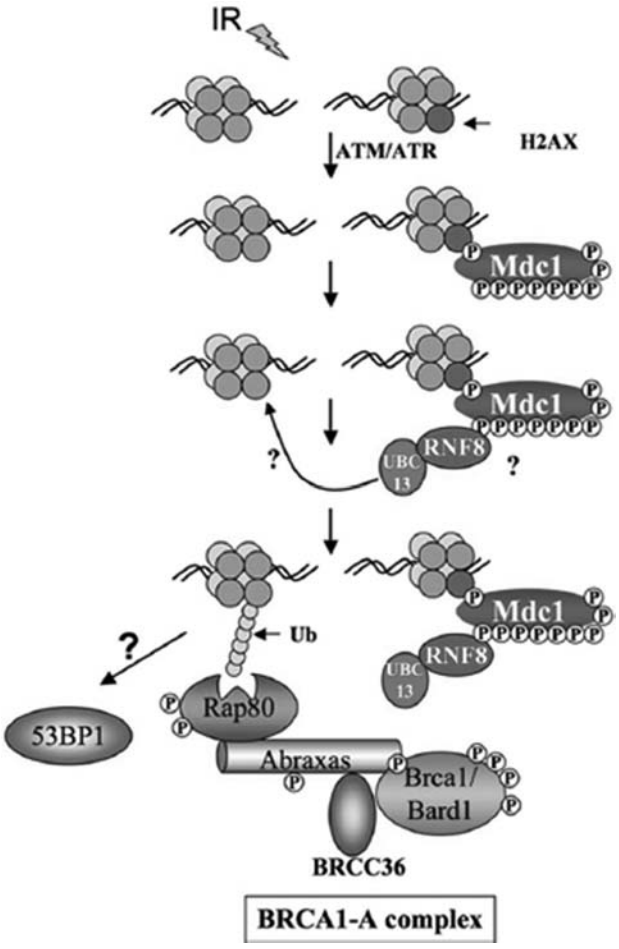

Figure 34.3. A model describing the BRCA1 assembly line in the repair of DNA damage.[87]

TABLE 34.1. Patient characteristics and response to erlotinib in 165 stage IV NSCLC patients with EGFR mutations

	All patients *N* (%)	First-line erlotinib *N* (%)	Second-line erlotinib *N* (%)	*p*
Number of patients	165	89 (53.9)	76 (46.1)	
Age, median (range)	65 (26–88)	66 (26–88)	63 (30–85)	0.09
Gender				0.39
Male	48 (29.1)	23 (25.8)	25 (32.9)	
Female	117 (70.9)	66 (74.2)	51 (67.1)	
Smoking status				0.39
Former	45 (27.3)	24 (27)	21 (27.6)	
Current	8 (4.8)	3 (3.3)	5 (6.6)	
Never	112 (67.9)	62 (69.7)	50 (65.8)	
ECOG PS				0.72
0	45 (27.3)	23 (25.8)	22 (28.9)	
1	97 (58.8)	55 (61.8)	42 (55.3)	
≤2	23 (13.9)	11 (12.4)	12 (15.8)	
Histology				0.29
Adenocarcinoma	124 (75.2)	65 (73)	59 (77.6)	
BAC	23 (13.9)	15 (16.9)	8 (10.5)	
Large-cell carcinoma	16 (9.7)	7 (7.9)	9 (11.8)	
Unspecified	2 (1.2)	2 (2.2)	0 (0)	
EGFR mutation				
Exon 19 del	103 (62.8)	52 (58.4)	51 (68)	0.26
Exon 21 L858R	62 (37.6)	37 (41.6)	25 (32.9)	0.26
Response				0.46
CR	20 (13.2)	11 (13.6)	9 (12.7)	
PR	91 (59.9)	52 (64.2)	39 (54.9)	
CR + PR	111 (73.1)	63 (77.8)	48 (67.6)	
SD	25 (16.4)	11 (13.6)	14 (19.7)	
PD	16 (10.5)	7 (8.6)	9 (12.7)	
NE	13	8	5	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, nonevaluable.

Customizing Chemotherapy Based on BRCA1 Expression in Stage IV NSCLC

Based on the plethora of evidence for the central role of BRCA1 in conferring differential sensitivity to irradiation and DNA-damaging drugs (cisplatin, carboplatin) and to antimicrotubule drugs (paclitaxel, docetaxel, vinorelbine),[73–75] we performed a study of customized treatment, mainly in adenocarcinoma, in which stage IV NSCLC patients with EGFR mutations received erlotinib and those without EGFR mutations were assigned to chemotherapy based on BRCA1 mRNA levels. Those with the lowest levels of BRCA1 received cisplatin plus gemcitabine, those with intermediate levels received cisplatin plus docetaxel, and those with the highest levels received docetaxel alone. Ninety-three patients were included, fifteen with EGFR mutations. More patients with EGFR mutations were female and never-smokers (Table 34.1).

The response rate in patients with EGFR mutations treated with erlotinib was higher than in those without mutations who received customized chemotherapy. Median survival was not reached in patients with EGFR mutations treated with erlotinib or in patients with low levels of BRCA1 treated with cisplatin plus gemcitabine. The multivariate analysis showed that patients with more than one metastatic site and those with higher levels of BRCA1 had shorter survival. These observations are along the lines of preclinical findings that loss of the let-7 miRNA[28] and/or activation of the T/t-antigen signature[69] lead to upregulation of BRCA1. In addition, MYC is required for the induction of BRCA1.[91] We can also speculate that patients whose tumors display the activated wound response signature[63,64] will exhibit high levels of BRCA1 mRNA. As in early NSCLC,[72] high levels of BRCA1 could be an independent prognostic marker in metastatic NSCLC.

The multivariate analysis also showed that levels of Rap80 were an independent prognostic marker. When patients with low levels of BRCA1 mRNA, in whom median survival was not reached, were divided according to their Rap80 mRNA levels, median survival was

not reached in patients with low or intermediate levels of Rap80 but was six months in patients with high levels. This result may be explained by preclinical evidence that overexpression or knockdown of Rap80, respectively, reduces or increases ionizing irradiation-induced cytotoxicity.[92] Rap80 was able to translocate to irradiation-induced foci in HCC1937 cells, which express a truncated BRCA1 that is unable to migrate to nuclear foci.[92]

When these findings are translated to the clinic, they indicate that some patients with low BRCA1 mRNA may have high Rap80 mRNA, as found in our study, and respond poorly to platinum compounds. To validate these findings, a large randomized trial in stage IV NSCLC is being carried out: BRCA1 Expression Customization (BREC; clinicaltrials.gov NCT00617656). Patients are randomized 1:3 to either the control or the experimental arm. Patients in the control arm receive docetaxel plus cisplatin, and those in the experimental arm receive chemotherapy based on their RAP80 and BRCA1 mRNA levels. Those with low levels receive gemcitabine plus cisplatin; those with intermediate levels, docetaxel plus cisplatin; and those with high levels, docetaxel alone.

FUTURE DIRECTIONS

DNA Pathways as Targets for Cancer Therapy

The shift away from the use of nonspecific cytotoxic agents in cancer therapy to more specific, molecularly targeted agents[93] (e.g., bevacizumab, erlotinib, gefitinib, cetuximab) can lead to the development of so-called Phase 0 trials, integrating biomarkers to identify the right target population.[94] Furthermore, novel methods of assessment will look at measures such as time to progression and will refine response criteria by including PET-CT for the adequate evaluation of response.[95,96] Paradoxically, large Phase III trials of molecularly targeted agents have demonstrated a rather limited benefit,[97–102] highlighting the need to identify the proper molecularly defined target population.

There are several ongoing clinical trials of small-molecule inhibitors of DDR and related signaling pathways.[103] ATM and ATR are central to cellular responses to DSBs.[83] When activated, ATM and ATR phosphorylate a multitude of proteins, initiating a cascade that induces cell-cycle arrest and facilitating DNA repair.[87,88] Posttranslational modifications of proteins include the addition of nucleotides, such as poly(ADP-ribosyl)ation. Poly(ADP-ribosyl)ation is critical for a wide range of processes, including DNA repair, regulation of chromosome structure, transcriptional regulation, mitosis, and apoptosis.[104]

Inhibitors of PARP have been tested in inherited breast, ovarian, and prostate cancers with BRCA1 or BRCA2 dysfunction (BRCA1 and BRCA2 mutated cells defective in homologous repair).[13] The recombination-defective tumor cells are 100- to 1000-fold more sensitive to PARP inhibitors than are the heterozygote or the wild-type cell lines, indicating their potential to be exploited as specific treatment of BRCA1- or BRCA2-defective tumors.[105,106] PARP inhibitors induce single-strand breaks that can result in DSBs as a consequence of stalled replication forks. Such lesions would normally be repaired by homologous recombination, but this is abrogated in BRCA1- or BRCA2-deficient cancer cells. The inhibition of PARP leads to the persistence of these DNA lesions.[105]

Resistance to PARP inhibitors has been demonstrated to be caused by acquired mutations in BRCA2.[107,108] PARP inhibitor-resistant clones were resistant to cisplatin but not to antimicrotubule drugs such as docetaxel[107] in tumors with acquired new BRCA2 mutations, because the secondary mutation in the recurrent tumor restored the wild-type BRCA2 reading frame.[108] Theoretically, it could be possible to resensitize these tumors to cisplatin and to PARP inhibitors by treatment with proteasome drug inhibitors that block RAD51 recruitment to sites of DNA repair.[109] Intriguingly, the wound response signature[64] merges with the proteasome signature[65] and could thus be predictive of resistance to PARP inhibitors.

The Wnt signaling pathway has recently emerged as an important target in the development of novel cancer therapeutics.[110] In the past five years, aberrant activation of the Wnt signaling pathway has been shown in the pathogenesis of NSCLC. For instance, increased expression of upstream Wnt signaling proteins (e.g., Wnt ligands and Dishevelled) and methylation silencing of the genes encoding Wnt antagonists have been demonstrated in NSCLC.[111–114] The activation of the Wnt signaling pathway in NSCLC is at least partly due to the epigenetic gene silencing of Wnt antagonists, such as the Wnt inhibitory factor and secreted frizzled-related protein (SFRP) families.[113,114]

Two classes of novel cancer therapeutics based on Wnt pathway inhibition are currently under preclinical study. The first class is the biological Wnt inhibitors, including some of the natural Wnt antagonists that bind Wnt ligands and block Wnt pathway activation at the cell surface.[115,116] For instance, Wnt inhibitory factor inhibits tumor growth in a lung cancer xenograft model and a soluble Wnt receptor Frizzled8CRD-hFc inhibits the growth of MMTV-Wnt1 tumors.[115,116] The second class of Wnt inhibitors for drug development is small molecules that disrupt protein–protein interactions in the Wnt canonical pathway – for example, two small-molecule Wnt inhibitors showed specificity and also activity in xenograft tumor models.[117,118] One small-molecule inhibitor, FJ9, based on rational design, disrupted the interactions between Frizzled7 and the

TABLE 34.2. Potential biomarkers for customizing treatment

Progression	Longer survival	Shorter survival	Shortest survival
T/t antigen signature	Low BRCA1 mRNA	Intermediate BRCA1 mRNA	High BRCA1 mRNA
Wound response signature	Low MYC and CSN5 mRNA	Intermediate MYC & CSN5 mRNA	High MYC and CSN5 mRNA
Proteasome signature	High BIM mRNA	Intermediate BIM mRNA	Low BIM mRNA
HSP90 mRNA	Low	High	High
miR-let- 7	miR-let-7	Low miR-let-7	Low miR-let-7
EGFR mutations	L858R // exon 19del	H1650 (exon 19del)	H1975 (T790M)
FBW7 ubiquitin ligase	FBW7 intact	FBW7 loss	FBW7 loss
Drug response	cisplatin +++ antimicrotubules – antiangiogenic – erlotinib +++	cisplatin + antimicrotubules ++ antiangiogenic + erlotinib +	cisplatin – antimicrotubules +++ antiangiogenic +++ erlotinib –

PDZ domain of Dishevelled.[117] Another small-molecule inhibitor, ICG-001, which disrupts the interaction of β-catenin with a transcription co-activator CBP, was identified through cell-based high-throughput screening.[118] In addition, some existing drugs, such as nonsteroidal antiinflammatory drugs, may have the potential to be adapted as Wnt pathway therapeutics.[119]

A complete review of antiangiogenic drugs and multiple targeted therapies[103,120–122] is beyond the scope of this chapter. However, insights into the many signaling pathways provide a basis for identifying subgroups of NSCLC patients who can obtain maximum benefit from these treatments.[83,123] The customization of chemotherapy in NSCLC is feasible,[25] and further endeavors are warranted to design clinical trials of individualized treatment. BRCA1 complexes are central to DDR,[83,87,88] and BRCA1 could be the key marker for customizing platinum- versus antimicrotubule-based treatment[73–75] (Table 34.2). BRCA1 may also be a prognostic marker.[28,69,72] Interestingly, MYC is required for the induction of BRCA1,[91] and the wound response signature is regulated by MYC and CSN5.[64] MYC and CSN5 also lead to invasion and angiogenesis. We therefore hypothesize that the wound response signature is closely related to BRCA1 mRNA expression and angiogenesis (Table 34.2).

One important clinical caveat to bear in mind when developing new trials of targeted therapies is the importance of classifying stage IV NSCLC patients according to the pattern of metastases, as different metastatic sites could have different expression levels of genes involved in response to chemotherapy or metastasis development.[124] For example, in addition to striking differences in survival according to the metastatic site,[8] bone metastases could display overexpression of angiogenic genes, such as CTGF and others,[124] which are ultimately regulated by SATB1. SATB1 is also involved in the regulation of MYC expression.[56] Therefore, in clinical studies of antiangiogenic drugs, we can hypothesize that response will be better in patients with bone and liver metastases with higher levels of BRCA1, than in patients with more common lung metastases.

We do not deny that there are uncountable target genes for angiogenesis and hubs of signaling pathways; however, in spite of the differences in the genes involved, gene signatures often occupy overlapping prognostic space,[48] facilitating the clinical work of identifying subgroups of patients at the prognostic and predictive level. Further details on the clinical management of NSCLC, shown in Table 34.2, have been described earlier in the chapter.

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35 Metastatic Thyroid Cancer: Evaluation and Treatment

R. Michael Tuttle

Appropriate surgical resection of the primary tumor and involved locoregional lymph nodes remains the mainstay of therapy for patients with thyroid cancer. Depending on the specific tumor histology and the estimated risk of recurrence/death, additional adjuvant therapies, such as radioactive iodine, levothyroxine suppression, or external beam irradiation, are often recommended. With these treatments, most patients with thyroid cancer can expect more than 90 percent disease-specific survival rates over a thirty-year follow-up period.

However, there is a subset of thyroid cancer patients with RAI refractory disease that can have survival rates as low as 30 percent over three to five years. Unfortunately, radioactive iodine (RAI)-refractory metastatic thyroid cancer has proven to be quite resistant to traditional cytotoxic chemotherapy. However, recent advances in our understanding of the molecular biology of thyroid cancer coupled with the availability of relatively specific tyrosine kinase inhibitors has dramatically changed our approach to the management of RAI-refractory, structurally progressive thyroid cancer.

In this chapter we review the initial presentation, risk stratification, and therapy of thyroid cancer with a specific emphasis on novel systemic therapy options for patients with structurally progressive disease that cannot be controlled with either local measures or RAI therapy.

PATTERNS OF METASTATIC SPREAD, ORGAN SPECIFICITY, TIMING OF RECURRENT DISEASE, AND COMPLICATIONS CONFRONTED WITH METASTASIS

Thyroid cancers arise either from thyroid follicular cells (papillary, follicular, and anaplastic thyroid cancers) or from other cells within the thyroid gland, such as lymphocytes (primary thyroid lymphoma) or neuroendocrine c-cells (medullary thyroid cancer). In most large studies, thyroid cancers arising from thyroid follicular cells account for 90 percent to 95 percent of all primary thyroid cancers. Of the thyroid cancers originating in thyroid follicular cells, papillary thyroid cancer accounts for the vast majority of cases (more than 90%), with the balance including follicular thyroid cancers (5%–8%) and anaplastic thyroid cancers (1%–2%). Rarely, malignancies such as renal cell carcinoma, lung cancer, breast cancer, or melanoma can metastasize to the thyroid gland, but this occurs usually only as part of widespread disseminated metastatic disease.

The biologic behavior of thyroid cancer ranges from a clinically insignificant discovery in 10 percent to 15 percent of elderly thyroid autopsy specimens to a life-threatening disease with less than a 50 percent five-year survival rate in older patients presenting with distant metastasis [1]. Fortunately, most patients present with low- to intermediate-risk disease; therefore, ten-year survival rates are usually in excess of 98 percent, with forty-year survival rates greater than 85 percent to 90 percent in most studies [2].

Despite this relatively low disease-specific mortality rate, the risk of recurrent disease over a thirty- to forty-year follow-up period can be as high as 20 percent to 30 percent [2, 3]. In an Ohio State follow-up cohort, 24 percent of the patients had a clinically evident recurrence at a median of seventeen years of follow-up [2]. Of these patients, 18 percent had local recurrence in the neck (74% lymph node metastasis, 20% thyroid bed recurrence, and 6% recurrence in neck muscle or trachea) and 8 percent had recurrence in sites outside the neck (distant metastases). Similar data from the Mayo Clinic demonstrate a 14 percent recurrence rate over a forty-year follow-up period, with similar excellent overall disease-specific survival rates [4].

At the time of diagnosis, thyroid cancer appears to be localized within the thyroid in approximately 59 percent of cases, with clinically apparent spread to regional lymph nodes in 34 percent of cases and spread outside

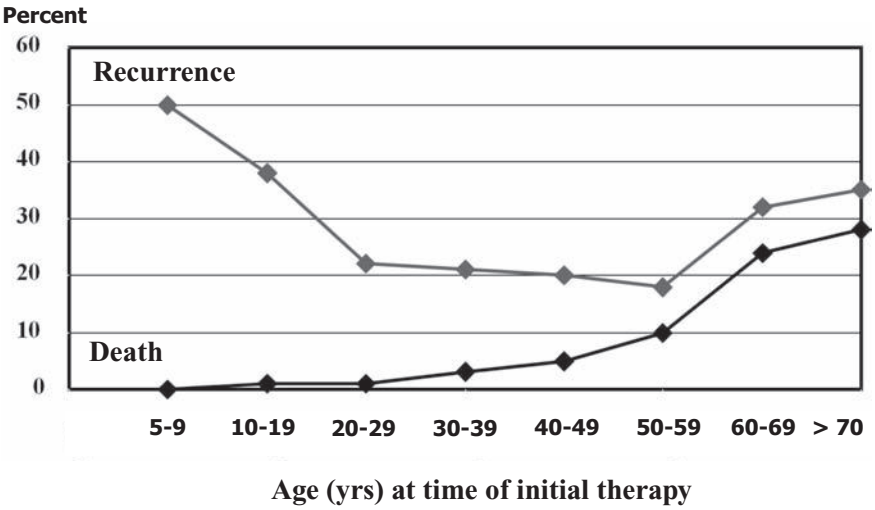

Figure 35.1. The risk of recurrence is high in the very young and the elderly. Despite a high risk of recurrence, disease-specific mortality is quite low in young patients (redrawn from Mazzaferri et al. [2]).

of the neck as distant metastases in 5 percent. These registry data are consistent with retrospective cohort studies showing clinically apparent spread to regional lymph nodes in 20 percent to 50 percent of patients who have undergone routine clinical examination [2, 5–8]. However, when meticulous neck dissections are done, 70 percent to 80 percent of patients may have microscopic lymph node metastases at diagnosis [9, 10]. Fortunately, distant metastasis at diagnosis is found in only 2 percent to 5 percent of cases, with a strong predilection to lung parenchyma as the primary site of distant spread (80%–85%) followed much less commonly by bone (5%–10%) and brain (1%) [2, 3, 8, 11].

The primary complications associated with locoregional recurrence are related to local invasion and compression of critical structures in the neck (trachea, esophagus, major vessels, and recurrent laryngeal nerves). Complications associated with distant metastases are often related to local compressive symptoms, such as postobstructive pneumonia, hemoptysis from pulmonary lesions, or direct impingement either on major vessels (superior vena cava syndrome) or on nervous systems structures (spinal cord compression, nerve root compression, or central nervous system metastases). Metastatic lesions to bone also predispose to pathologic fractures, which are not usually life-threatening but do often significantly affect quality of life.

Although most clinically evident recurrences can be treated adequately with additional surgery, RAI, or external beam irradiation, as many as 8 percent of patients with local recurrence and up to 50 percent of patients with distant metastatic recurrence will die of thyroid cancer [2]. The more sensitive follow-up testing currently used will enable the early detection of small-volume recurrent disease that may lead to specialized treatments, resulting in lower mortality rates.

STATE-OF-THE-ART DIAGNOSTIC/PROGNOSTIC TESTING

In many cancers, the risk of recurrence is tightly linked to the risk of disease-specific survival. In older thyroid cancer patients (more than 45 years of age at diagnosis), the risk of recurrence does parallel the risk of death from thyroid cancer (see Figure 35.1) [2]. However, long-term studies have shown that although the disease-specific mortality in patients less than 45 years of age at diagnosis is less than 1 percent to 2 percent, the risk of recurrence is as high as 30 percent. Therefore, staging systems designed to predict disease-specific mortality in young patients can significantly underestimate the risk of recurrence. To address this important clinical issue, we use a simple set of clinical–pathologic features to risk-stratify patients into low (classic papillary thyroid cancer, confined to the thyroid without vascular invasion or extrathyroidal extension), intermediate (microscopic extrathyroidal extension, regional lymph node metastases, aggressive histology or vascular invasion) or high (macroscopic gross extrathyroidal extension, incomplete primary tumor resection or distant metastases) risk of recurrence [12].

Once the diagnosis of thyroid cancer has been established (usually on the basis of thyroid fine-needle aspiration of an asymptomatic thyroid nodule), initial staging relies primarily on a high-quality preoperative neck ultrasound [13]. The goal of this neck ultrasound is not only to document the nodularity within the thyroid gland, but also, more importantly, to search for abnormal cervical lymph nodes that should be removed in

a compartment-oriented dissection at the time of the total thyroidectomy. Usually a chest radiograph is performed preoperatively as well. Unless there is a high clinical suspicion for distant metastases, no other cross-sectional imaging is routinely recommended.

Postoperatively, staging is usually facilitated by the measurement of serum thyroglobulin (thyroid–cancer-specific tumor marker) and by the use of RAI whole-body scans [14, 15]. Because most thyroid cancers continue to secrete thyroglobulin and to concentrate RAI through an intact sodium iodine symporter, these two tests can be used to identify persistent/metastatic disease. If the patient is at high risk for persistent disease (tumors larger than 4 cm, poorly differentiated thyroid cancer, or elevated thyroglobulin without identifiable disease on RAI scanning), additional cross-sectional imaging of the head, neck, chest, abdomen, and pelvis may be warranted, as well as 18-FDG PET scanning [13]. Because most young patients with persistent thyroid cancer have well-differentiated thyroid cancer that is usually of very small volume, 18-FDG PET scanning is seldom helpful in their follow-up. However, in older patients with non-RAI avid, poorly differentiated thyroid cancers, 18-FDG PET is important in determining the location of the disease and its prognostic characteristics [16].

Because medullary thyroid cancer arises from c-cells and not from thyroid follicular cells, the tumor markers used are calcitonin and CEA (not thyroglobulin). Additionally, RAI scanning is not helpful, as c-cells (and medullary thyroid cancer cells) do not concentrate RAI. Therefore, cross-sectional imaging from the neck through the pelvis is usually recommended for all but the smallest medullary thyroid cancers confined to the thyroid [17].

BIOLOGIC TARGETING OF CURRENT DRUGS, SPECIFIC GENES/RECEPTORS

Systemic Therapy Options

Traditionally, doxorubicin (60–75 mg/m^2 every three weeks, or 15–20 mg/m^2 weekly) administered either as a single agent or combined with other cytotoxic chemotherapy has been the standard recommendation for RAI-refractory progressive thyroid cancer that cannot be adequately treated with surgical resection or external beam irradiation [18]. However, because of the toxicity profile and the lack of clinically meaningful durable responses, traditional chemotherapies are seldom used in clinical practice. In addition, the ATA [13] and the NCCN [19] guidelines do not require that patients fail doxorubicin chemotherapy prior to their enrollment in clinical trials. Based on several Phase II clinical trials (reviewed later in this chapter), both the ATA and the NCCN guidelines note that commercially available tyrosine kinase inhibitors such as sorafenib (400 mg twice daily or sunitinib 50 mg once daily for four weeks of a six-week treatment cycle) can be considered for RAI-refractory thyroid cancer even though neither agent is FDA-approved for the treatment of thyroid cancer at this time.

For medullary thyroid cancer, both the NCCN [20] and the ATA [21] guidelines recommend clinical trials as the preferred treatment option for structurally progressive metastatic disease. Alternative treatment options recommended by the NCCN include a dacarbazine-based first-line therapy (such as dacarbazine plus 5-fluorouracil) or off-label use of sorafenib (400 mg twice daily) or sunitinib (50 mg once daily for four weeks of a six-week treatment cycle) [20].

Recent Clinical Trials Using Targeted Therapies

Over the past several years, our understanding of the pathophysiology of thyroid cancer has increased dramatically [22, 23]. It is becoming increasingly apparent that the tyrosine kinase/MAP kinase pathway that is being targeted in many other solid tumors is also quite important in the initiation and progression of thyroid cancer. The discovery of nonoverlapping mutations in ret/PTC, ras, and BRAF in approximately 70 percent of differentiated thyroid cancers further emphasizes the central role of the receptor tyrosine kinase/MAP kinase pathway in the pathophysiology of differentiated thyroid cancer. Furthermore, as in many other solid tumors, angiogenesis appears to play a key role in tumor growth, making inhibition of the vascular endothelial growth factor tyrosine kinase receptor pathway a primary target for many experimental therapies [24]. Other studies have demonstrated that epigenetic modifiers of gene expression (e.g., DNA methylation and histone acetylation) appear to have an important role not only in the functional aspects of thyroid physiology (e.g., expression of sodium iodine symporter) but also in cell cycle regulation [25].

Over the past ten years, at least nine Phase II clinical trials have been published that have examined the therapeutic effects of a wide variety of antineoplastic agents on structurally measurable disease in patients with RAI-refractory differentiated thyroid cancer [26]. The majority of the trials were composed of primarily differentiated thyroid cancer patients (papillary thyroid cancer, follicular variants of papillary thyroid cancer, follicular thyroid cancer, and other aggressive variants including poorly differentiated thyroid cancer), although the studies often included an exploratory arm that allowed enrollment of patients with medullary thyroid cancer or anaplastic thyroid cancer.

All the trials required structurally identifiable disease (usually at least one lesion greater than 1 cm in diameter), but the studies varied widely in the

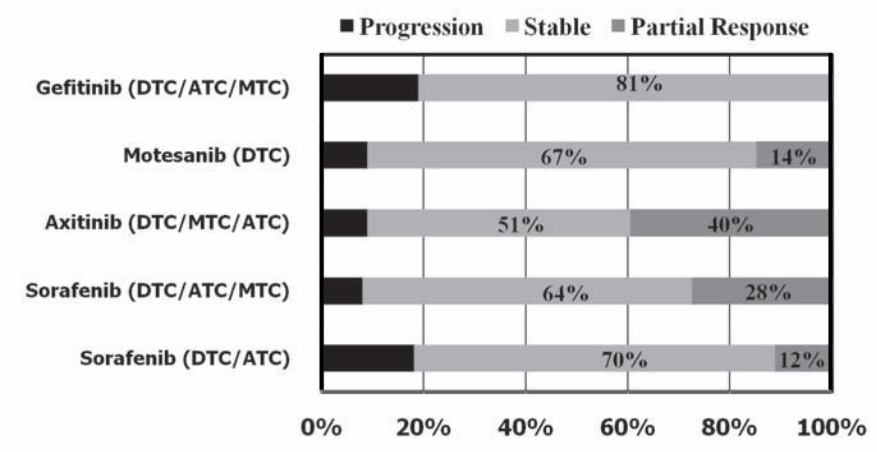

Figure 35.2. Stable disease was the most common response seen in each of the Phase II trials of oral tyrosine kinase inhibitors. Although none of the studies documented a complete response, partial responses by RECIST criteria were seen in a significant number of patients with each agent [27–31].

requirement and definition of disease progression prior to entry. Agents studied included paclitaxel, celecoxib, thalidomide, vorinostat, doxorubicin with interferon, gefitinib, motesanib, sorafenib, and axitinib. Only one complete response was seen in the 335 thyroid cancer patients enrolled in the nine trials. Partial responses (at least a 30% decrease in the longest diameter of a single tumor or the sum of longest diameters of multiple target lesions confirmed at four weeks) were more common, and ranged from 3 percent to 47 percent in the individual studies (see Figure 35.2 for the results of the Phase II trials evaluating oral tyrosine kinase inhibitors). However, a careful analysis of the waterfall plots (best obtained response expressed as percent change) demonstrates that most patients in the motesanib, axitinib, and sorafenib studies had some degree of tumor shrinkage as the best obtained response, even though the decrease usually did not reach the 30 percent decrease required for classification as a partial response [26].

In medullary thyroid cancer, specific agents that inhibit the RET tyrosine kinase are likely to be particularly effective. A point mutation in the RET gene is seen in the germline in hereditary medullary thyroid cancer (multiple endocrine neoplasia 2 and familial medullary thyroid cancer) and in the tumor itself in many sporadic medullary thyroid cancers. Not surprisingly, receptor tyrosine kinase inhibitors directed at the RET gene have had promising results in medullary thyroid cancer [17].

Although the precise rates of partial response and durable stable disease rates remain to be precisely defined for each of these agents, it is very clear that these novel agents are significantly more active than the traditional doxorubicin-based cytotoxic chemotherapy approaches. Unfortunately, it appears that these agents are more often cytostatic rather than tumoricidal, as the rates of tumor stabilization far exceed the rates of partial or complete tumor responses. Therefore, the most common responses that can be expected when patients are enrolled in these Phase II trials are either stabilization of disease or minor decreases in tumor size that do not meet the definition of a partial response.

FUTURE DIRECTIONS, PRIMARY NEEDS

Although a dramatic improvement has been seen in the past ten years in the effectiveness of systemic therapies in RAI-refractory thyroid cancer, more research is needed. Even though the Phase II trials of the various tyrosine kinase inhibitors demonstrated significant growth inhibition, none appears to have had a significant tumoricidal effect that may be expected to result in tumor regression or cure. Therefore, the primary emphasis in the management of metastatic, RAI-refractory thyroid cancer should be focused either on the development of novel agents that are likely to be tumoricidal or on the combination therapies that take advantage of the growth-inhibitor effects of the tyrosine kinase inhibitors while adding additional agents to improve cell killing. Like most solid tumors, it is likely that combination therapy (often multimodality) will be required to produce meaningful tumor reduction, and possible cure, in patients with aggressive metastatic RAI-refractory thyroid cancers.

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36 Metastatic Renal Cell Carcinoma

Jean-Jacques Patard, Stéphane Culine, and Alain Ravaud

Renal cell carcinoma (RCC) accounts for 2 percent of all cancers [1]. In Europe, 40,000 patients are diagnosed with RCC each year, leading to 20,000 deaths [2].

One-third of patients are initially diagnosed with locally invasive or stage IV disease [3]. Recurrence occurs in 25 percent of patients having surgical resection for localized disease with a curative intent [4]. The prognosis for patients with distant disease is poor, with a five-year survival rate of 10 percent or less [5].

A major breakthrough has recently occurred in the knowledge of the genetics and transduction pathways involved in RCC [6]. Novel targeted therapies directed against angiogenesis and mammalian target of rapamycin (mTOR) pathway are revolutionizing the treatment of metastatic RCC (mRCC).

This review covers the key molecular pathways and provides the latest data likely to modify current practice [6].

KEY MOLECULAR PATHWAYS FOR THERAPEUTIC TARGETING

A major breakthrough was obtained with recognition of the importance of the hypoxia-driven pathway involving hypoxia-inducible factor (HIF) and related knowledge on angiogenesis with vascular endothelial growth factor (VEGF). Furthermore, new insights on mechanisms of disease resistance in the HIF/VEGF pathway have led to the consideration of alternative pathways. The mTOR pathway seems to be an important primary or alternative pathway in RCC.

Hypoxia-Induced Pathway

Similar to other deprivation factors, hypoxia may affect cell growth. In normoxia, the subunit alpha of HIF (HIFα) is hydroxylated by a Von Hippel–Lindau protein (pVHL) complex unit and degraded through the proteasome [7]. In this scenario, there is no activation of the subsequent transcriptional events leading to the production of growth factors induced by hypoxia [8].

On the other hand, during hypoxia, HIF hydroxylation is inhibited. Depending on the level of oxygen deprivation, unhydroxylated HIF accumulates and no longer binds to pVHL. HIF1α is therefore stabilized by dimerization with the constitutively expressed HIF1β subunit and translocates to the nucleus. The HIF1α and HIF1β complex binds to hypoxia-inducible gene promoters, including the main growth factor genes implicated in angiogenesis, pH regulation, glucose transport, glycolysis, cell cycle, homing, and apoptosis [9]. HIF1α accumulates and may be detected in primary tumors and in metastatic sites (see Figure 36.1).

Hypoxia-Induced Pathway and RCC

RCC is composed of three major histological subtypes, including clear-cell, papillary, and chromophobe carcinomas. Clear-cell RCC is an example of the involvement of the HIF pathway in tumor proliferation and growth. Von Hippel–Lindau disease has been linked to inactivation of the tumor suppressor gene VHL and a defective pVHL, enabling the appearance of multiple tumors, including RCC [10].

In all patients with Von Hippel–Lindau disease and the majority of patients with sporadic RCC, both VHL gene alleles have been lost or inactivated. The consequences of a defective pVHL on HIF stabilization are similar to those of hypoxia. Genetic events leading to the inactivation of the VHL suppressor gene are known to induce accumulation of HIF-1α in the absence of hypoxia [11]. This activates the genes induced by the accumulation of HIF1α and HIF1β, with the production of increasing levels of VEGF and platelet-derived growth factor (PDGF) [7]. The significance of pVHL in RCC growth has been shown in RCC tumor mouse xenografts with pVHL-defective tumor cells, in which introduction of pVHL abolished tumor growth [12].

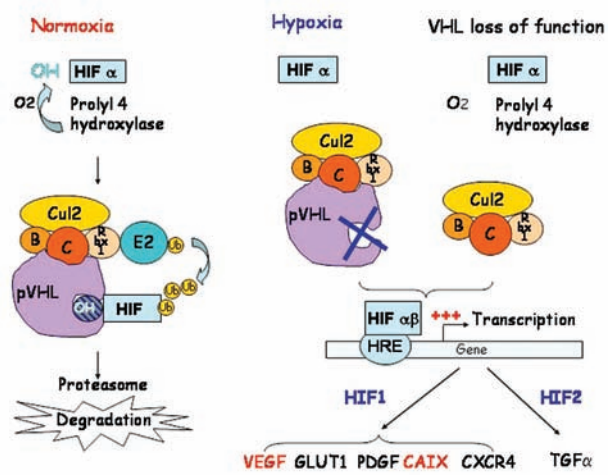

Figure 36.1. Hypoxia/VHL/HIF pathway in renal cell carcinoma.

Furthermore, by expressing an HIF variant or an HIF-derived peptide in RCC tumor cells, it was possible to avoid hydroxylation and RCC tumors were able to grow in mouse xenograft models [13]. The involvement of HIF is at present considered mandatory for the onset of RCC.

Furthermore, even if expression of the HIF1α protein is higher in clear-cell histology compared with non–clear-cell histology, a higher expression of HIF1α has been associated with a better outcome [14]. On the other hand, in sporadic RCC, the presence of a VHL mutation is considered an independent predictive factor for better disease-free survival and survival in RCC treated by nephrectomy, but not in mRCC [15]. In some studies, VHL mutations were more frequent in small low-stage or low-grade tumors [16].

Consequences of Hypoxia-Induced Pathway Involvement and RCC

In RCC, as in many other types of malignancy, a critical breakthrough in understanding the pathophysiology was the discovery of the link between the HIF pathway and angiogenesis.

VEGF production by induction of the HIF pathway is indeed a recent discovery in RCC. VEGF acts by binding to the VEGF receptors (VEFGR-1–3). VEGFR-2 is the main receptor for inducing the effects of VEGF activation. Activation of the VEGFR-2 signaling pathway leads to induction of metalloproteinases; increased vascular permeability; activation of endothelial cells, leading to their proliferation; and activation of anti-apoptotic events in endothelial cells and endothelial progenitor cells. Studies have shown that VEGF is overexpressed in RCC, which seems to be an adverse prognostic factor for survival in patients with mRCC [17].

PDGF is another peptide produced by the HIF-dependent gene. PDGF targets vascular pericytes and facilitates the modeling of tumor vessels. Transforming growth factor alpha (TGF-α) is a ligand for epidermal growth factor receptor (EGFR) and activates the EGFR pathway. In RCC, altered expression of TGF-α is associated with tumor development. The glucose transporter GLUT-1 is overexpressed in RCC and is implicated in the development or progression of RCC progression, as a low level of expression in tumor microarrays was associated with a trend for better survival in clear-cell and papillary tumors. pH regulation has been a special focus in RCC, showing that a decreased level of carbon anhydrase IX (CAIX) was correlated with a worse outcome in MRCC [53, 54].

mTOR Pathway

The mTOR pathway is implicated in angiogenesis and in the phosphatidylinositol-3 kinase (PI-3 kinase) pathway [18]. mTOR regulates the translation of eukaryotic translation initiation factor 4E binding protein (4EBP1) and ribosomal S6 kinase 1 (S6K1) [19]. The mTOR pathway is directly under the control of the PI-3 kinase pathway with downstream events but also acts in the regulation of this pathway. mTOR is considered to promote angiogenesis in RCC by VEGF production and by facilitating proliferation of endothelial cells through Akt activation and antiapoptotic mechanisms.

PI-3 kinase stimulates the activation of the kinase Akt, which inhibits the tuberous sclerosis complex TSC1 and TSC2 proteins turning to activation of mTOR. Moreover, mTOR is able to inhibit the PI-3kinase/Akt pathway.

The mTOR pathway has been reported to be more significantly altered in clear-cell RCC, high-grade tumors, and tumors with poor prognostic features [20].

Phosphatase and tensin homolog deleted on the chromosome Ten (PTEN) tumor suppressor gene is frequently mutated or deleted in a wide variety of solid tumors; these cancers are generally more aggressive. PTEN negatively controls Akt activity. Exploration of protein expression of PTEN in renal carcinogenesis has shown that PTEN is highly expressed in normal renal tissue specimens, whereas in RCC its expression is reduced to less than 10 percent compared with normal tissue [20].

EGFR Pathway

EGFR and its ligands, EGF and TGF-α, are overexpressed in RCC. Analysis has shown an overexpression of EGFR in RCC. Adding EGF to RCC cell lines increases tumor invasion and motility. When EFGR pathway inhibitors are added to RCC tumor cell lines

or given to mouse models, tumor angiogenesis is inhibited.

VEGF INHIBITION

A tremendous step forward in the therapeutic management of mRCC has been achieved with antiangiogenics [6, 21].

Sunitinib

Sunitinib is an orally administered tyrosine kinase inhibitor of multiple targets, especially VEGFR-1, 2, and 3 and PDGFR-α and β. Two Phase II trials in patients with cytokine-refractory mRCC showed an objective response (OR) rate higher than 35 percent and a prolonged progression-free survival (PFS) compared with previous results [22, 23]. A Phase III study comparing sunitinib with interferon (IFN)-α as first-line therapy was planned [24]. Seven hundred patients with mRCC were randomized to receive sunitinib, at a dose of 50 mg/day for four weeks every six weeks, or IFN-α. The median PFS was eleven months in the sunitinib group and five months in the IFN-α group (hazard ratio [HR]: 0.42, 95% confidence interval [CI]: 0.32–0.54; $p < 0.001$). The OR was 31 percent in the sunitinib group (95% CI: 26–36) and 6 percent for the IFN-α arm (95% CI: 4–9; $p < 0.001$). Based on these results, sunitinib is now a new reference standard for first-line treatment of clear-cell mRCC [21, 25].

Recently, survival data have became available (Motzer et al, 2009) [26]. Median overall survival (OS) was 26.4 and 21.8 months in the sunitinib and in the IFN-α arms, respectively (HR: 0.82, 95% CI:0.67–1.00, $p = 0.05$). When patients who crossed over from the IFN-α arm to the sunitinib arm were excluded ($n = 25$), median survival times were 26.4 versus 20.0 months for sunitinib versus IFN-α treatment arms, respectively (HR: 0.8, 95% CI: 0.66–0.98, $p = 0.03$). Finally, when considering OS in patients who did not receive any post-study treatment, median OS reached 28.1 months for sunitinib-treated patients, compared with 14.1 months in patients who received only IFN-α (HR: 0.64, 95% CI: 0.48–0.87, $p = 0.003$).

An analysis of prognostic factors showed that the benefit of sunitinib extends across all prognostic risk factor groups [27]. In patients with favorable, intermediate, and poor risk outcomes, the median PFSs were fourteen, nine, and four months with sunitinib, respectively, compared with eight, four, and one months with IFN-α. Furthermore, in a retrospective analysis, sunitinib exposure, measured by the steady-state area under the curve, had an influence on efficacy and varied by fourfold for a fixed dose [28].

The continuous administration of sunitinib at 37.5 mg/day has been assessed. In a Phase II trial, 21 (20%) and 56 (52%) of 107 patients had a partial response or a stable disease, respectively [29]. The median PFS was thirty-six weeks. An ongoing trial is comparing the two schedules of administration of sunitinib.

Bevacizumab

Bevacizumab is a humanized monoclonal antibody that binds isoforms of VEGF-A. Bevacizumab was evaluated in patients refractory to immunotherapy and showed a gain in OR (10%) and in PFS compared with placebo at a dose of 10 mg/kg every two weeks [30]. Therefore, in a double-blind Phase III trial, 649 patients with mRCC received bevacizumab or placebo added to IFN-α [31]. PFS was significantly increased from 5.4 to 10.2 months (HR: 0.63; $p < 0.0001$) with addition of bevacizumab to IFN-α compared with IFN-α alone. The benefit of bevacizumab plus IFN-α was seen in patients with both good (12.9 vs. 7.6 months) and intermediate (10.2 vs. 4.5 months) prognosis but was not detected in the group of poor-risk (2.2 vs. 2.1 months) patients. OR was 31 percent in the bevacizumab-plus-IFN-α group versus 13 percent in the placebo-plus-IFN-α group ($p < 0.0001$). No mature data are available on survival.

The efficacy of first-line bevacizumab alone has also been evaluated in a randomized Phase II trial with OR of 13 percent and PFS of 8.5 months [32]. Based on the results from the Phase III trial, the combination of bevacizumab plus IFN-α has been considered as an additional option to first-line treatment.

Sorafenib

The activity of sorafenib was reported in a randomized discontinuation trial in patients having received at least one previous treatment [59]. This study was followed by a placebo-controlled Phase III trial in patients who had failed cytokine therapy [33]. Sorafenib significantly prolonged PFS compared with placebo (24 weeks vs. 12 weeks; HR: 0.44; $p < 0.000001$). OR was only 10 percent. The gain was observed in all prognostic risk factor groups. Patients were subsequently unblinded, and 216 of the 452 patients receiving placebo crossed over to sorafenib.

At the final analysis, OS for the intent-to-treat population did not differ significantly between sorafenib (17.8 months) and placebo (15.2 months) [34]. Sorafenib has also been investigated as a first-line treatment; PFS did not differ between sorafenib and IFN-α (5.7 vs. 5.6 months) [35]. A more intensive administration of sorafenib was tested with a preplanned dose escalation: among 44 evaluable patients, 8 patients had a complete response, 14 experienced a partial response,

and 14 had a stable disease for at least 3 months [36]. This study is compelling owing to the highest response rate described to date with sorafenib and a complete response rate never observed with antiangiogenics, thereby justifying a similar ongoing multicenter study.

mTOR INHIBITION

Temsirolimus

Temsirolimus, an mTOR kinase inhibitor, is effective in patients with poor-prognosis mRCC [37, 38]. In the pivotal Phase III trial, patients with poor-prognosis mRCC were randomized to receive first-line treatment with temsirolimus or IFN-α alone or in combination. In the temsirolimus group, overall survival was 10.9 months (95% CI: 8.6–12.7), which was significantly longer than in the IFN-α group (median: 7.3 months; 95% CI: 6.1–8.9), with an HR of 0.73 (95% CI: 0.57–0.92; $p < 0.0069$). However, OS in the combination group (median 8.4 months) was not significantly improved. The benefit of temsirolimus on PFS and OS was more pronounced in patients with non–clear-cell histology tumors but was not seen in the subpopulation of patients 65 years of age and older [39]. Based on these results, temsirolimus has been recommended as first-line treatment in patients having at least three poor-prognostic factors.

Everolimus

Everolimus, an oral mTOR inhibitor, has shown activity in a Phase II trial of patients with mRCC [40]. Patients with no more than one prior therapy received everolimus 10 mg/day. Twelve patients (23%) exhibited a partial response and fourteen (38%) had stable disease. PFS was 11.2 months. The results of a Phase III study comparing everolimus and best supportive care (BSC) versus placebo and BSC in patients who failed previous anti-VEGFR treatment has been recently reported [60]. Median PFS time in the everolimus arm was 4 months, compared with 1.9 months in the placebo arm (HR = 0.30, 95% CI 0.22–0.40], $p < 0.001$).

EGF INHIBITION

Lapatinib

Lapatinib, a dual inhibitor of EGFR (ErbB-1) and ErbB-2 receptors, was compared with hormone treatment in a Phase III trial after failure of first-line cytokine therapy [42]. Median time to progression (TTP) and median OS did not differ between the two groups. However, patients with high tumor EGFR expression showed a significantly longer OS (46. vs. 38 weeks; HR 0.69; $p = 0.02$).

TOXICITY

Knowledge of the toxicity of antiangiogenics has increased in recent years [43]. The main toxicities associated with antiangiogenic drugs are fatigue, hypertension, nausea, stomatitis, hand-foot syndrome, and diarrhea. There is still major concern about how these treatments should be administered in elderly patients, in patients with recent vascular events, or in patients taking drugs that potentially interact with hepatic drug metabolism [44].

Less is known about the mTOR inhibitors, although there is a large body of literature on organ transplantation. mTOR inhibitors appear to be less toxic than antiangiogenics.

SEQUENTIAL TREATMENT AFTER ONE LINE OF ANTIANGIOGENICS

At present, no validated treatment has resulted from randomized Phase III trials. Nevertheless, most data point toward the absence of cross-resistance between antiangiogenics. Therefore, alternative antiangiogenic agents may be administered after failure of a previous one while awaiting definitive results of Phase III trials.

Antiangiogenics Following Sunitinib

A retrospective study demonstrated that sorafenib following sunitinib was only moderately efficient, with an impact frequently limited to stabilization [45]. In twenty-two patients progressing under sunitinib and then receiving sorafenib, one patient had a partial response and another stabilized.

Antiangiogenics Following Bevacizumab

In a Phase II trial, sunitinib was given to bevacizumab-refractory patients. Among sixty-one patients, fourteen (23%) had a partial response and thirty-six (59%) had stable disease [46]. Median PFS was 30.4 weeks.

Antiangiogenics Following Sorafenib

A retrospective study has shown that sunitinib following sorafenib may induce stabilization or a partial response [45]. In sixty-eight patients progressing under sorafenib and receiving sunitinib, ten (14.7%) had a partial response, and thirty-four (50%) had stable disease. More interestingly, among ten patients having progressed under sorafenib, two and three patients achieved partial response and stable disease, respectively.

COMBINATION AND TARGETED THERAPIES

Antiangiogenics represent a breakthrough in the treatment of mRCC; however, despite initial benefits, most patients experience progression in less than twelve months. Preclinical findings justify increasing the inhibition on the angiogenic pathway in a direct way. Therefore, associations of drugs for vertical inhibition have been proposed associating monoclonal antibodies of the VEGF pathway or tyrosine kinase inhibitors of the VEGFR pathway or mTOR inhibitors. The other way to inhibit tumor cell growth is to inhibit multiple pathways involved in the process. To date, only angiogenesis and the mTOR pathway have been shown to be major targets and to support the association of related drugs.

Antiangiogenics and Antiangiogenics

The association of bevacizumab and sunitinib has been explored in a Phase I study [47]. Nineteen patients with mRCC received escalating doses of sunitinib from 25 to 50 mg daily with fixed-dose bevacizumab (10 mg/kg iv). Dose-limiting toxicity (DLT) was grade 4 hemorrhage in one patient in each of the higher doses and one fatal myocardial infarction at the highest dose. All levels of treatment induced a 37 percent partial response. The recommended dose for a further Phase II trial was considered to be sunitinib and bevacizumab at the standard dose when given alone.

The association of bevacizumab and sorafenib has been explored in a Phase I trial [48]. Sixteen patients were included for a dose escalation. Grade 3 proteinuria and uncontrolled grade 3 hypertension were DLTs at the highest level, corresponding to the maximum tolerated dose (MTD), and the recommended dose was sorafenib at 200 mg twice daily (BID) and bevacizumab at 5 mg/kg.

Antiangiogenics and Interferon Alpha

In Phase I trials, patients received sunitinib or sorafenib and IFN-α with DLT that included fatigue and myelosuppression. Only inferior dosages of both sunitinib or sorafenib and IFN-α, compared with optimal dosages in monotherapy, were manageable. Phase II studies have evaluated the association of sorafenib and IFN-α at standard or low dose with inconstant gain for the association [55, 56, 60, 61].

Antiangiogenics and Other Drugs

Antiangiogenics have been combined with mTOR inhibitors to enhance the inhibition of angiogenesis while using the mTOR pathway involved in angiogenesis and/or to inhibit various pathways when using the larger spectrum of mTOR activity.

Temsirolimus and bevacizumab were associated without MTD to the standard dose of each drug in a Phase I study [49]. During the Phase I trial using up to a recommended weekly dose of temsirolimus at 25 mg/kg iv and bevacizumab at a dose of 10 mg/kg/2 weeks, DLTs encountered were grade 3 stomatitis and hypertriglyceridemia. Among twelve evaluable patients, eight partial responses were reported.

Temsirolimus and sorafenib have been evaluated [50]. Patients were treated with escalating continuous oral doses of sorafenib (200 and 400 mg BID) and weekly iv temsirolimus (15 mg, 25 mg). Thirty-three evaluable patients showed DLTs including grade 3 hand–foot syndrome, mucositis, rash, thrombocytopenia, neutropenia, and creatinine elevation. The full recommended dose of both drugs appeared unachievable, owing mainly to mucositis.

PREDICTIVE FACTORS FOR EFFICACY OF TARGETED DRUGS IN mRCC

The prognosis of patients with mRCC at the diagnosis of metastatic spread is usually based on the Memorial Sloan-Kettering Cancer Center (MSKCC) classification; however, little is known about how to identify the patients who have a fair chance from benefiting of "standard" antiangiogenics. In this respect, the pivotal study using sunitinib as a first-line therapy showed that low hemoglobin ($p = 0.004$), hypercalcemia > 10 mg/mL ($p = 0.001$), ECOG < 0 ($p = 0.0005$), more than one metastatic site ($p = 0.0064$), and interval between diagnosis and treatment < 1 year ($p = 0.0002$) were independent adverse prognostic factors [27].

NON–CLEAR-CELL HISTOLOGY

To date, most mRCC patients included in clinical trials have had a predominant clear-cell histology; there are no available prospective data on other histologies, especially papillary (PRCC) and chromophobe (ChRCC) features. A multicenter retrospective study analyzed forty-one files of patients with PRCC and twelve with ChRCC [51]. Two patients with PRCC had partial responses and were treated with sunitinib. Twenty-seven (68%) patients had a stable disease for at least 3 months. PFS was 7.6 months with a trend for better PFS with sunitinib than sorafenib (11.9 vs. 5.1 months). For patients with ChRCC, three (25%) had a response (two under sorafenib and one under sunitinib) and all the others had at least a stable disease for a minimum of 3 months. PFS was 10.6 months, and patients treated with sorafenib had a trend for more prolonged PFS (27.5 months).

Another study evaluated the influence of histological subtype on the efficacy of temsirolimus as first-line therapy [38]. In the Phase III trial comparing temsirolimus

TABLE 36.1. Suggested treatment algorithm based on available Phase III studies

Setting		Phase III trial
Treatment-naïve	Good or intermediate risk*	Sunitinib Bevacizumab + IFN-α
	Poor risk*	Temsirolimus
Previously treated	Prior cytokine	Sorafenib
	Prior VEGFR–TKI	Everolimus
	Prior mTOR inhibitor	Clinical trial

*Memorial Sloan-Kettering Cancer Center classification risk status.

with IFN-α, 18 percent were non–clear-cell RCC. PFS and overall survival were 7 and 11.6 months, respectively, in PRCC patients, whereas they were 5.5 and 10.6 months in clear-cell RCC patients.

IMPACT OF RECENTLY PUBLISHED PHASE III STUDIES ON PRACTICE

Recent findings have considerably clarified the first-line treatment for mRCC patients. The current treatment algorithm based on available Phase III studies has been summarized in Table 36.1.

Only in specific circumstances should patients with a good performance status and only one site of metastasis be given immunotherapy. This represents fewer than 5 percent of the population of mRCC patients [4, 21, 52].

All other patients should be offered antiangiogenic therapy, based on the results of Phase III trials that suggest sunitinib as the standard treatment and bevacizumab plus IFN-α as an alternative option.

For patients with poor MSKCC performance characteristics, temsirolimus has shown an advantage in survival. Sunitinib or sorafenib have shown some efficacy and therefore represent an optional treatment.

As second-line therapy, sorafenib is the drug of choice following immunotherapy, whereas sunitinib is an option.

As second-line therapy after an anti-VEGFR TKI treatment failure, everolimus should be recommended. Finally, patients who failed a previous first-line treatment with mTOR inhibitors should be included in clinical trials.

CONCLUSION

The past few years have been especially promising for the treatment of mRCC, with published Phase III trials on sunitinib, sorafenib, temsirolimus, bevacizumab, and everolimus. Preliminary data on sequential treatments and the association of targeted drugs exist. From now on, it is clear that survival has at least doubled compared with survival in the immunotherapy era. Progress will continue by associating surgery with targeted therapies and by adding the survival benefits of different lines of effective therapies. Major data are pending on the ability of targeted-based therapy to induce a durable complete response in a substantial subset of patients, and on the impact of these novel agents in the adjuvant setting.

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37 Bladder Cancer

Neveen Said and Dan Theodorescu

Bladder cancer is the most common malignancy affecting the urinary system. In the United States, an estimated 68,810 new cases, with a male-to-female ratio of 4:1 and approximately 13,750 deaths, were expected to occur in 2008 [1]. The incidence of bladder cancer in the United States is higher in whites than in people of African or Asian descent, native Americans, or Latinos. However, survival is longer in white men than in men of other ethnic groups or in women. The disease has a median age at presentation of 70 years [1]. In the United States, the most common type of bladder cancer is urothelial carcinoma (UC), formerly known as "transitional cell" carcinoma (TCC).

UC arises from the mucosal lining of the bladder and is frequently multifocal. Numerous factors, including chromosomal markers, genetic polymorphisms, and genetic and epigenetic alterations, may be involved in tumorigenesis, progression, and metastasis. Seventy percent to eighty percent of patients with UC present with no muscle invasion (formerly known as "superficial disease"), and 20 percent to 30 percent present with muscle-invasive disease (Figure 37.1A). Despite a good prognosis for patients with non–muscle-invasive UC, recurrence is common and is associated with development of muscle-invasive disease in up to 30 percent of patients. In addition, 50 percent of patients presenting with muscle-invasive UC have occult distant metastases and a poor five-year survival rate.

ETIOLOGY AND PATHOGENESIS

Bladder cancer is commonly initiated by prolonged exposure to carcinogens that cause cumulative DNA damage in the urothelium (Figure 37.2). These carcinogens are excreted in the urine, in which they can be activated by hydrolyzing enzymes and then stored in the bladder. Consequently, the entire urothelium is at risk, a phenomenon known as *field cancerization*, which likely contributes to the multifocality of the disease [2, 3]. Risk factors include tobacco smoking, occupational exposure to aromatic amines, consumption of arsenic-laced water, chronic infections, radiation therapy of neighboring organs, and therapeutic use of alkylating agents or analgesics, such as acetaminophen and phenacetin [2–6].

Host genetic factors further modify the susceptibility to UC development, as demonstrated by familial aggregates of UC in populations with similar smoking history and occupational or environmental exposure [6–8]. Inherited genetic polymorphisms in the enzymes regulating the metabolic pathways involved in the in vivo activation, detoxification, or inactivation of carcinogens have been identified to modify the risk of developing UC (Figure 37.2) [5]. Case–control studies confirmed a significantly higher percentage of UC in cigarette smokers with genotypes consistent with extensive metabolic activators and high levels of enzymatic activity of P450 cytochrome enzymes (CYP1A2, CTP2D6, and CTP3A4), which are involved in *N*-oxidation of arylamines to *N*-hydroxylated metabolites, the initial step of arylamine activation into carcinogens [5, 9, 10].

N-acetylation is one of the best described detoxification pathways for arylamines [3, 5, 8–11]. Genetic polymorphisms of *N*-acetyltransferases *NAT1* and *NAT2* alter the metabolic rate at which procarcinogens are neutralized and result in acetylation phenotypic (slow or fast) *NAT1* and *NAT2* variants that modify bladder cancer risk. Slow *NAT2* acetylation polymorphism is a relatively common and varies according to race, being lowest in Asians compared with Africans and Caucasians [12]. An increased risk of bladder cancer has been associated with a slow acetylator phenotype in individuals with environmentally or occupationally related aromatic amine-induced UC [3, 5, 8–11].

Glutathione S transferases *GSTT1* and *GSTM1* are involved in the elimination of carcinogens in the body,

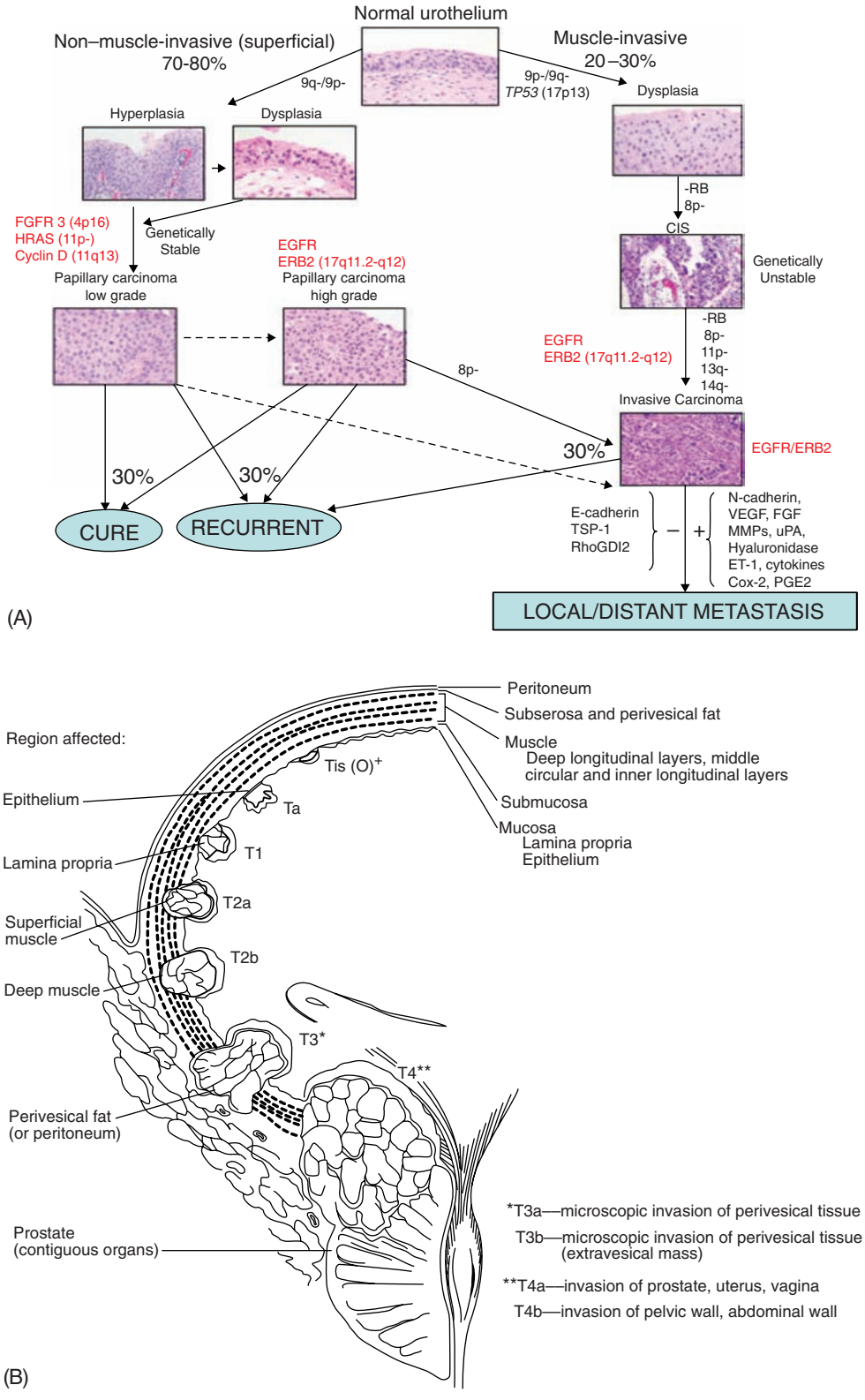


Figure 37.1 (A) Divergent pathways of UC development. At presentation, most tumors (70%–80%) are diagnosed as non–muscle-invasive disease (superficial), and 20%–30% as muscle-invasive disease. For the non–muscle-invasive disease, the preneoplastic lesions progress from hyperplasia/dysplasia, with stable genetic alterations, into superficial low- or high-malignant UC. The preneoplastic lesions of muscle-invasive UC are severe dysplasia/CIS that accumulate unstable genetic alterations and progress into invasive, high-grade UC (hematoxylin–eosin, original magnification ×400; histopathology images are modified from Joe Kronz, MD, http://www.hopkinsmedicine.org.) (B) Staging of bladder cancer.

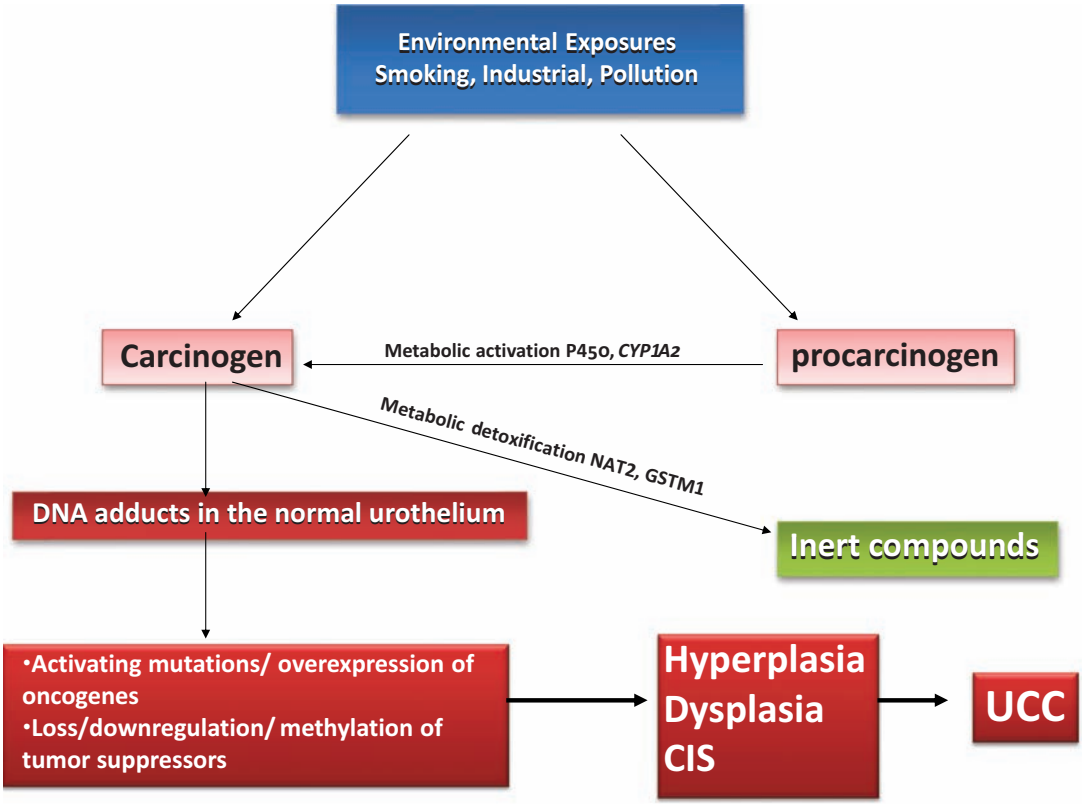


Figure 37.2. Hypothetical model of carcinogen activation and detoxification, and the resulting cellular consequences in patients with abnormal detoxification mechanism.

such as products of oxidative stress and polycyclic aromatic hydrocarbons from tobacco smoke [2, 5, 6, 12]. Deletion of the *GSTT1* and *GSTM1* genes results in a variant called *GSTT1/GSTM1* null and a complete loss of enzymatic activity, impaired ability to detoxify carcinogens, and an increased risk of cancer, potentially affecting multiple cancer sites, including the bladder [2, 5, 6].

CLINICAL MANIFESTATIONS

The classic presentation of patients with UC is painless, intermittent macroscopic or microscopic hematuria [13–17]. Voiding symptoms, such as frequency, urgency, and dysuria, are the second most common presentation, usually associated with diffuse carcinoma in situ (CIS) or invasive bladder cancer. Obstructive symptoms resulting from local extension, including flank pain, lower extremity edema, and pelvic mass, may be encountered. Symptoms of advanced metastatic disease include weight loss and abdominal or bone pain [16]. Patients with extensive local tumors may have a palpable mass. Hepatomegaly and supraclavicular lymphadenopathy can be signs of metastatic disease. Lymphedema from occlusive pelvic lymphadenopathy and hydronephrosis if the tumor obstructs the ureteral orifice occasionally occur. Metastases can occur in bones, lungs, and unusual sites such as the skin, presenting as painful nodules with ulceration [18].

DIAGNOSIS

The diagnosis of primary UC can be suspected by cystoscopy, a visual inspection of the whole bladder urothelium, but pathological examination of the lesion is required to confirm the diagnosis [19]. This is most commonly carried out by a transurethral resection of bladder tumor (TURBT) procedure. Cytologic evaluation of exfoliated urothelial cells in either voided urine or bladder washings can also suggest the diagnosis in some cases, but this is not definitive, as the sensitivity of urine cytology is greatest for CIS (at about 90%) but the false-negative rate is high in low-grade tumors [20].

A host of urinary biomarkers have also been described as diagnostic tools. Urinary biomarkers include immunocytochemistry, molecular and proteomics assays [21–23], molecular cytogenetics, telomerase expression [24], tumor-associated intracellular or secreted products, oncogene mutations, and markers of apoptosis [9, 19, 25–33]. Recently, multiplex RNA profiling and surface-enhanced laser desorption/ionization time-of-flight spectroscopy (SELDI-TOF) have also identified different proteins that can distinguish bladder cancers from normal urothelium [34–37].

TABLE 37.1. American Joint Committee on Cancer (AJCC) bladder cancer staging [18, 41, 42]

Primary tumor (T)			
TX	Primary tumor cannot be assessed		
T0	No evidence of primary tumor		
Ta	Noninvasive papillary carcinoma		
Tis/CIS	Carcinoma in situ: "flat tumor"		
T1	Tumor invades subepithelial connective tissue		
T2 T2a T2b	Tumor invades muscle Tumor invades superficial muscle (inner half) Tumor invades deep muscle (outer half)		
T3 T3a T3b	Tumor invades perivesical tissue Microscopically Macroscopically (extravesical mass)		
T4 T4a T4b	Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall Tumor invades prostate, uterus, vagina Tumor invades pelvic wall, abdominal wall		
Regional lymph nodes (N)			
Regional lymph nodes are those within the true pelvis; all others are distant lymph nodes.			
NX	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in a single lymph node, 2 cm or less in greatest dimension		
N2	Metastasis in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension; or multiple lymph nodes, none more than 5 cm in greatest dimension		
N3	Metastasis in a lymph node more than 5 cm in greatest dimension		
Distant metastasis (M)			
MX	Distant metastasis cannot be assessed		
M0	No distant metastasis		
M1	Distant metastasis		
Stage grouping			
Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a T2b	N0 N0	M0 M0
Stage III	T3a T3b T4a	N0 N0 N0	M0 M0 M0
Stage IV	T4b Any T Any T Any T Any T	N0 N1 N2 N3 Any N	M0 M0 M0 M0 M1

Source: AJCC Cancer Staging Manual, Sixth Ed. (2002) New York: Springer-Verlag.

CLINICAL STAGING

Computed tomography (CT) with intravenous contrast is the most common staging modality and provides information regarding extravesical extension; pelvic or retroperitoneal nodal involvement; visceral, pulmonary, or osseous metastasis; tumor involvement; or obstruction of upper tract function (Table 37.1). Important limitations of this technology are the difficulty to distinguish inflammatory or iatrogenic edematous changes from true extravesical tumor extension and the relative low sensitivity of CT for identification of

TABLE 37.2. Treatment options for bladder cancer [38, 39]

Cancer Stage	Initial Treatment Options
Tis	Complete transurethral resection (TUR) followed by intravesical Bacille Calmette-Guérin (BCG)
Ta (single, low-to-moderate grade, not recurrent)	Complete TUR
Ta (large, multiple, high-grade, or recurrent)	Complete TUR followed by intravesical chemo- or immunotherapy
T1	Complete TUR followed by intravesical chemo- or immunotherapy
T2–T4	Radical cystectomy
	Neoadjuvant chemotherapy followed by radical cystectomy
	Radical cystectomy followed by adjuvant chemotherapy
	Neoadjuvant chemotherapy followed by concomitant chemotherapy and irradiation
Any T, N+, M+	Systemic chemotherapy followed by selective surgery or irradiation

nodal involvement [38]. Other imaging studies, such as MRI and bone scintigraphy, may be appropriate to evaluate for extravesical extension and distant metastatic lesions when indicated, but they are not routinely carried out [38, 39].

PATTERNS OF INVASION AND METASTASIS

Contiguous spread occurs in 60 percent of tumors and is characterized by cancer cells directly invading the lamina propria and muscularis propria beneath the primary mucosal lesion, with either tentacle-like invasion (25%) or lateral spread under normally appearing mucosa (10%) (Figure 37.1A, B). The depth of invasion of the muscularis propria is correlated with hematogenous and lymphatic invasion with subsequent metastasis to regional lymph nodes or distant sites [40].

Muscle-invasive disease is accompanied by involvement of the prostate and prostatic urethra in 40 percent of men undergoing cystectomy. About 40 percent of patients with prostatic involvement have invasion of the stroma and 6 percent have stromal involvement without prostatic urethral involvement [40]. Despite radical surgery, patients with stromal invasion have a high incidence of subsequent distant metastases. Lymphatic metastases often occur earlier and may be independent of hematogenous metastases as manifested by the observation that 10 percent to 15 percent of patients with lymphatic metastasis are cured with surgery alone [41, 42].

The extent of the local tumor, as well as of the nodal metastases, directly affects survival after surgical excision [40]. The most common sites of nodal metastases are the pelvic lymph nodes, with involvement of the paravesical (16%), the obturator (74%), the external iliac (65%), and the presacral nodes (25%). Juxtaregional common iliac lymph nodes are involved in about 20 percent of patients, concomitant with involvement of the previously mentioned regions [40]. The common sites of visceral metastases are liver (38%), lung (36%), bone (27%), adrenal glands (21%), and intestine (13%) [43–45]. Bone metastases are more common with bilharzial bladder cancer [40]. Bone lesions arising from UC usually manifest as osteoblastic or mixed osteoblastic–osteolytic lesions [43]. Despite advances in treatment of systemic urothelial cancer, few patients survive five years [1].

CLINICAL MANAGEMENT OF INVASIVE BLADDER CARCINOMA

Non–muscle-invasive tumors are typically treated by TURBT, an endoscopic technique that removes the cancer and preserves bladder function. Depending on multiple clinical and pathologic factors, additional intravesical therapies may be used adjuvantly in this setting [46, 47]. This chapter focuses on clinical aspects of muscle-invasive and metastatic disease (see Table 37.2).

Patients with resectable, localized, muscle-invasive UC are treated with radical cystectomy with urinary diversion, or bladder-sparing protocols with a combination of radiation and chemotherapy [47]. Recurrence or persistence rates after bladder-sparing protocols approach 50 percent but can be reduced by careful patient selection. Survival of patients with localized muscle-invasive UC undergoing cystectomy has been improved by treatment with neoadjuvant therapy with methotrexate, vinblastine, Adriamycin, and cisplatin (MVAC) [30, 33, 46, 48–57]. Radical cystectomy includes wide excision of the bladder and prostate in male patients and typically the bladder, uterus, ovaries, and anterior vaginal wall in females [58], and has a perioperative mortality of 1 percent in most centers. The five-year disease-free survival is 65 percent to 80 percent for pT2 tumors and 37 percent to 61 percent for pT3 tumors (Table 37.1), and further decreases to 5 percent

to 20 percent, depending on the number and extent of local lymph node involvement [47]. Pelvic recurrence rates after cystectomy range from 2 percent to 10 percent and depend on the stage of the primary tumor as well as the presence of pelvic nodal involvement [58].

CLINICAL MANAGEMENT OF METASTATIC UROTHELIAL CARCINOMA

MVAC is associated with a response rate of 15 percent to 35 percent [59]. Complete responses are seen in approximately 13 percent of patients, and mean survival is eight to twelve months [59]. However, the significant toxicity associated with MVAC [30, 33, 55–57] has led to the increasing use of gemcitabine and cisplatin (GC) as a more tolerable alternative palliative treatment [59]. Sustained long-term survival was observed only in some patients with locally advanced cancer with lymphatic metastases, but not in patients with visceral metastatic disease [60]. Combined paclitaxel, carboplatin, gemcitabine, and trastuzumab (a humanized monoclonal antibody against human epidermal growth factor receptor-2, Her-2/neu) has been tried with a 70 percent overall response rate, and a median time to progression of 9.3 months and overall survival of 14.1 months [57]. This was based on the identification of the molecular pathways of advanced UC [61] (Figures 37.1 and 37.3a).

MOLECULAR MECHANISMS OF INVASIVE BLADDER CANCER

Epidermal Growth Factor Receptor

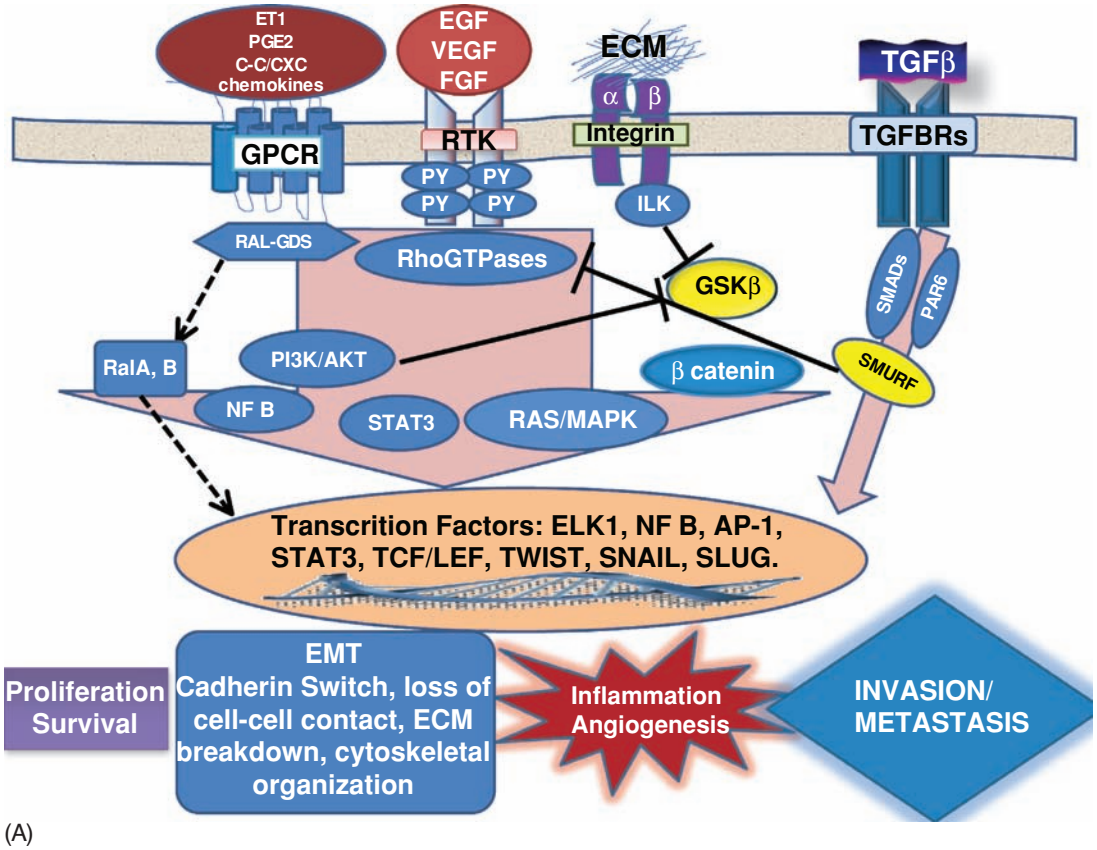
Epidermial growth factor receptor (EGFR) expression levels in UC have been positively correlated with tumor progression, increasing pathologic grade and stage [62, 63], and higher rates of recurrence [64]. Increased EGFR expression in tumor tissues was inversely correlated with patient survival. However, when the comparison of survival was limited to patients with invasive UC, no significant difference was found between patients with high levels of EGFR expression and those with low EGFR values, suggesting that EGFR overexpression might be associated with the phenotypic transition from non–muscle-invasive to invasive forms of the disease [65]. Interestingly, gene amplification and gene rearrangement does not appear to be a common mechanism for EGFR overexpression in UC [66]. However, when non–muscle-invasive human UC cells were engineered to overexpress either mutated or normal *HRAS* (***RAS****: Rat sarcoma oncogene), Harvey (the HRAS oncogene), Kirsten (KRAS),* or *neuroblastoma (NRAS),* they overexpressed EGFR at both the mRNA and protein levels, suggesting a role of HRAS in transcriptional regulation of EGFR, in addition to its role in EGFR signal transduction (Figure 37.3) [67]. In vitro studies implicated the role of EGFR in multiple steps involved in tumor cell motility and invasion, supporting the notion that EGFR overexpression is causally related to tumor progression and not merely an epiphenomenon [67–69]. Amplification and protein overexpression of the *ERBB2* gene, located on 17q11.2–q12, has been suggested as a prognostic marker for patients with recurrent progressive UC [70]. Importantly, EGF, the ligand for EGFR, is found at tenfold greater concentrations than those found in blood and likely potentiates the consequences of EGFR overexpression [13, 64].

VEGF and VEGFRs

The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF. In humans, VEGF-A exists in three isoforms, 121-, 165-, and 189-amino acid, binds and activates VEGFR-1 and VEGFR-2, two receptor tyrosine kinases, and is a potent inducer of angiogenesis [71]. PlGF and VEGF-B bind and activate only VEGFR-1. Under pathological conditions, increased PlGF and VEGF-A recruit bone-marrow–derived monocytes/macrophages via VEGFR-1 to cancer tissues or inflammatory lesions and significantly enhance pathological angiogenesis. VEGFR-3 is expressed mainly in lymphatic endothelial cells and ligation by VEGF-C and -D regulates lymphangiogenesis [71]. VEGF-C or VEGF-D–overexpressing tumors are lymphangiogenic and highly metastatic to lymph nodes [71].

High levels of VEGF mRNA expression predicted earlier recurrence and increased risk of progression in patients with low or intermediate grade T1 UC [72]. Increased VEGF-A immunostaining was correlated with increasing stage, and serum VEGF levels positively correlated with stage, grade, vascular invasion, and the presence of CIS. VEGF levels ≥400 pg/mL were found to be highly predictive of metastatic disease [73–75]. The T24 bladder tumor cell line expresses both VEGF-A isoforms and VEGFR-2 (KDR/Flk-1), and autocrine and paracrine VEGF-induced mitogenic signaling loops have been identified [24]. This mitogenic VEGF pathway involves the activation of PKC, sphingosine kinase (SPK), Ras (H-Ras and N-Ras activation, but not K-Ras activation), and mitogen-activated protein kinases (ERK1/2). VEGF-induced Ras activation was mediated through Ras-GAP activities, independent of Ras-GEF interaction [24, 76, 77].

A tissue microarray (TMA) study of 286 archival cystectomy tumor blocks found that VEGF-D overexpression was positively correlated with tumor stages and regional lymph node metastasis, and negatively correlated with disease-free survival. Overexpression of VEGFR-3 was particularly present in the subgroup of high-grade tumors and was associated with a shorter



(A)

Figure 37.3. (A) Signaling pathways in UC. Receptor tyrosine kinases (EGFR, VEGFR, FGFR) contain a cytoplasmic tyrosine kinase domain, a single transmembrane domain, and an extracellular domain that bind to cognate ligands and activate downstream signaling through simultaneous activation of multiple pathways, which lead to activation of Ras/MAPK, AKT/PI3K, with subsequent activation of multiple transcription factors resulting in cell proliferation/survival, EMT, inflammation, angiogenesis, and lymphangiogenesis. Crosstalk between ECM-integrin-ILK, TGFβ, GSKβ/β-catenin directly affects cell adhesion and/or the cytoskeletal dynamics, through their effect on Rho GTPases. The convergence of these signaling pathways eventually leads to cancer cell invasiveness and metastasis.

disease-free survival. In multivariate analysis, VEGF-D and VEGFR-3 expression were independent prognostic parameters of tumor stage and lymph node metastasis [78, 79]. Comparison of lymph node metastases to corresponding primary tumors indicated that expression of VEGF-D and VEGFR-3 were significantly higher in the former [78, 79]. Thus, VEGF-D and VEGFR-3 expression could be useful prognostic tools for tumor progression and metastasis, as well as therapeutic targets of the latter [78, 79].

Fibroblast Growth Factors and Receptors

Two isoforms of the potent angiogenic factor fibroblast growth factor (FGF) implicated in the pathogenesis of bladder cancer are the acidic FGF1 (aFGF) and the basic FGF2 (bFGF). Both are tightly bound to heparan sulfates of the extracellular matrix (ECM) and are thought to be released by proteases as a consequence of ECM degradation during cancer progression [80, 81]. Both FGF1 and FGF2 contribute to a more aggressive phenotype in UC, and their immunohistochemical determination may be helpful in identifying tumors that are more likely to progress [80, 81]. FGF2 was found to be overexpressed in CIS, earlier than VEGF, which was upregulated at later stages in the development of muscle-invasive UC [80, 82–84].

High-molecular-weight (HMW) forms of FGF2 contain a nuclear localization signal and have been implicated in the promotion of cancer metastasis. In a rat carcinogen-induced bladder cancer cell line (NBT-II), HMW-FGF2 NBT-II–expressing clones exhibited increased tumorigenicity with increased lung metastases, compared with low-molecular-weight (LMW) cytoplasmic FGF2 expressing LMW-FGF2 NBT-II clones or parental NBT-II cells that do not metastasize to the lung [85]. HMW-FGF2 NBT-II clones showed no increase in the FGF2-specific receptor, suggesting that nuclear FGF2 has novel targets, inducing the survival program responsible for the growth of lung metastases [85].

FGFs and their cognate receptors have been shown to play a role in reciprocal signaling from epithelial to mesenchymal compartments [86]. Furthermore, ligands for the epithelial-specific FGFRs are typically

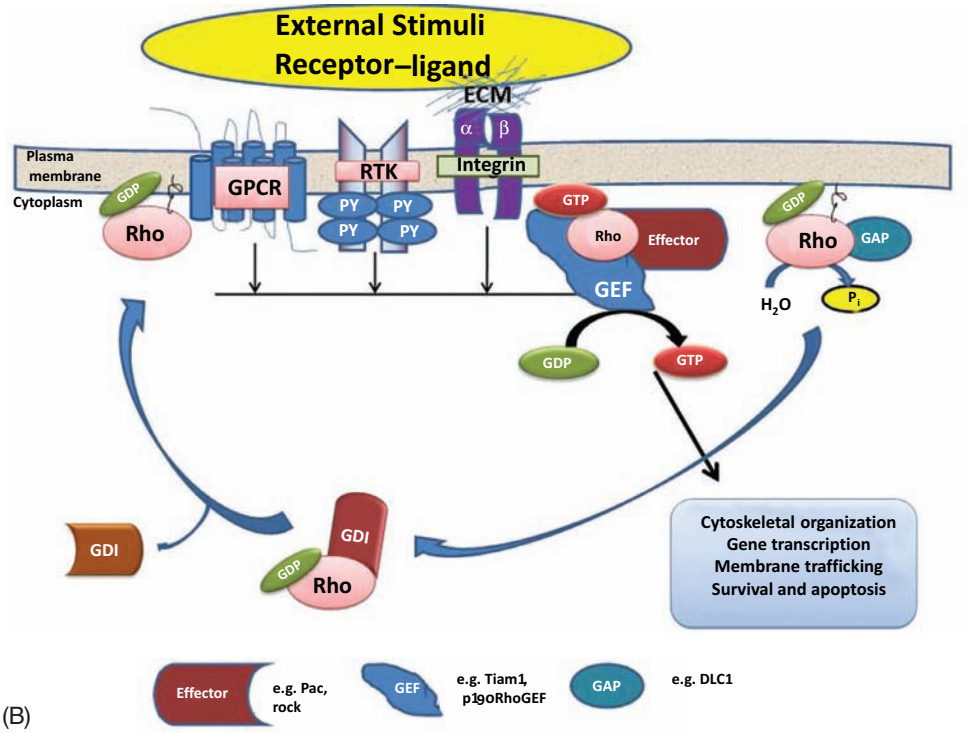


Figure 37.3. (B) Regulation of GTPase cycle: GDP-bound inactive GTPases are mainly cytoplasmic, maintained there by GDIs masking the C-terminal tail required for plasma membrane localization. On dissociation of the GDI, posttranslational modification can take place and GTPases translocate to the plasma membrane, where they can be activated by GEFs on external stimuli from surface ligand–receptor systems such as adhesion receptors (ECM-integrins), G-protein coupled receptors (GPCRs), and receptor tyrosine kinases (RTKs). On activation by GEFs, Rho GTPases can bind different effector proteins, selection of which can be mediated by GEFs and induce downstream signaling pathways. GAPs inactivate the Rho GTPases and switch off the downstream signaling.

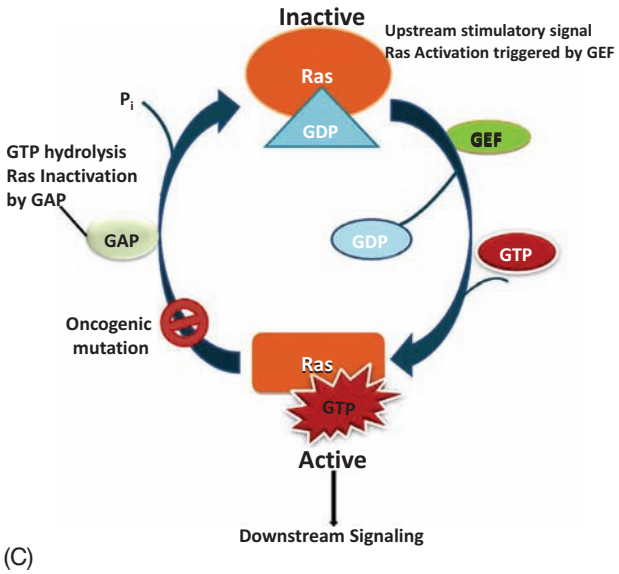


Figure 37.3. (C) Ras activation cycle. Ras is a single-subunit small GTPase that functions as binary signaling switch with "on" and "off" states. In the "off" state it is bound to the nucleotide GDP, whereas in the "on" state, Ras is bound to GTP. Activation and deactivation of Ras and other small G proteins are controlled by cycling between the active GTP-bound and inactive GDP-bound forms. The exchange process and hence Ras activity is regulated by the activity of GEFs and GAPs. Ras has an intrinsic GTPase activity, but this process is too slow for efficient function, and is stabilized by bound RasGAP. Thus, GAPs regulate Ras inactivation. GEFs facilitate Ras activation. In the GTP-bound conformation, Ras has high affinity for numerous effectors that allow it to carry out its functions, PI3K, and MAPK. Constitutively active Ras with mutations that prevent GTP hydrolysis, thus locking Ras in a permanently "on" states, are very common and have been shown to play a key role in signal transduction, proliferation, and malignant transformation.

located in the stromal component, whereas ligands for the mesenchymal-specific receptors are often restricted to cells of epithelial origin.

FGFR2IIIb mRNA is distributed throughout normal urothelium except for the umbrella cells, and low levels of FGFR2IIIc are detected in the stroma, whereas bladder carcinoma cell lines generally express no FGFR2IIIc isoform and very little FGFR2IIIb compared with normal urothelium [87]. In bladder carcinoma, low or complete loss of FGFR2IIIb expression is associated with poor prognosis [88, 89]. Introduction of FGFR2IIIb into the human T24 bladder carcinoma cell line led to a decrease in proliferation and decreased tumor growth after subcutaneous inoculation in nude mice [87].

Transforming Growth Factor-β Family

The transforming growth factor (TGF)-β family of proteins includes TGF-β1 to β5, Müllerian inhibitory substance, inhibin, and activin [90]. Whereas TGF-βs were originally found to assist the malignant transformation of rat fibroblasts [90], in most cases they are inhibitors of cellular proliferation, at least in part through stimulating p27 and p15, nuclear proteins that inhibit the phosphorylation of retinoblastoma (Rb) protein by various cyclin-dependent kinases [91]. TGF-β1 and TGF-β2 mRNA expression was significantly higher in the more indolent urothelial tumors than in aggressive ones [91]. In contrast, Miyamoto and colleagues [92] reported that UCs had higher TGF-β1 expression than did normal urothelium. TGF-βs have potent angiogenic activity, with TGF-β1 concentrations in sera of patients with invasive and/or grade 3 tumors significantly higher than those in patients with non–muscle-invasive cancers, [93, 94]. Furthermore, TGF-β1 overexpression was found to be associated with angiogenic and inflammatory markers, advanced pathological stage, and risk of disease progression in patients undergoing radical cystectomy for UC [95]. However, decreased expression of its membrane-bound receptors TGF-β-RI and/or TGF-β-RII was found to be associated with bladder cancer stage, grade, progression, and survival [32, 95–97]. These seemingly conflicting data on the expression of TGFβs and their putative receptors may be attributed to their multiple cellular sources, differential expression, downstream effectors, and hence the function of TGFβ, in the reactive tumor microenvironment, at both the primary and secondary metastatic sites.

Deregulation of p53 Pathways

The p53 tumor suppressor encoded by the *TP53* gene located on chromosome 17p13.1 [98] inhibits phase-specific cell cycle progression (G1-S) through the transcriptional activation of $p21^{WAF1/CIP1}$ [99]. Most UCs exhibit a loss of a single 17p allele, and an additional mutation in the remaining allele can inactivate *TP53,* leading to increased nuclear accumulation of the mutant protein [100]. Overexpression of nuclear p53 protein, as determined by immunohistocytochemistry (IHC), is thus frequently used as a surrogate marker for detection of mutant p53. Based on such assays, p53 overexpression has been associated with an increased risk of progression, or mortality in non–muscle-invasive and muscle-invasive tumors, respectively, independent of tumor grade, stage, and lymph-node status [46, 98, 99, 101–110]. *TP53* deletion was significantly correlated with grade and stage [111–115]. Mutations in the *TP53* gene that result in a truncated form of the protein (or no protein), homozygous deletion of both alleles of the gene, or gene silencing by methylation of the promoters of both alleles cannot be detected by nuclear accumulation of p53 protein [116].

The discordance in the identification of p53 as an independent prognostic marker for UC progression, recurrence, mortality, and response to therapy is believed to be due to the genetic and epigenetic status of patients, the number of cases in each study, the technical variability in the assays used, and the statistical analyses. A study of 995 patients with non–muscle-invasive UC confirmed that p53 overexpression correlated with higher grade and stage of the disease [117]. However, the prognostic significance of this marker was lost on multivariate analyses that included tumor size, stage, grade, multiplicity, and patient age, sex, and treatment. In a recent study [21, 118], the influence of the *TP53* genetic status on non–muscle-invasive tumor recurrence and progression was evaluated using a highly effective electrophoretic technique. The frequencies of tumor recurrence and progression were significantly higher in patients with *TP53* mutation, compared with wild-type tumors. Progression-free survival was significantly shorter in patients with *TP53* mutated tumors. However, there was no statistically significant difference with regard to recurrence frequency and time to recurrence.

Deregulation of Retinoblastoma Pathway

Deletions of the long arm of chromosome 13, including the *Rb* locus on 13q14, have been detected in muscle-invasive tumors [119]. Altered Rb expression was detected in patients with muscle-invasive tumors, positively correlated with proliferative indices, and negatively correlated with patient survival [105, 120, 121]. Normal expression of Rb and p53 proteins favors good prognosis in patients with T1 tumors, whereas patients with abnormal expression of either or both proteins had a significant increase in progression [122]. Therefore, *P53* and *Rb* nuclear protein status could potentially be

used in stratification of non–muscle-invasive (Ta, T1, and CIS) bladder cancer patients [123, 124] so patients with normal protein expression for both genes may be managed conservatively, whereas patients with alterations in one or both genes may require more aggressive treatment.

Analysis of multiple cell cycle regulatory proteins (pRb, p53, and $p21^{WAF1/CIP1}$, with or without $p16^{INK4a}$) predicts the outcome of UC more accurately than any single marker, and independent of standard clinicopathologic prognostic factors [125, 126]. Other than cell cycle deregulation, Rb inactivation contributes to tumor progression through repression of E-cadherin [127]. Thus in UC, loss of heterozygosity (LOH) of 13q14 ([81] and references cited therein) and loss of Rb protein expression, frequently detected in tumors of high grade and stage [125], may further contribute to loss of E-cadherin expression.

Ras and Rho Family GTPases and Their Regulators

The larger Ras superfamily in humans comprises more than 100 small (20–30 kDa), related monomeric guanine nucleotide-binding proteins, including six subfamilies: Ras, Rho, Arf, Rab, Ran, and Rad. The H-Ras molecule stimulates the activation of other downstream signaling pathways, which are associated with enhanced cell motility and invasion [128]. Underlying the functional diversity in the Rho family is a common guanosine triphosphate (GTP)/guanosine diphosphate (GDP) cycle (Figure 37.3B,C). Small G proteins cycle between a GTP-bound state and a GDP-bound state. In vivo, this cycle is tightly regulated by guanine nucleotide exchange factors (GEFs), which stimulate the exchange of GDP for GTP, and GTPase activating proteins (GAPs), which increase the rate of GTP hydrolysis [129]. The Rho subfamily has been implicated as a nexus for signal transduction pathways that affect cell adhesion, migration, cell-cycle progression, cell survival, membrane recycling, and gene expression. Crosstalk between Ras and Rho proteins was observed in several biological processes, including cell transformation, cell migration, and epithelial–mesenchymal transition (EMT) [130, 131].

RAS

In the early 1980s, the *HRAS* gene, which codes for p21Ras, a small GTPase, was the first named human oncogene found to be mutated in the T24/T24T urothelial cell lines [132, 133]. However, its precise role in urothelial cancer remains unclear [134]. In the normal urothelium, normal HRAS protein diminishes with differentiation, with highest staining intensity in the basal (progenitor) cells of the multilayered transitional epithelium, whereas the superficial (differentiated) compartments stained to a much lesser degree [135]. In UC, some studies have shown an association of the *HRAS* mutations with low-grade, noninvasive non–muscle-invasive papillary UC [136], whereas others have implicated *HRAS* mutations in several of the steps of tumor invasion in UC [67, 137]. *HRAS* codon 12 mutations were reported in approximately 40 percent of bladder tumors [138, 139], and a positive correlation between HRAS protein immunostaining in tumor tissues and invasiveness was also reported [140].

These data suggested that hyperactive HRAS in UC can be caused by activating mutations, or via either overexpression of the *HRAS* gene and/or increased signaling by upstream receptor tyrosine kinases (RTKs) [132, 133]. The role of HRAS in UC induction is further supported by results demonstrating that transfection of an *HRAS* gene will convert SV40 immortalized human urothelial cells into invasive transitional cell carcinomas [141, 142]. Ras interacts with Raf, a serine/threonine kinase, which is activated in tumor cells containing enhanced growth signaling pathways in non–muscle-invasive, muscle invasive, and metastatic disease with subsequent activation of MAPK [143]. The activation of Ras depends on the addition of a lipid (farnesyl) moiety to its carboxy terminal; thus, farnesyl transferase inhibitors and MAPK inhibitors may be of therapeutic potential [143] (Table 37.1).

Ral GTPases

Ras-like (Ral) guanyl nucleotide-binding proteins, RalA and RalB, are two members of the Ras family of monomeric G proteins that share 85 percent amino acid identity [144, 145]. Ral proteins are involved in endocytosis, exocytosis, actin cytoskeletal dynamics, and transcription. Ral involvement in these processes has been shown to be mediated through effectors such as Ral binding protein 1 (RalBP1), Sec5, filamin, phospholipase D1 (PLD1), as well as other unidentified effectors. Recent studies have also indicated roles for Ral proteins in tumorigenesis and cancer progression [144, 145]. Ral-guanyl nucleotide exchange factors (Ral-GEFs) with biochemical specificity for Ral proteins were identified as direct effectors of oncogenic Ras. In cell culture model systems, both gain-of-function and loss-of-function studies identified Ral activation as a proximal consequence of Ras expression that could contribute to Ras-induced oncogenic transformation [146, 147]. Using small interfering RNA (siRNA), it was shown that RalA and RalB have roles in regulating anchorage-independent proliferation, survival, and migration in several human cancer cell lines, including bladder [146, 148, 149].

Ral activation has been reported as a mediator of epidermal growth factor (EGF)-stimulated migration in human bladder cancer cells [150]. Investigation of Ral GTPase activation, mutation status, and expression in human bladder cancers and cell lines revealed that

GTP-bound, activated RalA and RalB are present in cell lines derived from bladder cancers, and that the activation state is higher in lines harboring the *G12V HRAS* oncogene [151]. Overexpression of Ral effectors such as RalBP1 and the metastasis-associated protein CD24 in bladder cancer [151], support the role of Ral GTPases in progression and suggest that targeting them or their effectors may be a rational therapeutic approach.

Rho-GTPases and Their Regulators

The first report on significant association of the Rho/ROCK pathway (reviewed in [131, 152, 153]) with invasion and metastasis of bladder cancer has been reported by Kamai et al. [130], demonstrating significantly higher expression of RhoA, RhoC, and ROCK proteins in primary tumors and lymph node metastases than in non-neoplastic bladder and normal lymph node samples. This high expression in tumors was associated with poor differentiation, muscle invasion, lymph node metastases, and poor survival. In contrast, RhoB expression was inversely related to grade and stage.

Rho GDP dissociation inhibitors (GDIs) bind to Rho proteins in the cytoplasm, conferring aqueous solubility and blocking activation or function through inhibiting the dissociation of bound nucleotides and their interactions with GEFs, GAPs, and effectors. Three RhoGDIs have been identified: GDI1, GDI2, and GDI3. RhoGDI1 was first identified on the basis of its ability to inhibit GDP dissociation from RhoA, CDC42Hs, and Rac1 (reviewed in [129, 153]). At present, there is little evidence for a role for RhoGDI1 or RhoGDI3 in cancer initiation, invasion, migration, or metastasis [131, 153]. In contrast, RhoGDI2 (also known as D4-GDI or Ly-GDI), which shares 67 percent amino acid identity with RhoGDI1 and was initially believed to be exclusively expressed in hematopoietic cells [154], is highly expressed in the epithelium of the genitourinary tract [153]. Importantly, reduced expression of RhoGDI2 correlated with increasing invasive and metastatic activity in human bladder carcinoma lines [155, 156]. Expression analysis and follow-up mechanistic work on a novel animal model of bladder cancer metastasis identified RhoGDI2 as a metastasis suppressor gene [157]. In human bladder tumors, the RhoGDI2 level inversely correlated with development of metastatic disease, and multivariate analysis identified RhoGDI2 as an independent prognostic marker of tumor recurrence following radical cystectomy [155].

Cadherins and Catenins

E-cadherin, a calcium-dependent cell adhesion molecule, mediates the homotypic interactions that maintain tight epithelial integrity and urine impermeability in the normal urothelium [80, 158]. In UC, loss of E-cadherin function through genetic or epigenetic mechanisms has been implicated in the progression and invasive phenotype [80]. Methylation of the E-cadherin promoter, and subsequent gene silencing, have been reported in early and late UC, and the frequency of methylation significantly correlated with progression and poor prognosis [159, 160]. In another study, E-cadherin promoter hypermethylation and LOH in 16q were significantly correlated with tumor grade [161]. Immunohistochemical studies revealed that E-cadherin expression is inversely correlated with tumor grade and stage [162, 163], depth of muscle invasion, lymph node metastasis [164–167], tumor recurrence [168], and five-year survival [169]. Thus, E-cadherin status can be considered as a predictor of disease progression in patients treated with cystectomy [169, 170], either as an independent marker or in conjunction with other adhesion-related markers [171].

Concomitant with loss of E-cadherin expression, N-cadherin, which is not expressed in normal urothelium, appeared in stage pT1, and increased in pT2–pT3 tumors. Progression-free survival and multivariate analyses revealed that N-cadherin expression is an independent prognostic marker for the progression of non–muscle-invasive to invasive UC [172]. N-cadherin has a key role in determining the invasive capacity of UC cells via activation of PI3 kinase/Akt signaling [172, 173]. Abnormal expression of E-cadherin, β-catenin, and p120ctn was associated with high grade and high stage of UC and poor patient survival [36]. The loss of membranous expression of one or more of those glycoproteins has been unanimously attributed to an aggressive phenotype of UC [36, 80]. Urine- and plasma-soluble E-cadherin (sE-cadherin) were found to be higher in patients with UC than in healthy subjects and were elevated in patients with metastases to regional and distant lymph nodes. Preoperative sE-cadherin was independently associated with metastases to regional lymph nodes and disease progression, but not with mortality [32, 33].

Reduced E-cadherin has been reported in NBT-II nitrosamine-induced rat bladder cancer cells as a consequence of Snail activation and AKT-mediated nuclear factor-κB (NF-κB) activation [174]. NF-κB induced Snail expression. Expression of the NF-κB subunit p65 was sufficient for EMT induction [158], validating this signaling module during EMT. NF-κB pathway activation is associated with tumor progression and metastasis of several human tumor types. Thus, this signaling and transcriptional network linking AKT, NF-κB, Snail, and E-cadherin during EMT is a potential target for antimetastatic therapeutics [158, 174].

Recently, Twist, a basic helix–loop–helix transcription factor, has been reported to play a key role in the metastatic progression of UC. In TMA generated from 226 bladder tissue specimens including nonmalignant

bladder tissues, primary bladder cancer tissues, and matched lymph node metastatic lesions, Twist protein expression was significantly higher in UC specimens compared with nonmalignant tissues, and was positively associated with tumor stage and grade. Furthermore, Twist expression was much higher in the metastatic lesions compared with their primary sites, and, more importantly, Twist was negatively correlated with membranous expression of E-cadherin [175]. These findings were further supported by an independent study of the seventy bladder tumors, in which Twist expression was positively associated with current smoking status, tumor stage, and grade, negatively correlated with E-cadherin expression, and predicted poorer progression-free survival [176].

Extracellular Matrix Turnover in Bladder Cancer

Several studies have addressed the expression of basement membrane (BM) proteins and receptors in UC. Aberrant glycosylation of the Sialosyl-Lewis^X (SLe^X) epitope, which increased adhesion of tumor cells to endothelial E-selectin, was reported in invasive/metastatic bladder tumors and was associated with lymph node and distant metastases and a poor five-year survival rate, whereas tumors that did not express the aberrantly glycosylated epitope rarely showed distant metastases [177].

Both laminin and type IV collagen have been used as markers of BM; however, their prognostic value is not universally accepted [80]. Within high-stage urothelial tumors, defects in type IV collagen correlated with poor survival, whereas defects in laminin correlated with metastasis [178]. The concentrations of several BM components (laminin, elastase, and fibronectin) in tissue homogenates were significantly higher in UC than in normal urothelium.

Serum and urine laminin concentrations have been demonstrated to possess a high predictive value in the diagnosis of invasive UC, and together with interruption of the BM laminin staining pattern, suggest BM breakdown and loss [80]. Laminin-5 (LN5), which anchors epithelial cells to the underlying BM, was found to be inactivated owing to promoter methylation in resected bladder tumors and exfoliated cell samples (bladder washes and voided urine). The methylation index and frequency were significantly correlated with several parameters of poor prognosis (tumor grade, stage, growth pattern, muscle invasion, and ploidy pattern), and helped to distinguish invasive from noninvasive tumors [179].

Integrins in Urothelial Cancer

The most commonly expressed integrin in the basal layer of the normal urothelium is $\alpha_6\beta_4$. Altered expression of $\alpha_6\beta_4$ has been reported as an early event in the development of UC, as evidenced by a marked reduction of the integrin β_4 subunit and a predominance of the $\alpha_6\beta_1$ heterodimer in CIS [180]. Few immunohistochemical studies have shown a promising prognostic role of the loss of $\alpha_6\beta_4$ expression with the acquisition of invasive and metastasis phenotype [181, 182]. Conversely, α_v integrin subunit shows a grade- and stage-dependent overexpression in UC, suggesting its importance in cell proliferation and migration [183]. Recently the $\alpha_5\beta_1$ integrin (a fibronectin receptor) has been proposed as the initiating cellular signaling for BCG and gene therapy in UC [184, 185].

Proteases in Urothelial Cancer

High levels of MMP-2 and MMP-9 mRNA were found in invasive than in non–muscle-invasive UC and were positively associated with decreased survival [186], and their urinary levels were significantly linked to high tumor grade and stage, as well as with tissue polypeptide-specific antigen (TPS) and nuclear matrix protein 22 (NMP22) [25, 187]. Levels of MMP-1 positively correlate with disease progression and reduced survival [25, 26, 31, 187–189]. Decreased expression of tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 and TIMP-2 (inhibitors of MMP-9 and MMP-2, respectively), was associated with advanced stage and grade [25, 186, 187, 189–192]. High expression of tissue and urokinase plasminogen activator (tPA and uPA), urokinase plasminogen activator receptor (uPAR), and plasminogen activator inhibitor (PAI)-1 correlate with an unfavorable prognosis in invasive UC [193, 194].

Hyaluronic Acid/Hyaluronidase/Hyaluronic Acid Synthase

Hyaluronic acid (HA), a nonsulfated glycosaminoglycan, is an important component of the ECM [195]. In tumor tissues, elevated HA levels are contributed by both the tumor-associated stroma and tumor cells. HA is degraded by tumor–cell-derived endoglycosidase hyaluronidase (HAase), specifically hyaluronidase 1 (HYAL1) [196, 197]. In tumor xenografts, HA was exclusively localized in tumor-associated stroma, whereas HYAL1 was expressed by tumor cells [30].

The secretion of HAase by tumor cells has been shown to induce angiogenesis through cleavage of HA into angiogenic hyaluronic acid fragments. The presence of these angiogenic hyaluronic acid fragments in the urine of grade 2 and 3 bladder cancer patients suggests that the HA system is active in UC [198]. Urinary HA and HAase levels correlate with their levels in tissues and are elevated in the urine of UC patients, and together they serve as an accurate diagnostic marker

[28, 29, 196, 197]. HA synthesis occurs at the plasma membrane by a transmembrane HA synthase (HAS1, HAS2, or HAS3) [199–202]. HAS1 expression in tumor tissues is a predictor of UC recurrence and treatment failure [37, 203, 204].

HA regulates cell adhesion, migration, and proliferation by interacting with receptors such as CD44. Pericellular HA produced by tumor cells binds CD44 and induces a lipid–raft-associated signaling complex containing phosphorylated ErbB2 (p-ErbB2), PI3-kinase, and CD44, the first two of which have been shown to be important in UC progression. Regarding the third, CD44 is a family of transmembrane glycoproteins involved in cell–cell and cell–matrix interaction, and has been implicated to play an important role in tumor metastasis [195]. In UC, progressive loss of both standard CD44 and the CD44v6 variant were correlated with advanced pathological stage, but their value as independent predictive prognostic markers in invasive UC is still unclear [80, 205–209].

CD24

CD24 is a glycosyl phosphatidyl inositol-linked surface protein that has been identified as a downstream target of Ral signaling by profiling the expression of RalA/B-depleted bladder carcinoma cells [151, 210]. CD24 is highly expressed in bladder as well as other cancers. Loss of CD24 function in cancer cell lines was found to be associated with decreased cell proliferation and anchorage-independent growth, changes in the actin cytoskeleton, and induction of apoptosis [210]. Evaluation of CD24 expression by immunostaining of bladder cancer TMA showed that increased CD24 expression significantly correlated with shorter patient disease-free survival [210]. Choi et al. [211] evaluated 56 pTa, 29 pT1, 19 pT2, and 31 pT3 UC specimens immunohistochemically using an anti-CD24 monoclonal antibody. In normal urothelium, CD24 was localized to the cytoplasm of the luminal cell layer with very low intensity. CD24 expression was upregulated in noninvasive UC, and a high level of expression was correlated with the tumor grade. CD24 expression increased with stromal/muscle invasion, stage, and grade.

Endothelin Axis in UC

Endothelins (ETs) are a family of three 21-amino-acid peptides, ET-1, ET-2, and ET-3, which mediate their action by activating two G-protein–coupled receptors (GPCRs) of $G\alpha_q$ and $G\alpha_s$ subtypes, ETA receptor (ET_AR) and ETB receptor (ET_BR). The endothelin axis has a relevant role in various cancer and stromal cell interactions, leading to autocrine/paracrine loops with subsequent aberrant proliferation, escape from apoptosis, new vessel formation, immune modulation, invasion, and metastatic dissemination.

The importance of the ET axis in UC has been identified by virtue of ET-1 being regulated by RhoGDI2. The loss of RhoGDI2 expression in UC cell lines was correlated with upregulation of ET-1 expression [128]. Independent studies [212, 213] investigated ET-1, ET_AR, and ET_BR expression in samples obtained by radical cystectomy at two urologic institutions by reverse-transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry, and promoter methylation. The ET axis showed positive signals in the majority of bladder cancer samples, compared with the negative normal urothelium. In contrast to ET-1 and ET_AR, ET_BR expression was associated with a more favorable prognosis [212]. ET_BR was found to be methylated in UC as compared with normal urothelium. The frequency of methylation was correlated with tumor grade and stage and was inversely associated with aggressive tumors and poor outcome of the disease [213].

Other Mediators of Urothelial Cancer Progression and Metastasis

Interleukin (IL)-8 expression is increased in muscle-invasive tumors and carcinoma in situ when compared with noninvasive papillary tumors [74, 214]. Cyclooxygenase-2 (COX-2) is not expressed in normal bladder urothelium [215], but was found to be overexpressed at the molecular level in invasive UC. COX-2 expression was also associated with an aggressive disease, increased angiogenesis, lymph node metastasis, increased risk of disease recurrence and disease-specific mortality [213, 215–217]. Thrombospondin (TSP)-1 is an endogenous constituent of the ECM that possesses a tumor suppressor function through inhibition of tumor neovascularization [75, 218]. In an experimental study of UC cell lines, angiogenesis was inhibited by TSP [75]. The intensity of TSP immunostaining of UC tissues was inversely correlated with stage [75], mean vascular density [219], and five-year survival rate, suggesting its usefulness as a prognostic marker [220].

Animal Models of Bladder Cancer

Carcinogen-Induced Bladder Cancer

N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) [221], a downstream metabolite of N-nitrosodibutylamine, found in tobacco smoke and different food and industrial products, is carcinogenic in mice and rats. BBN-induced murine tumors exhibit features of both urothelial and squamous morphology and are often muscle-invasive and metastatic, whereas in rats, BBN induces

almost exclusively papillary tumors that are not muscle-invasive [222, 223]. Consistently, most BBN-induced murine tumors contain p53 mutations, whereas most of the rat tumors do not [221, 223].

Similar to human bladder cancer, H-Ras mutations are observed at low frequencies in both rodent models [221, 223]. BBN-induced carcinogenesis occurs more efficiently in H-Ras transgenic mice and p53 heterozygous mice, than in their wild-types, with 50 percent of the tumors that develop in p53 heterozygous mice displaying loss of the remaining wild-type allele [115, 222, 224]. BBN-induced tumors also display elevated levels of EGFR and COX-2 [223, 225], making them an attractive model system to study the effects of EGFR inhibitors and nonsteroidal antiinflammatory drugs (NSAIDs) on tumor growth and metastasis. Allelic losses within mouse chromosome 4 (syntenic to human 9p21–p22) are also common, mirroring the loss of 9p21–p22 that occurs in human cancer [226].

Comparing gene expression profiles associated with UC for humans, mice, and rats [227] revealed that many human genes homologous to those differentially expressed in carcinogen-induced rodent tumors are also differentially expressed in human disease and are preferentially associated with progression from non–muscle-invasive to muscle-invasive disease. The overall gene expression profiles of rodent tumors corresponded more closely with those of invasive human tumors.

The compound 4-aminobiphenyl (4-ABP), found in tobacco smoke as well as in certain industrial products, has also been linked to human UC [222–224]. 4-ABP forms DNA adducts and induces bladder tumors in mice [228]. After oral administration for about four weeks, a clear time- and dose-dependent incidence of DNA adducts and bladder cancer has been reported. However, because the incidence of the p53 mutational signature of this model does not resemble that of the primary human tumor, this model does not serve as a surrogate of the human disease [11, 229].

Transgenic Mouse Models

Transgenic/knockout mice allow for reproducible generation of models to study carcinogenesis and tumor progression in UC [230]. Furthermore, the cooperative activities among specific oncogenes and tumor suppressor genes can be examined by generating bi- or even tri-transgenic animals that harbor two or more distinct genetic alterations in well-defined genetic backgrounds, averting the tremendous diversity of genetic backgrounds in humans [231, 232]. Moreover, transgenic/knockout mice have proved to be excellent preclinical models for assessing novel diagnostic, preventive, and therapeutic strategies [124, 233, 234].

The ability to drive urothelium-specific expression of specific genes relies on urothelium-specific gene promoter, uroplakin (UP), which is conserved across mammalian species and is expressed in a urothelium-specific fashion [235–246]. UPII promoter has been used to drive urothelium-specific, concentration-dependent expression of oncogenes, mutated tumor suppressors, and growth factor receptors in the urothelium, yielding information regarding the in vivo roles of specific genetic alterations in the multistage bladder carcinogenic process – for example, the urothelial expression of constitutively active and mutated H-Ras oncogene [246–248] inactivation of p53 and Rb pathways in the urothelium by overexpression of SV40-large T antigen and overexpression of EGFR [249, 250].

Human Tumor Xenograft Models

Xenografts of human bladder cell lines in immunodeficient mice have the advantage of using the well-established and well-characterized human bladder cancer cell lines, as well as enabling more rapid and less costly experimentation than carcinogen-induced or UPII-driven spontaneous carcinogenesis. Orthotopic models that recapitulate the two major pathways of bladder cancer presentation (non–muscle-invasive versus invasive), have been established that have proven valuable in preclinical therapeutic studies. Furthermore, metastasis models have been developed to understand the process as well as to test therapeutic approaches.

Orthotopic Xenografts

Human tumor cells are instilled directly into the bladders of nude mice to allow for engraftment without any manipulation of the normal epithelial layer of the bladder. Tumor burden can be quantified either by non-invasive imaging techniques (contrast-enhanced MRI, Fd-glucose PET, various CT methods), or transfecting tumor cells with either luciferase or green fluorescent protein (GFP), and then measuring luciferase activity or GFP fluorescence by intravital imaging, or estimating tumor burden by monitoring GFP fluorescence in voided urine [251, 252]. Such models have been used more extensively to develop novel methods for intravesical delivery of viral gene therapy to the urothelium [124, 134, 233, 234, 251–257].

Sequential orthotopic inoculation has been employed to isolate invasive and metastatic variants of the human 253J TCC cell line [258]. Two such variants, 253J B-V and 253J lung-IV, which grew invasively in the bladder or metastasized to the lung, respectively, were isolated. Orthotopic approaches can also recapitulate the invasive characteristics of the original primary human tumor from which cell lines

were derived much better than ectopic (subcutaneous) approaches can. For example, orthotopic inoculation of RT4 bladder tumor cells maintains their non–muscle-invasive characteristics, whereas inoculation of EJ leads to invasive disease in the mouse [259, 260]. Although these behaviors mirror those of the original primary tumors from which the cell lines were derived, subcutaneous inoculation of both cells does not provide any distinction among them.

Metastasis Assays

Spontaneous Metastasis Assays

Spontaneous metastases arise from primary transplanted "orthotopic" tumor cells in the urothelium [258], from autochthonous models as carcinogen-induced UC, or urothelium-specific (UPII-driven) overexpression SV40-large T antigen with oncogenes [246–250]. Orthotopic injection transplantation has resulted in tumor models that may recapitulate human cancer, including tumor histology, vascularity, gene expression, and metastatic biology [261]. However, such an approach involves mechanical disruption of the target tissue during the implantation, allowing escape of tumor cells into the circulation, seeding distant sites at the onset of the experiment. Transplanted tumors may also suppress secondary tumor growths via diffusible factors [262], suppressing the secondary lesions, and masking the analysis of the earliest phases of the metastatic process [261, 262]. Furthermore, implantation relies on serial in vitro and in vivo passages of cultured cells, allowing selection of metastatic variants in an artificial or foreign milieu [261].

Unfortunately the majority of the autochthonous tumors (carcinogen-induced or genetically engineered models [UPII-SV40-T]) metastasize with a relatively long latency and low frequency, precluding easy and efficient analysis [261].

Experimental Metastasis Assays

Experimental metastasis involves direct injection of a bolus of tumor cells into the venous circulation, eliminating the need for cells to spontaneously escape from a primary tumor, intravasate, and circulate [263, 264]. This assay evaluates the terminal phases of the metastatic cascade, allowing colonization in the secondary site over a short period of time [263, 264]. Reiterative repetitions of tail vein injection [265] of the T24T cell line, a highly tumorigenic and metastatic variant of the T24 line [150, 157, 266] was employed to create the increasingly metastatic cell line FL ("from lung") series, FL1, FL2, and FL3 [267]. Similar approach was adopted for luciferase-transduced UMUC3 cells (UMUC3-Luc) generating lung metastasizing UMUC3-Lul 1–3 series (Theodorescu, unpublished data). Correlating gene expression profiles with clinical data by gene expression analysis led to the discovery of RhoGDI2 as a metastasis suppressor [266]. Variations of the same methodology allowed the identification of two down-regulated candidate genes downstream of RhoGDI2. These two genes, endothelin-1 (ET-1) [128] and neuromedin U (NmU) [268], could be excellent targets for drug therapy to treat metastatic bladder cancer. A novel model of bladder bone metastasis consisting of the T24/TSU-Pr1 cell line and two sublines designated TSU-Pr1-B1 and TSU-Pr1-B2, was generated through successive in vivo intracardiac cycling of bone metastatic lines [269]. This model allowed undermining the molecular mechanisms of metastatic colonization at different secondary sites, and represented the first model with the osteoblastic–osteolytic bone phenotype observed in UC patients [270–272].

SUMMARY AND FUTURE DIRECTIONS

The major problem of metastatic UC is poor prognosis and survival. Molecular understanding of the proteins that drive the invasive and metastatic process outlined in this chapter has begun to bear fruit by providing rational targets of therapy. Continued genetic and epigenetic studies of primary tumors, urine, or serum, as well as available UC cell lines, will allow further identification of biomarkers predictive of grade, stage, and survival. These biomarkers can be used in nomograms in conjunction with conventional clinical and pathological markers to further improve risk stratification and identify high-risk patients, and determine the appropriate treatment regimens – either intravesical therapy, selective adjuvant chemotherapy after cystectomy, or neoadjuvant chemotherapy. Furthermore, novel concepts have been applied to genetic and transcriptional data in bladder cancer that has allowed individualized prediction of response to therapy and drug discovery [273]. These promise to revolutionize personalized medicine and drug discovery in bladder and other cancers.

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38 Bone Complications of Myeloma and Lymphoma

G. David Roodman

OVERVIEW OF MYELOMA BONE DISEASE

Multiple myeloma (MM) is the most common cancer to involve bone, with up to 90 percent of patients developing bone lesions [1]. The bone lesions are purely osteolytic in nature and do not heal in the vast majority of patients. Up to 60 percent of patients develop pathologic fractures over the course of their disease [2]. Bone disease is a hallmark of MM, and myeloma bone disease differs from bone metastasis caused by other tumors. Although myeloma and other osteolytic metastases induce increased osteoclastic bone destruction, in contrast with other tumors, once myeloma tumor burden exceeds 50 percent in a local area, osteoblast activity is either severely depressed or absent [3]. The basis for this severe imbalance between increased osteoclastic bone resorption and decreased bone formation is currently a topic of intensive investigation.

The clinical and economic impact of myeloma bone disease in patients with myeloma can be catastrophic. Saad and coworkers [4] retrospectively assessed the impact of pathologic fractures on survival of patients with malignant disease. Patients with myeloma had the highest incidence of fracture (43%) compared with patients with breast cancer, prostate cancer, and lung cancer, respectively. Myeloma patients who experienced pathologic fractures had at least a 20 percent increased risk of death compared with myeloma patients without pathologic fractures. Further, patients who had a prior skeletal-related event, which included pathologic fracture, spinal cord compression syndrome, surgery to bone, or radiation therapy to bone, were more likely to develop new pathologic fractures as compared with patients who did not have a prior skeletal-related event.

CLINICAL MANIFESTATIONS OF MYELOMA

Bone destruction in MM can involve any bone, with the spine, skull, pelvis, and ribs being more frequently involved [5]. The most common radiographic findings of bone involvement included osteolysis, osteopenia, and/or pathologic fractures. Eighty percent of patients experience bone pain. Hypercalcemia occurs in approximately 15 percent of myeloma patients [6], and results predominantly from widespread bone resorption in patients with impaired renal function. Parathyroid hormone-related protein (PTHrP), the major mediator of the humoral hypercalcemia of malignancy [7], is increased in only a minority of patients with myeloma and is not a frequent cause of hypercalcemia in myeloma [6].

PATHOPHYSIOLOGY OF MYELOMA BONE DISEASE

In contrast to normal bone remodeling, which involves the resorption of bone by osteoclasts and the deposition of new bone by osteoblasts at sites of previous resorption, in myeloma, bone resorption is increased and bone formation is suppressed or absent. Furthermore, growth factors released by the increased bone resorption process also increase the growth of myeloma cells [8]. This creates a "vicious cycle" with the bone resorption process increasing myeloma cell tumor burden, which then results in further bone destruction.

Recent studies have identified several important factors produced by myeloma cells in vivo that have been implicated in the osteolytic bone resorptive process. These include the receptor activator NF-κB (RANKL), macrophage inflammatory protein 1-alpha (MIP-1α), interleukin (IL)-3, and IL-6 [9–12].

FACTORS INVOLVED IN OSTEOCLAST ACTIVATION IN MYELOMA

The RANK/RANKL signaling pathway is a critical component of both normal and pathological bone remodeling processes. RANK is a transmembrane signaling

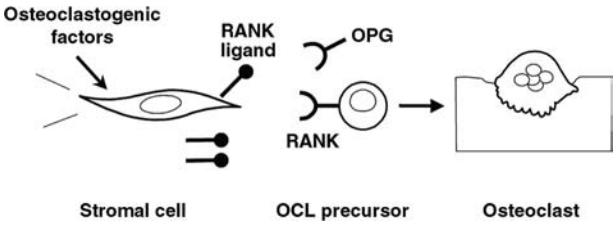

Figure 38.1. Local expression of stromal cell RANK ligand (RANKL), osteoclast (OCL) formation, and bone resorption. Locally secreted osteoclastogenic factors (e.g., cytokines and growth factors) in the bone marrow microenvironment stimulate the formation of both membrane-bound and soluble RANKL by bone marrow stromal cells. After it binds membrane-expressed RANK on adjacent preosteoclasts, RANKL induces OCL formation and OCL-mediated bone resorption. This process may be regulated by the local secretion of osteoprotegerin (OPG), a soluble decoy receptor that blocks the effects of RANKL. However, contact between myeloma cells and stromal cells in the bone marrow microenvironment decreases stromal cell production of OPG, thereby initiating high levels of osteolysis.

receptor, which is a member of the tumor necrosis receptor superfamily. It is found on the surface of mature osteoclasts and their precursors [13, 14]. RANK ligand (RANKL) is expressed as a membrane-bound protein on marrow stromal cells and osteoblasts, and secreted by activated lymphocytes. Its expression is augmented by cytokines that stimulate bone resorption [15], such as PTH, 1,25 $(OH)_2$ vitamin D_3, and prostaglandins (Figure 38.1) [16, 17]. RANKL binds to RANK receptor on osteoclast precursors and induces osteoclast formation. Rank signals through the NF-κB and JunN terminal kinase pathways and induces increased osteoclastic bone resorption and enhanced osteoclast survival [8]. The important role of RANKL in normal osteoclastogenesis has been clearly demonstrated in RANKL or RANK gene knockout mice. These animals lack osteoclasts and as a result develop severe osteopetrosis [18, 19].

Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and is a member of the TNF receptor superfamily [20]. It is produced by osteoblasts as well as other cell types and blocks the interactions of RANKL with RANK, thereby limiting osteoclastogenesis. In normal subjects, the ratio RANKL/OPG greatly favors OPG. Knockout of the OPG gene in mice results in severe osteopenia and osteoporosis [19–23]. Pearse and coworkers demonstrated that RANKL expression was increased in bone marrow biopsies of MM patients, whereas OPG expression was decreased [24], and Terpos and coworkers showed that circulating levels of OPG and RANKL correlated with clinical activity of myeloma, severity of bone disease, and poor prognosis [25]. Further, inhibition of RANKL prevented bone destruction in either the murine SCID-hu model or the T2 MM syngeneic model of myeloma [25, 26]. These studies revealed that blocking RANKL decreased bone destruction and tumor burden. In addition, myeloma cells have been reported to express RANKL, which may further contribute to the bone-destructive process.

MIP-1α is a chemokine that is produced by MM cells in 70 percent of MM patients and is a potent inducer of human osteoclast formation. MIP-1α can increase osteoclast formation independently of RANKL and can potentiate both RANKL and IL-6–stimulated osteoclast formation [27]. Magrangeas et al. have shown by gene expression profiling that MIP-1α is the gene most highly correlated with bone destruction in myeloma [28]. Further, Abe and coworkers have shown that elevated levels of MIP-1α also correlate with an extremely poor prognosis in myeloma [29]. In vivo models of myeloma have demonstrated that MIP-1α can induce osteoclast formation and bone destruction and that blocking MIP-1α expression in myeloma cells injected into SCID mice or treating the animals with a neutralizing antibody to MIP-1α results in decreased tumor burden and bone destruction [30, 31]. MIP-1α also increases adhesive interactions between myeloma cells and marrow stromal cells by increasing expression of β_1 integrins, which takes place through $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrins and adhesive molecules such as VCAM-1. This results in production of RANKL, IL-6, VEGF, and TNFα by marrow stromal cells, which further enhances myeloma cell growth, angiogenesis, and bone destruction. Further, Masih-Khan et al. reported that the t4:14 translocation results in a constitutive expression of the FGFR3 receptor, which results in high levels of MIP-1α [32]. Patients with the t4:14 translocation have a very poor prognosis, which may reflect the increased MIP-1α production in this patient population.

IL-3, in addition to RANKL and MIP-1α, is also significantly elevated in bone marrow plasma of MM patients as compared with normal controls [12]. IL-3 can induce osteoclast formation in human bone marrow cultures at levels similar to those measured in myeloma patient samples, and osteoclast formation induced by marrow plasma from MM patients could be inhibited by using a blocking antibody to IL-3 [12]. IL-3 also indirectly influences osteoclastogenesis by enhancing the effects of RANKL and MIP-1α on the growth and development of osteoclasts and can stimulate myeloma cell growth directly [12].

IL-6 has been long recognized as a proliferative factor for plasma cells and is an osteoclastogenic factor, but it is unclear whether IL-6 levels correlate with disease status [33]. Levels of IL-6 are elevated in MM patients with osteolytic bone disease when compared with MM patients without bone disease, as well as in patients with monoclonal gammopathy of unknown significance (MGUS) [34]. Most studies support the

idea that IL-6 is produced by cells in the bone marrow microenvironment through direct contact with myeloma cells rather than myeloma cells. The cells producing IL-6 most likely are osteoclasts and stromal cells, but increased osteoblast production of IL-6 has been also reported in co-cultures of human osteoblasts with MM cells [35]. Although the precise role of IL-6 in myeloma bone disease has not been determined, IL-6 production by osteoclasts can increase tumor burden, leading to enhanced bone destruction, as well as act as an autocrine/paracrine factor to increase osteoclast formation [36].

OSTEOBLAST INHIBITION IN MYELOMA

Histomorphometric studies have shown that bone remodeling is uncoupled in MM with increased bone resorption and decreased or absent bone formation. Thus, MM patients have low levels of bone formation markers, such as alkaline phosphatase and osteocalcin [37]. This explains why bone scans underestimate the extent of MM bone disease, as bone scans reflect new bone formation.

In the past few years, signaling pathways involved in osteoblastic differentiation have been identified, which provide a better understanding of the inhibition of osteoblast activity in myeloma. In addition, these studies have identified several potential therapeutic targets for treating MM bone disease.

The formation and differentiation of osteoblasts from mesenchymal cells require the activity and function of the transcription factor Runx2/Cbfal [38]. Runx2/Cbfal–deficient mice (Runx2) completely lack osteoblasts and bone formation [38]. Human osteoblast differentiation is associated with increased Runx2/Cbfal activity without a change in Runx2 protein levels, although it has been reported that Runx2/Cbfal overexpression can also impair bone formation. These results indicate that a time-dependent expression of Runx2 drives osteoblast differentiation and plays a critical role in this process.

Inhibition of Runx2/Cbfal activity in MM bone disease has recently been demonstrated [39]. When MM cells were co-cultured with osteoprogenitor cells, the MM cells inhibited osteoblast differentiation and reduced numbers of both early osteoblast precursors, as well as the more differentiated precursors [39]. Interestingly, this effect was mediated by blocking Runx2/Cbfal activity in osteoprogenitor cells. In addition, because Runx2/Cbfal stimulated secretion of the RANKL decoy receptor, OPG, in osteoprogenitor cells [40], it is possible that inhibition of Runx2/Cbfal activity also increases osteoclastogenesis. The interaction between Runx2/Cbfal and MM cells appears to be mediated by cell-to-cell interaction between MM cells and osteoprogenitors. This cell-to-cell interaction is dependent on VLA-4 on MM cells and VCAM-1 on osteoblast precursors, because neutralizing anti-VLA-4 antibodies reduce the inhibitory effect of MM cells on Runx2/Cbfal activity [39].

IL-3 appears to play a dual role in the bone-destructive process in myeloma. As noted previously, it can stimulate osteoclast formation and bone resorption and, in addition, indirectly inhibit osteoblast formation. Treatment of primary mouse or human marrow stromal cells with IL-3–inhibited bone morphogenic protein (BMP)-2–stimulated osteoblast formation, and marrow plasma from myeloma patients that expressed high IL-3 levels inhibited osteoblast differentiation, which was reversed by an anti–IL-3 antibody.

IL-7 also can inhibit osteoblasts in MM. IL-7 levels are increased in marrow plasma samples from patients with MM compared to normal controls [39]. IL-7 is a very potent inhibitor of osteoblast differentiation and can affect osteoblast formation in several ways, including interfering with Runx2 activity [39, 41, 42].

WNT SIGNALING PATHWAY INHIBITORS IN MM BONE DISEASE

The Wnt signaling pathway plays an important role in skeletogenesis by promoting the proliferation, expansion, and survival of premature and immature osteoblastic cells [43]. Osteoblasts produce several soluble inhibitors of the canonical Wnt pathway, including Dickkopf (DKK)-1, secreted frizzled-related protein (sFRP), and Wnt inhibitor factor (Wif-1).

Tian and coworkers reported the production of DKK-1 by primary $CD138^+$ MM cells but not by plasma cells from MGUS patients, and also demonstrated that levels of DKK-1 mRNA correlated with focal bone lesions in patients with myeloma [44, 45]. In contrast, patients with advanced disease, as well as some human myeloma cell lines, did not express DKK-1, suggesting that such an inhibitor may mediate bone destruction only in the early phases of disease [44]. Anti-DKK1 antibody administration to SCID-hu mice injected with primary myeloma cells inhibited myeloma cell growth and increased bone formation in the implanted fetal bone. Myeloma cells also produce sFR2 [46], which may suppress osteoblast differentiation in MM.

In addition to osteoblastogenesis inhibition, elevated DKK-1 levels also appear to enhance osteoclastogenesis. Wnt signaling in osteoblasts upregulates expression of OPG [47] and downregulates the expression of RANKL [48], suggesting a possible mechanism by which inhibition of Wnt signaling in osteoblasts would indirectly increase osteoclastogenesis. Taken together, these studies indicate that DKK-1 is a key regulator of bone remodeling in both physiological and pathological

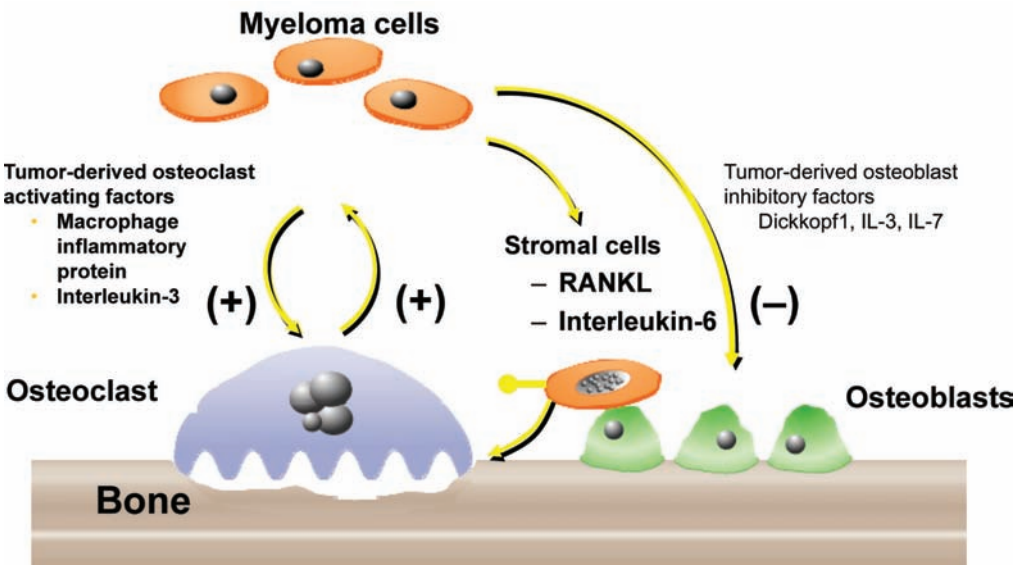

Figure 38.2. Mechanisms responsible for myeloma bone disease. Myeloma cells produce factors that directly or indirectly activate osteoclasts, such as MIP-1α and IL-3. In addition, they induce RANK ligand and IL-6 production by marrow stromal cells to enhance osteoclast formation. The bone-destructive process releases growth factors that increase the growth of myeloma cells, further exacerbating the osteolytic process. Myeloma cells also produce DKK1, IL-3, soluble frizzle-related protein-2, and IL-7, which suppress osteoblast differentiation and new bone formation.

conditions and that blocking this factor may contribute to both stimulation of osteoclastogenesis and inhibition of osteoblasts in myelomatous bones. Thus, multiple stimulators of osteoclast activity and suppressors of osteoblast differentiation are present in myeloma and together result in the devastating bone disease present in these patients (Figure 38.2).

EVALUATION OF BONE INVOLVEMENT IN MYELOMA

Myeloma lesions are characterized by discrete lytic lesions without evidence of reactive bone formation (Figure 38.3). Almost 80 percent of patients with myeloma will have radiologic evidence of skeletal involvement on metastatic bone surveys, with the vertebrae, ribs, skull, shoulders, pelvis, and long bones being the most frequently involved [49]. However, plain radiography has relatively low sensitivity and can demonstrate lytic bone disease only when at least 30 percent of trabecular bone has been lost [50]. If conventional radiography is inconclusive or negative in the setting of high clinical suspicion for bone disease, CT without contrast, PET/CT, or MRI may be used. These modalities are more sensitive than conventional radiography for detecting occult bone disease.

TREATMENT OF MYELOMA BONE DISEASE

Treatment of myeloma bone disease involves treatment of the underlying malignancy and its manifestations. Current treatments include use of chemotherapy and autologous hematopoietic stem cell transplantation for myeloma, localized radiation therapy to control pain or impending fracture or treat solitary plasmacytoma, kyphoplasty or vertebraplasty for vertebral fractures, and surgery to bone and inhibiting bone resorption and osteoclast formation with bisphosphonate therapy.

Bisphosphonate therapy, which inhibits osteoclast formation and activity, is the mainstay of treating myeloma bone disease [51]. Bisphosphonate therapy decreases bone pain and progression of lytic lesions, prevents development of new pathologic fractures, and may improve survival. Intravenous pamidronate or zoledronate given once monthly is the mainstay of bisphosphonate therapy in the United States. Zoledronate (or zoledronic acid) is the most potent bisphosphonate used in the management of myeloma bone disease; it has similar efficacy to pamidronate but can be given over a shorter period of time (15 minutes vs 2 hours) [52].

Current recommendations suggest starting bisphosphonate therapy for myeloma when there is evidence of bone involvement [53]. The optimal duration and frequency of bisphosphonate therapy in myeloma are not well understood and are currently being studied. ASCO guidelines currently recommend using either pamidronate or zoledronate in patients with lytic destruction of bone or spinal cord compression on imaging or with diffuse osteopenia [53]. Patients with renal impairment should receive pamidronate over a longer infusion time.

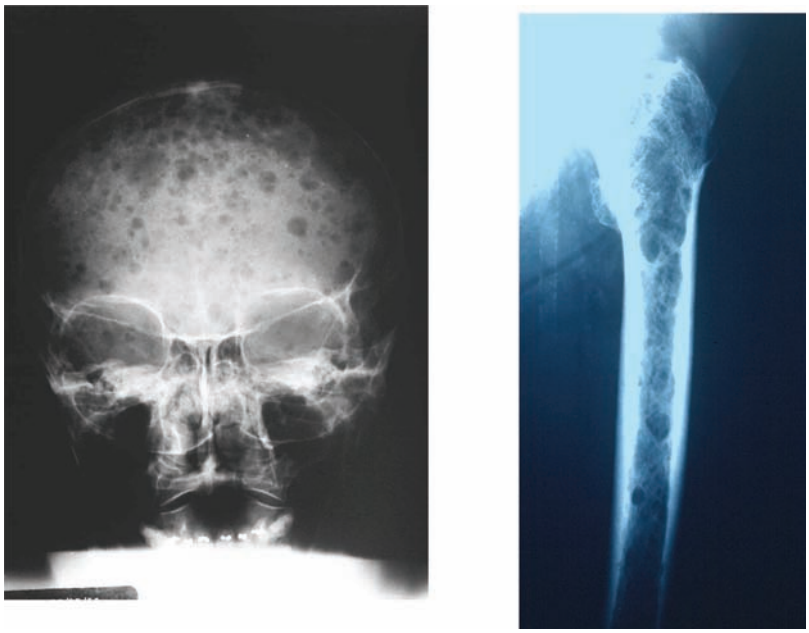

Figure 38.3. Plain-film radiographs of lytic bone lesions in multiple myeloma. Courtesy of Henry J. Mankin, MD, at Massachusetts General Hospital.

OSTEONECROSIS OF THE JAW ASSOCIATED WITH BISPHOSPHONATE THERAPY

An emerging complication associated with bisphosphonate therapy is osteonecrosis of the jaw (ONJ), although a cause-and-effect relationship has not been clearly demonstrated. Patients with myeloma have been reported to have the highest incidence of ONJ (1.6%–11%; reviewed in [54]), whereas patients with postmenopausal osteoporosis treated with oral bisphosphonates have an incidence of ONJ of 1/10,000 to 1/100,000 patient-treatment years [55]. Bisphosphonate-associated ONJ is defined as the presence of exposed bone in the mandible or maxilla in patients receiving bisphosphonate therapy that does not heal within eight weeks of appropriate dental management in the absence of local metastatic disease or previous radiation therapy [54]. Patients can have single or multiple lesions, with the mandible more frequently involved than the maxilla. Most patients have only exposed bone, although fistulas to the maxillary sinus or the skin can occur, and pathologic fractures of the mandible have been reported [54]. The risk factors associated with development of ONJ for patients on bisphosphonate therapy appear to be the duration of bisphosphonate therapy, presence of active myeloma, and a previous dental extraction or dental surgery. Current treatment of ONJ associated with bisphosphonate therapy is conservative management with oral rinses and antibiotic therapy for weeks to months.

Stopping or continuing bisphosphonate therapy in myeloma patients who develop ONJ remains a major question. Stopping bisphosphonate therapy does not accelerate healing in patients with ONJ, and patients can heal with continued bisphosphonate therapy. Furthermore, bisphosphonates have an extremely long half-life in bone, which has been estimated to be more than ten years, so stopping bisphosphonates may not have any effect on ONJ. However, several consensus statements have suggested stopping or considering stopping bisphosphonate therapy in patients who have received two years of bisphosphonate therapy and are in plateau phase or are in complete remission [53, 56]. In patients who have progressive bone disease, reinstitution or continuation of bisphosphonate therapy should be considered after the risks and benefits have been discussed with the patient.

BONE INVOLVEMENT IN LYMPHOMA

Hodgkin Disease

Bone involvement in Hodgkin disease (HD) occurs with a frequency of 10 percent to 15 percent [57]. Bone lesions in HD are often multiple and seldom seen in early stages of the disease [58]. Clinically, pain is the most common symptom from bone involvement in HD. Sites of involvement include the spine, pelvis, femur, humerus, ribs, sternum, scapula, and base of the skull [59]. However, as with non-Hodgkin lymphoma (NHL), vertebral and femoral involvement are the most common sites affected [60]. Hypercalcemia, when it occurs, is the result of excess production of $1,25(OH)_2$ vitamin D_3 or PTHrP by the lymphoma cells [61, 62]. Radiographic findings include a vertebral sclerotic pattern, along with periosteal reaction and hypertrophic pulmonary osteoarthropathy [60]. Bone disease in patients with HD can be lytic, blastic, or mixed, with the mixed form of bone disease the most prevalent [63–66].

Non-Hodgkin Lymphoma

Approximately 7 percent to 25 percent of patients with NHL will develop bone involvement during their course of disease [67]. About 4 percent to 9 percent of patients present with bone destruction at time of initial diagnosis [68, 69]. Bone lesions can range from lytic to densely osteoblastic lesions, but lytic lesions predominate [68, 69]. The more highly aggressive and poorly differentiated the lymphoma, the more lytic the bone

metastases with little or no sclerosis. NHL has a predilection for the axial skeleton, with about 75 percent of bone involvement in NHL being in the axial skeleton [70]. Overall, patients with diffuse rather than nodular patterns of involvement more frequently have lytic lesions.

Adult T-Cell Leukemia/Lymphoma Bone Disease

Adult T-cell leukemia/lymphoma (ATL) is an uncommon aggressive peripheral T cell neoplasm of $CD4^+$ T cells associated with infection by the human T-lymphotropic virus, type 1 (HTLV-1) [71]. The cumulative risk of developing ATL in patients harboring HTLV-1 is approximately 2.5 percent over seventy years.

Approximately 70 percent of patients with ATL develop hypercalcemia at some point during the course of their disease [72]. Hypercalcemia is a major cause of morbidity and mortality in patients with ATL. Hypercalcemia in ATL is multifactorial in etiology; humoral hypercalcemia of malignancy seems to be the predominant mechanism by which hypercalcemia occurs in ATL. Several studies found that a significant number of patients with ATL had low phosphate levels, hypercalcemia, and low levels of 1,25-$(OH)_2$ vitamin D_3. These laboratory values were consistent with humoral hypercalcemia of malignancy and are secondary to increased production of PTHrP by ATL cells. PTHrP induces production of RANKL in marrow stromal cells, thus promoting differentiation and maturation of osteoclasts. The increased osteoclast formation leads to increased bone resorption and increased release of calcium. PTHrP also acts on the kidney to increase distal tubular reabsorption of calcium, further increasing serum calcium levels. This increased reabsorption of calcium by the kidney is thought to be the primary mechanism by which PTHrP induces hypercalcemia of malignancy.

The pathophysiology of the increased bone resorption in ATL patients is similar to that of patients with myeloma bone disease, with cytokines or factors secreted by the lymphoid tumor cells or bone marrow stromal cells responsible for increasing the activity of osteoclasts. The factors associated with increased osteoclast activity in ATL include IL-1, IL-6, TNF-$\alpha\beta$, and MIP-1αMIP-1β, which can increase bone resorption [73]. MIP-1α has been reported to increase bone resorption by stimulating production of osteoclastogenic factors such as IL-6, RANKL, and PTHrP by osteoblasts and bone marrow stromal cells (BMSCs) in patients with ATL [27]. IL-1, 1,25-$(OH)_2$ vitamin D_3, and PTHrP have been reported to be elevated in patients with ATL and associated with increased osteoclast activity and increased bone resorption in vitro [74].

The mainstay of treatment of bone involvement in ATL is management of the underlying disease and decreasing tumor burden. Hypercalcemia associated with ATL is also managed by treating the underlying disease in an effort to decrease tumor burden, as well as using intravenous bisphosphonates.

SUMMARY

Bone involvement is very common in myeloma and very uncommon in lymphomas. Treating bone involvement in myeloma, in addition to targeting the underlying disease, also involves treatment of patients with bisphosphonates, such as zoledronic acid or pamidronate, to block osteoclast activity and induce osteoclast apoptosis. Treatment of bone involvement in lymphomas focuses predominantly on treating the underlying disease, although hypercalcemia in ATL is treated with bisphosphonates. More importantly, the identification of the underlying pathophysiology of bone involvement in myeloma and lymphoma has led to the development of novel agents to treat this devastating complication of myeloma and lymphoma. New agents already in clinical trials include denosumab, an antibody to RANKL, as well as an antibody to DKK-1. In the future, small-molecule antagonists to receptors for the cytokines and hormones responsible for the bone involvement produced should emerge from preclinical studies and be available to the clinic to help reverse bone disease in these patients.

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39 Breast Metastasis

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PATTERNS OF METASTATIC SPREAD

Background

Breast cancer is an important public health problem, with more than one million new cases worldwide annually.[1] In the developed world, early-stage disease represents an increasing proportion of incident breast cancer diagnoses, whereas metastatic breast cancer (MBC) without a preceding diagnosis of early-stage disease is a rare event. However, despite an early-stage diagnosis, up to one-third of patients will experience distant relapse. MBC is treatable but incurable, with a median survival of two to three years. Consequently, clinicians often recommend systemic adjuvant therapy for patients with early breast cancer (EBC), as it prevents or delays the development of incurable metastatic disease. Adjuvant therapy may comprise hormonal therapy, cytotoxic chemotherapy, and targeted therapy, depending on the breast cancer subtype, the individual's risk of recurrence, and various patient-specific factors including age, menopausal status, and comorbid conditions.

Breast cancer is a heterogenous disease with respect to natural history and response to therapy. More than 95 percent of breast cancers arise from the breast epithelium and are carcinomas. The two most common histological subtypes are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), which account for approximately 75 percent and 15 percent of breast cancers, respectively.[2] IDC cells are typically clustered in well-formed glandular structures, whereas ILC cells are frequently organized in single file. These distinct cellular patterns are commonly used to distinguish the two histologic subtypes. Staining for the transmembrane glycoprotein, E-cadherin, inactivation of which is strongly associated with lobular breast cancer, is also increasingly used in histologic evaluation to distinguish these two subtypes.

Endocrine-Responsive Breast Cancer

The majority of breast cancers are dependent on the steroid hormones estrogen and progesterone for growth, at least initially. This effect is mediated through binding of the hormones to the cytosolic estrogen and progesterone receptors, respectively. Estrogen binding to the estrogen receptor (ER) triggers a signaling cascade leading to transcription of genes involved in proliferation, inhibition of apoptosis, and the promotion of angiogenesis, invasion, and metastasis. Overall, approximately 75 percent of invasive breast cancers express ER, and 75 percent of these will also express the progesterone receptor (PR).[3] When expressed, the PR is usually a surrogate marker for an intact ER; therefore, all PR-positive tumors should also express ER.[3] In a recent series of more than 5000 cases, there were no tumors classified as ER-negative/PR-positive (Table 39.1). The expression of ER is both a prognostic marker of favorable outcome and a strong predictor of response to endocrine therapy. ILCs are more likely than IDCs to express ER. In fact, almost 100 percent of true ILCs express ER.[2,3]

Increasing age at diagnosis is associated with increased expression of ER and PR and other favorable biological characteristics.[4,5] During the 1990s, the proportion of all incident hormone–receptor-positive breast cancers in the United States increased, and was temporally associated with an increased use of hormone replacement therapy (HRT). Following the reporting of the association between HRT and incident breast cancers in the Women's Health Initiative study, HRT prescriptions declined dramatically.[5,6] Thereafter, a significant decline in incident hormone-sensitive EBCs was reported among postmenopausal women.

Immunohistochemistry (IHC) using anti-ER and anti-PR antibodies is currently the most common method for ER and PR testing in clinical practice.[7]

TABLE 39.1. Status of ER and PR in 5,497 cases of infiltrating mammary carcinoma in histologic specimens

Receptor	No. (%)
ER+	4,100 (75)
PR+	3,016 (55)
ER+/PR+	3,016 (55)
ER+/PR–	1,084 (20)
ER–/PR–	1,397 (25)
ER–/PR+	0(0)

ER, estrogen receptor: PR, progesterone receptor; +, positive; –, negative. From Nadji et al. 2005.[3]

Hormone receptor status may be expressed both as a percentage of cells staining positive and the intensity of staining. Differences exist among individual laboratories as to what defines ER and/or PR positivity, and several scoring systems have been developed. The widely used Allred system uses semiquantitative IHC scoring, but the optimal scoring strategy is uncertain, as two recent studies of almost 7000 breast cancers found that the distribution of ER values using current IHC techniques was bimodal, with more than 90 percent of tumors being either completely ER-negative or unequivocally ER-positive.[3,8,9] The American Society of Clinical Oncology (ASCO) does not recommend a specific cutoff for considering a tumor ER-positive, and guidelines from the U.S. National Institutes of Health suggest that any ER staining should be sufficient to consider the patient a possible candidate for endocrine therapy.[10,11] This leads to some degree of clinical uncertainty in terms of predicting true hormone-responsiveness.

HER2

The human epidermal growth factor receptor 2 (HER2) is a member of the family of epidermal growth factor receptors that regulate cell growth and survival.[12] HER2 signaling promotes cell proliferation through the RAS–MAPK pathway. In addition, HER2 signaling inhibits cell death through the phosphatidylinositol 3′-kinase–AKT–mTOR pathway.[12] Approximately 20 percent to 30 percent of breast cancers are classified as HER2-"positive."[12] Breast cancer is considered HER2-"positive" when the *HER2neu* gene is amplified and/or the HER2 protein is overexpressed. *HER2neu* gene amplification is frequently detected by fluorescent in situ hybridization (FISH), whereas HER2 protein expression is typically detected by IHC. IHC is a semiquantitative method whereby HER2 status is scored on a scale of 0 to 3+, where 3+ is considered positive, 0 or 1+ are considered negative, and 2+ is considered equivocal. For patients with 2+ staining by IHC, FISH is recommended.[13] By FISH, a gene copy number of $\geq$2.0 defines HER2-positive disease.

Because of the reported inconsistencies in HER2 status determination in several of the large, randomized adjuvant trastuzumab trials, other methods for determining HER2 status, such as chromogenic in situ hybridization (CISH) and representational oligonucleotide microarray analysis (ROMA), are also being investigated.[13,14] A panel of ASCO and the College of American Pathologists (CAP) experts recently published a set of guidelines on HER2 testing.[13] The expert panel strongly recommended that laboratories offering HER2 testing should be accredited on an annual basis.[13] Furthermore, it is notable that although HER2 is overexpressed in other malignancies, gene amplification is rare outside breast cancer. Consequently, HER2-targeted therapy is currently indicated mainly (outside of research protocols) in the treatment of breast cancer.

Breast Cancer Gene Expression Profiles

Based on gene expression patterns, breast cancer has been classified into at least five subtypes, including luminal A and B, HER2-positive, basal-like, and normal breastlike.[15,16] The main distinction in this classification system is between tumors that express genes characteristic of luminal epithelial cells (including ER) and tumors that are negative for these genes (basal-like tumors). Luminal tumors are further divided into luminal A (ER-positive, HER2-negative) and luminal B (ER-positive, HER2-positive). Basal-like tumors are so called because their genetic expression profiles more closely resemble basal epithelial cells than luminal cells. Normal basal epithelial cells lie closest to the basement membrane of the mammary gland epithelium and hence furthest from the lumina of lactiferous ducts. Classically, basal-like tumors do not express ER, PR, or HER2 and are termed "triple negative," although not all basal-like tumors are triple negative and vice versa.

Patterns of Recurrence

Gene expression profiles provide important prognostic information about the natural history of specific cancer subtypes. For example, ER-negative breast cancers are associated with an early peak of recurrence at two to three years that declines dramatically thereafter (Figure 39.1). In contrast, ER-positive tumors are associated with a more modest recurrence peak at about two to three years and a relatively constant recurrence risk beyond five years. Consequently, although recurrences are rare beyond five years for women with ER-negative breast cancer, late recurrences are not uncommon among women with ER-positive

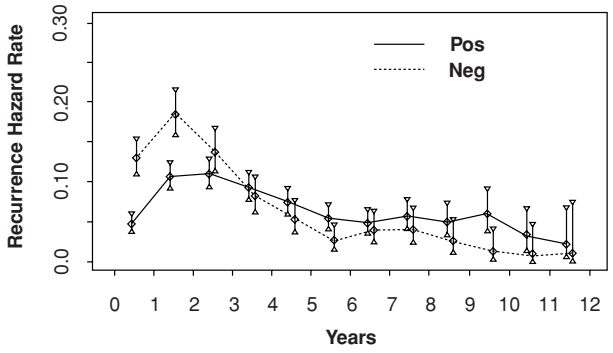

Figure 39.1. Annual hazard of breast cancer recurrence by ER status (Saphner et al. *JCO* 1996).[17]

breast cancer. In fact, in the Oxford overview, a meta-analysis that included data from 10,386 patients with hormone-sensitive breast cancer, 33.2 percent of patients experienced a recurrence despite five years of adjuvant tamoxifen, and more than half these recurrences occurred between years 6 and 15 after diagnosis (Figure 39.2).[17]

Historically, HER2 overexpression was considered an adverse prognostic variable and was associated with a more aggressive phenotype. The prognostic impact of HER2 status has since changed with the development of trastuzumab, a humanized monoclonal antibody directed against HER2. In HER2-positive EBC, trastuzumab in combination with chemotherapy followed by maintenance trastuzumab for up to one year of therapy has resulted in significant survival benefits in this population.[18–21]

Sites of Metastases

Although breast cancer can potentially spread to any distant site, common sites of metastases include bone, liver, lungs, and lymph nodes. In ER/PR-positive breast cancer, late relapses many years after diagnosis tend to involve bone preferentially. This so-called bone-dominant MBC tends to be more indolent and to respond to serial endocrine therapies. From a clinical perspective, these patients form an important subgroup, with a better overall prognosis and greater number of therapeutic options. The definitive reasons for this pattern of metastases have not been completely elucidated but probably relate to an interplay between factors from the primary tumor and the microenvironment of the metastatic site. For more than one hundred years it has been recognized that various tumors express a preference to metastasize to certain organs, and that this must imply a hospitable environment in those tissues.[22,23] It was Paget who proposed that tumor cells ("seeds") must be predisposed to arrest and proliferate only in those anatomical sites ("soil") that provide the appropriate microenvironment, leading to the "seed-and-soil" hypothesis.[23] Recently, genetic profiling of some breast cancers has added weight to this hypothesis by linking the expression of certain genes with site-specific metastases.[22] For example, a specific gene signature has been proposed that appears to predict the development of lung metastases in a xenograft breast cancer model.[24] Likewise, it seems likely that in ER/PR-positive bone-dominant MBC there is a specific interplay between the genetic profile of tumor cells and various growth factors.[24]

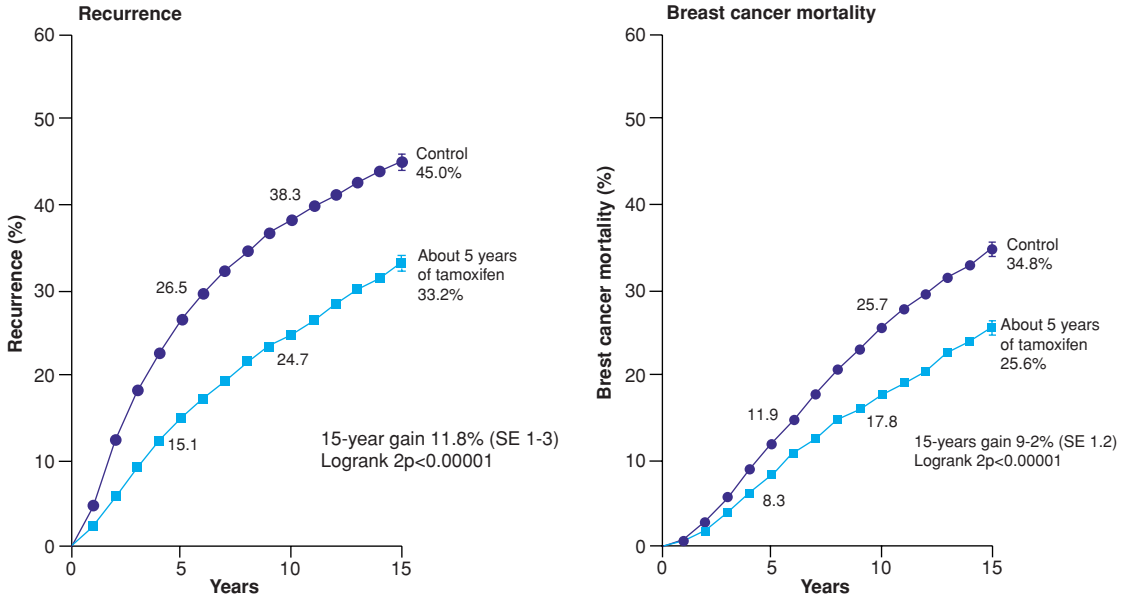

Figure 39.2. Five years of Tamoxifen reduces the risk of late occurrences (EBCTCG lancet 05).[17]

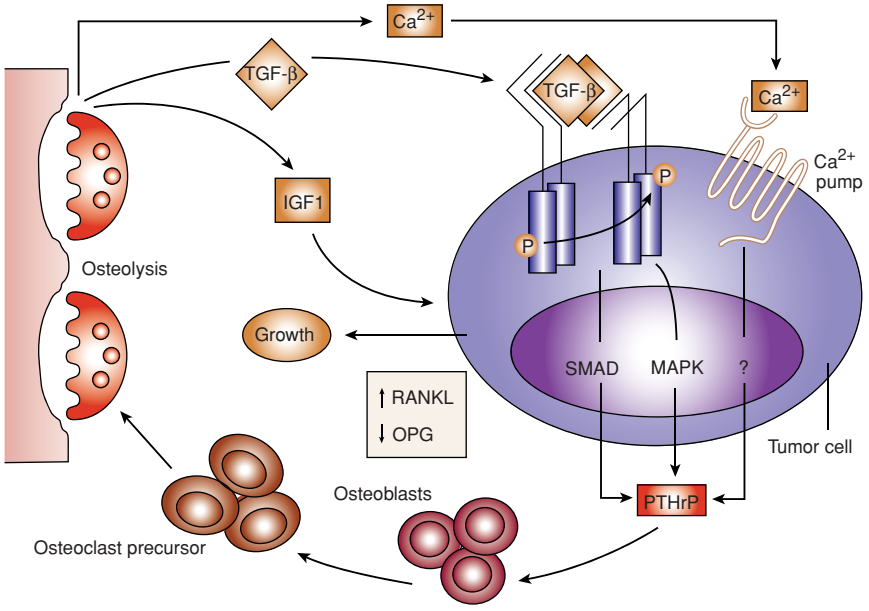

Figure 39.3. "Vicious circle" hypothesis leading to the growth of bony metastases (Mundy et al. *Nat Rev Cancer*).[113] Ca^{2+}: calcium; PTHrP: parathyroid hormone-related peptide; RANKL: receptor activator of nuclear factor-κB ligand; OPG: osteoprotegrin; TGF-β: transforming growth factor-β; IGF1: insulinlike growth factor 1; P: autophosphorylation; MAPK: mitogen-activated protein kinase.

It has been proposed that tumor cells produce parathyroid–hormone-related peptide (PTHrP) and, through a series of steps, cause the release of growth factors from bone, which ultimately lead to autophosphorylation of tumor cells and the "vicious circle" of cell growth (Figure 39.3).

In brief, tumor cells produce PTHrP, which activates osteoblasts to produce receptor activator of nuclear factor-κB ligand (RANKL) and downregulate osteoprotegerin (OPG). In turn, this activates osteoclast precursors, leading to osteolysis and the release of bone-derived growth factors, such as transforming growth factor (TGF)-β and insulinlike growth factor (IGF)-1, and raising extracellular calcium (Ca^{2+}) concentrations. These growth factors bind to receptors on the tumor cells, activate autophosphorylation, and then promote tumor cell proliferation and further production of PTHrP, completing the "vicious circle."

Clinical experience indicates that ILCs are more likely to metastasize to atypical locations than IDCs.[25] For example, ILCs are more likely than IDCs to spread to the surface linings of the body, such as the skin, peritoneum, gastrointestinal tract, and meninges, and are less likely to spread to the lungs.[25] As a result, patients with metastatic ILC are prone to a different set of clinical problems, such as ascites and subacute bowel obstruction. The molecular mechanisms for this pattern of metastases have not been elucidated. It has been suggested that the loss of the E-cadherin function, a cell–cell adhesion molecule frequently altered in ILC, may be important.[25] Theoretically loss of cell–cell adhesion could account for the ability of ILC tumor cells to spread along epithelial linings rather than spreading as bulky tumor aggregations within organs.[25] High levels of the transcription factor Twist are seen in ILC.[26] Overexpression of Twist appears to play a key role in metastasis in a mouse model of human ILC.[26]

Complications Associated with Metastasis

Symptoms of MBC vary broadly, depending on the location of the metastases. Several serious clinical situations warrant urgent treatment with chemotherapy or local therapy. Visceral crises, such as hepatic dysfunction from overwhelming liver metastases, dyspnea from pulmonary disease or pleural effusions, or abdominal distension from ascites may require urgent chemotherapy in addition to definitive local procedures such as biliary, pleural, or ascitic fluid drainage. Spinal cord compression is a medical emergency, which requires high doses of corticosteroids in addition to radiotherapy or decompressive surgery if permanent neurological dysfunction is to be avoided. Brain metastases can present in a variety of ways, such as seizures, visual disturbances, focal neurological dysfunction, altered mental state, or stroke, and typically require corticosteroids and urgent local therapy, such as surgery, stereotactic radiosurgery, or whole-brain radiotherapy. In the developed world, it is rare that any of these complications are the first presentation of MBC and they tend to occur more commonly in patients with long-standing metastatic disease.

STATE-OF-THE-ART DIAGNOSTIC/PROGNOSTIC TESTING

Pathologic Confirmation of Metastatic Breast Cancer

A biopsy of at least one metastatic lesion to confirm the diagnosis of MBC is ideal but not always possible. Pathologic review of the metastatic specimen and comparison with the primary breast cancer specimen often improves clinical certainty about the origin of a newly diagnosed metastasis. If the histological appearance of the metastasis is similar to the breast primary cancer, IHC testing for ER/PR and HER2 is often, but not always, repeated. ASCO recommends that the ER and PR should be measured on metastatic lesions if the results would influence treatment planning.[11]

At many institutions, IHC is repeated on specimens when a diagnosis of MBC is suspected not only for confirmation of diagnosis but also because a metastatic IHC profile may be different from that of the primary cancer. In patients with ER/PR-positive primary breast cancer, apparent loss of ER or PR expression in the metastatic lesion is well described.[27,28] For example, in one small series this phenomenon occurred in 36 percent of cases.[28] There are numerous possible explanations for this, including sampling error relating to tumor heterogeneity and lab variability associated with the technical limitations of IHC. Theoretically, in ER/PR-positive primary tumors, ER/PR-negative tumor cells could escape the cytotoxicity of adjuvant endocrine therapy, leading to a selection of ER/PR negative cells in the metastatic lesion. Similar theories about HER2-positive tumors exist. In practical terms, these apparent anomalies do not significantly affect clinical management. Treatment decisions regarding ongoing endocrine and anti-HER2 therapy are based on a complex assessment of patient and tumor-related characteristics, as detailed later, and not simply on a single IHC result.

Tumor Predictive and Prognostic Testing

In EBC, gene expression profiling of tumors is used in selected situations to guide adjuvant therapy. In this way, studying which genes are actively transcribed into messenger RNA has led to the development of prognostic and predictive models. For example, the "Amsterdam" 70-gene profile was developed in low-risk patients treated with locoregional therapy alone and has been subsequently validated as a predictor of breast cancer survival, independent of lymph node status.[29] However, until recently, a limitation of this technique, was the requirement for fresh-frozen tissue, which is typically not available in the United States and other countries.

A 21-gene recurrence score prognostic indicator has also been developed.[30] This assay evaluates sixteen cancer-related genes chosen from a panel of 250 candidate genes and five reference genes.[30] This signature was validated on a proportion of tumors from a large randomized controlled trial, which compared the outcome of patients treated with adjuvant tamoxifen versus observation.[30] The relative expression of these cancer-related genes allows a continuous variable "recurrence score" to be assigned to an ER/PR-positive tumor, which correlates with risk of breast cancer recurrence.[30] Subsequently, this assay was tested on a proportion of tumors from another large randomized controlled trial assessing the addition of chemotherapy to tamoxifen, and it was found that the expression of these sixteen cancer-related genes correlated with the magnitude of benefit from adjuvant chemotherapy.[31] Therefore, currently some patients with ER/PR-positive EBC and a low recurrence score by this assay are able to avoid chemotherapy and receive adjuvant hormonal therapy alone.

As noted, both these gene expression profiles have not been prospectively validated, and many clinicians are cautious about their more widespread use. Therefore, ongoing clinical trials in EBC are assessing whether these gene expression profiles can be used prospectively to predict prognosis and whether treatment can be directed based on the predictive response to systemic therapy. In MBC there is a need for similar validated models to predict response from various agents. Ultimately, such models could help direct therapy, although at present this is investigational and would have greater urgency if specific choices were associated with vastly different outcomes in subsets of patients not currently identifiable by ER, PR, and HER2 testing.

Radiology

Computed tomography (CT) and bone scintigraphy with technetium methylidene diphosphonate remain the most frequently used imaging modalities for assessing breast cancer tumor burden and treatment response. In the staging of newly diagnosed MBC, the National Comprehensive Cancer Network (NCCN) recommends chest imaging, bone scan, and consideration of either CT or magnetic resonance imaging (MRI) of the abdomen.[32] In addition, it is recommended that patients with pain in long or weight-bearing bones or those with abnormal findings on bone scan should be imaged with plain radiographs.[32] Positron emission tomography (PET) scanning using fluorodeoxyglucose (FDG) is not routinely recommended in the initial workup of MBC.[32] To date, PET scanning has used FDG based on the principle that FDG is taken up by cells in proportion to their rate of glucose metabolism, and hence is a marker of cell proliferation.[33] PET scanning is useful to investigate equivocal findings on

other imaging modalities and should be considered complementary to these techniques.[34] For example, it can be useful in discriminating between scarring from surgery and metabolically active tissue, such as tumor recurrence.[34] Numerous small studies have shown that PET can be an early marker of response or lack of response to treatment.[33] However, this approach has not yet been validated in large prospective studies, so early changes on PET scanning cannot now be used to direct therapy. Therefore, the role of PET imaging in the diagnosis and surveillance of patients with MBC has not yet been fully defined.[34]

The specificity of FDG-PET scanning is limited by false-positive readings from other metabolically active (nonmalignant) tissue that actively takes up glucose. A further problem is that many indolent breast cancers may not be FDG-avid on PET. In general, a higher level of FDG uptake is seen in IDC than in ILC, leading to more false-negative results in ILC.[35,36] Beyond FDG there are many new PET tracers under development in breast cancer, which aim to target cellular processes and receptors such as ER and HER2.[33] In the future, these tracers could lead to more specific and sensitive monitoring of the tumor burden. Theoretically, this could allow a more accurate assessment of response to therapy, leading to improvements in treatment selection.

In the assessment of a patient with MBC, additional imaging is clinically directed based on symptoms. Examples of this include the use of MRI to examine the central nervous system or spinal cord for suspected metastases. Screening mammograms are not routinely used in MBC given that patients already have an incurable malignancy and detection of another early (and possibly curable) breast cancer will generally fail to have an impact on survival but could cause interruptions in systemic therapy, although in long-term survivors with very indolent MBC mammography may potentially be appropriate.

Blood Tests

The tumor markers carcinoembryonic antigen (CEA), CA 15–3, and CA 27–29 may be elevated in patients with MBC and in some patients correlate with disease course. CEA is a glycoprotein that is elevated in a variety of cancers, including breast and colorectal cancer.[37] CA 15–3 and CA 27–29 are assays that detect circulating MUC-1 antigen in peripheral blood.[11] Only 50 percent to 60 percent of patients with MBC will have elevated levels of CEA, compared with 75 percent to 90 percent who have elevated levels of CA 15–3 and CA 27–29.[11] The ASCO guidelines suggest that tumor markers may be a useful adjunct to the clinical and radiological assessment of treatment response, but should not be used in isolation.[11] In the absence of readily measurable disease, increasing tumor markers may be useful as early markers of treatment failure.[11] Spurious elevations in tumor markers can occur with hepatic impairment, vitamin B_{12} deficiency, megaloblastic anemia, thalassemia, and sickle cell disease. In addition, a transient rise in tumor markers can be seen in the first few weeks after the initiation of appropriate therapy in patients whose disease is responding, presumably relating to tumor cell lysis.[38] Therefore only very rarely should treatment decisions be based solely on rising tumor markers. In the follow-up of patients with EBC, the routine monitoring of these markers is not recommended.[11]

Circulating Tumor Cells

For more than a century, circulating tumor cells (CTCs) have been identified in the peripheral blood of patients with cancer.[39] More recently, the advent of techniques such as quantitative real-time polymerase chain reaction (PCR) and cell enrichment using immunomagnetic separation technology has led to the detection of CTCs with much higher sensitivity and specificity.[40,41] In MBC, it has been suggested that patients with higher numbers of CTCs before and during treatment appear to have a shorter progression-free and overall survival compared with patients with fewer CTCs, and, therefore, that CTCs are an independent prognostic factor.[42,43] At present it is unknown whether CTCs have superior prognostic value compared with other standard factors. CTCs cannot be used to direct or change therapy, as the technique has not been validated in this setting.

BIOLOGIC TARGETING OF CURRENT DRUGS

Because MBC is, except for anecdotes, incurable, treatment strategies should be aimed at improving or maintaining quality of life while optimizing disease-specific outcomes such as tumor response and progression-free and overall survival. Because breast cancer is one of the most chemosensitive solid organ tumors, a wide range of cytotoxic agents is available. Therapy recommendations are usually based on both patient and tumor characteristics, which include patient comorbidities, residual treatment-related toxicities, and prior therapies. Some cytotoxic agents used for breast cancer have specific targets. For example, anthracyclines intercalate between DNA base pairs on the double helix and inhibit topoisomerase IIα. Taxanes exert their cytotoxic effect by binding microtubules. Despite numerous efforts, it is not currently possible to direct therapy based on tumor expression of these targets.[44]

In the treatment of EBC, stepwise advances have led to the development of various complex combination chemotherapy regimens. These have included

combinations of anthracyclines, alkylating agents, and taxanes. Further advances have included the administration of chemotherapy regimens at shorter dose intervals in a dose-dense fashion with growth factor support.[45] In contrast, cytotoxic chemotherapy for MBC often involves the sequential use of single agents, sometimes at weekly intervals. Dose-dense combination therapy is not a standard approach for MBC. In fact, there remains controversy about the use of combination chemotherapy versus sequential single-agent chemotherapy for MBC. The rationale for the combination approach is that combining two or more agents with different mechanisms of action and differing side effect profiles could improve clinical efficacy. However, combination chemotherapy has not demonstrated consistent long-term advantages over sequential single-agent strategies. Consequently, some physicians reserve combination chemotherapy for patients with impending visceral crisis or rapidly progressive disease, whereas others routinely use combination therapy up front to optimize response rates, although generally at a cost of greater toxicity.

In all probability, this combination versus single-agent debate is not resolvable as a general rule but instead is drug- and situation-dependent. In MBC, chemotherapy is often continued indefinitely until tumor progression or dose-limiting toxicity. An alternative approach is the use of courses of chemotherapy over a few months, interrupted by so-called drug holidays. Again, there are benefits to each approach, and physician and patient-related factors should be taken into account, along with regimen and drug-specific toxicities.

ER/PR

In contrast to the use of cytotoxic chemotherapy, where biomarkers are typically not useful in guiding therapeutic recommendations, initial treatment decisions are typically made based on tumor expression of ER/PR and HER2. Estrogen deprivation (endocrine therapy) is a well-established treatment strategy for patients with hormone-sensitive breast cancer. In EBC, endocrine therapy is usually given after or instead of chemotherapy for a period of five or more years, and may involve selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs). Endocrine therapy remains an appropriate treatment strategy for MBC, particularly for patients with long disease-free periods between the primary breast cancer and the development of metastatic disease, and those who have minimal related symptoms, modest disease burden, and low risk of visceral crisis. These clinical factors are the currently the best predictors of response to endocrine therapy and are more useful than the level of tumor expression of ER and PR. There are currently no other validated biological markers to predict response in MBC.

For decades, tamoxifen has been one of the most widely used anticancer drugs. The activity of tamoxifen in premenopausal women with MBC has been demonstrated in numerous Phase II studies.[46] This has been confirmed in Phase III studies comparing tamoxifen with surgical oophorectomy or medical ovarian suppression with gonadotropin-releasing hormone (GnRH) agonists and in the Oxford overview meta-analysis.[17,47–49] Tamoxifen has been successfully combined with ovarian suppression, resulting in higher response rates but (as expected in the initial treatment of a relatively indolent disease with multiple subsequent treatment options) no proven survival benefit over tamoxifen monotherapy.[49–51] Because of the ease of administration, oral tamoxifen monotherapy remains the preferred choice in this setting.

Although the ovaries are the major source of estrogen in premenopausal women, postmenopausal women produce estrogen from androgen precursors in adipose tissue, the adrenal glands, and elsewhere. A critical step in this estrogen production is catalyzed by the enzyme aromatase. Several oral AIs have now been developed and are divided into two nonsteroidal formulations (letrozole and anastrozole), which bind reversibly to aromatase, and the steroidal formulation (exemestane), which binds irreversibly. Although these agents offer slight improvements over tamoxifen in terms of tumor response and progression-free survival, again (as expected) no survival benefit for an AI over tamoxifen has been demonstrated in an adequately powered randomized study in the first-line MBC setting.[52–54] However, a notable advantage of AIs is that they are not associated with the rare, but serious, tamoxifen-related side effects of venous thromboembolism and uterine carcinoma, which reflect its mixed agonist–antagonist mechanism of action. However, AIs are commonly associated with arthralgias and declines in bone mineral density over time (particular concerns in the adjuvant setting, as opposed to in metastatic disease).

Women with endocrine-responsive MBC may be suitable candidates for serial endocrine manipulation at each time of disease progression. There are no proven biological markers to predict response to second-line endocrine therapy, and the decision to use this strategy is based on clinical factors such as prior response and overall disease burden. Recent data suggest that the steroidal AI exemestane and the steroid-based pure antiestrogen fulvestrant are equally efficacious after progression of disease on first-line nonsteroidal AI (Figure 39.4).[55] Second-line therapy with an AI is advantageous because of the oral route of administration. Fulvestrant, conversely, is administered as an intramuscular injection every twenty-eight days and, as a pure antiestrogen, is not associated with an increased risk of

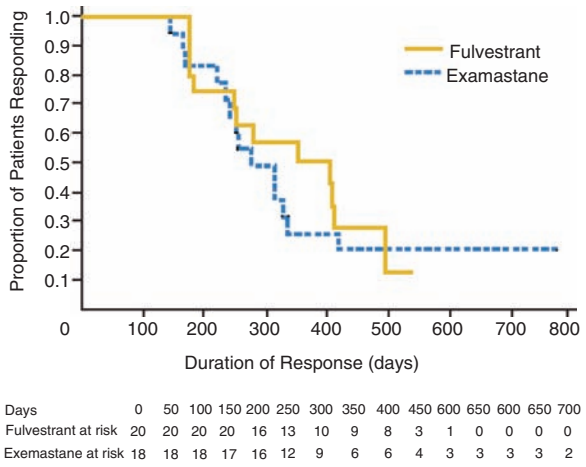

Figure 39.4. Duration of response for second-line exemestane and fulvestrant (from Chia et al. *JCO* 2008).[55]

venous thromboembolism or uterine carcinoma. Fulvestrant has also demonstrated equivalent activity to tamoxifen in the first-line setting in MBC.[56] Toremifene is another SERM with a consistently similar activity and side effect profile to tamoxifen.[57,58] High doses of the progestin, medroxyprogesterone acetate, have also shown similar benefits to tamoxifen.[59,60] Although there is no single algorithm to guide recommendations regarding endocrine therapy, because of the oral route of administration for most of these agents and the generally favorable toxicity profiles, clinicians frequently attempt to extend the hormone manipulation strategy for as long as it appears safe and feasible.

EGFR

One possible marker for endocrine resistance in hormone sensitive breast cancer is tumor expression of the epidermal growth factor receptor (EGFR).[61] Blockade of EGFR with concurrent endocrine therapy has been active in vitro, and this strategy has been the source of substantial clinical interest. In addition, EGFR expression has been identified in basal-like tumor cells, which are sensitive in vitro to inhibitors of EGFR.[62] However, results of clinical studies of EGFR-targeted therapies have been inconsistent and largely disappointing to date. The EGFR tyrosine kinase inhibitor erlotinib appears to have minimal activity as monotherapy in unselected patients with MBC.[63] Clinical experience in MBC with the monoclonal antibody cetuximab has also been disapointing.[64] Monotherapy with cetuximab seems to be associated with a low response rate even in triple-negative breast cancer.[65] However, emerging data suggest that some patients with triple-negative breast cancer may benefit from the combination of cetuximab and carboplatin.[65,66] Likewise, one recent study suggested a possible benefit for the addition of the EGFR tyrosine kinase inhibitor gefitinib to an aromatase inhibitor in newly diagnosed hormone-sensitive MBC.[67] Although some studies are limited by unselected patient groups, other attempts to select patients on the basis of EGFR expression have been disappointing.[64] The role of mutations in EGFR and k-ras remains unknown in this setting, unlike the case in non–small-cell lung cancer and colorectal cancer, where it is better characterized. Importantly, some ongoing studies have incorporated serial tissue biopsies into the design to help find potential biomarkers to identify a subset of patients with MBC who may benefit from EGFR inhibition.[65]

HER2

HER2 is an important target for an increasing array of drugs in MBC. As noted, the humanized monoclonal antibody trastuzumab has revolutionized the treatment of HER2-positive breast cancer. Trastuzumab binds to the extracellular domain of HER2, although the specific mechanisms by which it exerts its effects have not yet been determined.[12] Early studies in MBC suggested that the only patients to benefit from trastuzumab were those with tumors that showed 3+ staining by IHC or who had gene amplification by FISH and, similarly, that there was no benefit for patients with normal HER2 expression.[68–70] In MBC, trastuzumab has been successfully combined with a range of cytotoxic agents, including paclitaxel, docetaxel, capecitabine, gemcitabine, and vinorelbine, with significant and consistent clinical benefits.[71–76] Limited recent evidence has suggested that trastuzumab remains active with successive cytotoxic agents, a strategy that is commonly used in clinical practice.[77]

There is also evidence that HER2-positive breast cancer may be associated with a greater relative benefit from anthracyclines and to relative resistance to cyclophosphamide, methotrexate, 5-fluorouracil (CMF)-like regimens.[78] In contrast, other data have suggested that patients whose tumors overexpress HER2 do not derive benefit from anthracyclines.[44,79] The close proximity of HER2 and topoisomerase IIα (the putative target of anthracyclines) on chromosome 17q have been implicated as the underpinning of those associations, although this is controversial.[78] These studies are limited, for example, by the sensitivity of commercially available FISH probes for HER2 and topoisomerase IIα, which may extend beyond the genes of interest and are therefore subject to false-positive results. Techniques designed to more accurately and consistently determine HER2 and topoisomerase IIα status are ongoing.[14] Anthracyclines and trastuzumab are not routinely administered concurrently for MBC, as co-administration is associated with a significant rate of cardiotoxicity (27%).[80] As noted, at present it is

not possible to select specific cytotoxic chemotherapy with trastuzumab based on tumor expression (or lack of expression) of topoisomerase IIα. In addition, the level of gene expression of HER2 by FISH cannot be used to determine possible response or duration of response to trastuzumab, nor can it be used to direct therapy.

Despite the dramatic benefits of trastuzumab, a significant proportion of women with HER2-positive MBC eventually experience disease progression. The precise mechanisms for this resistance are poorly understood, although PTEN deficiency appears to play an important role. It is hoped that increased understanding of these pathways of trastuzumab resistance will translate into successful therapeutic innovation. There is evidence that cyclooxygenase-2 (COX-2) may be upregulated in HER2-positive breast cancer, and this may be a potential target for inhibiting tumor growth.[81] However, the COX-2 inhibitor celecoxib does not appear to reverse trastuzumab resistance.[81]

There is also evidence that HER2 overexpression is associated with relative resistance to endocrine therapy.[78] For the small group of patients with ER-positive, HER2-positive (luminal B) MBC, it is unclear whether trastuzumab should precede, follow, or be combined with endocrine therapy. One randomized trial has shown a benefit for the addition of trastuzumab to an AI, although the optimal sequencing of these agents was not studied.[82]

Whereas trastuzumab is a well-established therapy for HER2-positive MBC, a number of other agents are at varying stages of development. Lapatinib is an orally active, small-molecule tyrosine kinase inhibitor that targets HER2 and EGFR (or HER1). Lapatinib in combination with capecitabine has proven activity in patients pretreated with trastuzumab, as demonstrated in one Phase III study.[83] In one small study, coexpression of HER2 and HER3 predicted for a favorable response to lapatinib.[84] Further characterization of the patients likely to benefit from lapatinib is needed. Numerous other studies involving lapatinib are ongoing, including some in the adjuvant setting, although currently lapatinib is not a standard adjuvant treatment for HER2-positive breast cancer. As a small molecule, lapatinib may be particularly useful in preventing or treating brain metastases, which occur more frequently in patients with HER2-positive MBC. Similar advantages are postulated for neratinib (HKI 272), a pan-HER tyrosine kinase inhibitor recently shown to have significant single agent activity in HER2-positive breast cancer regardless of prior trastuzumab treatment.[85]

Newer antibodies targeting HER2 are in clinical trials. Pertuzumab is a monoclonal antibody with a binding site on HER2 distinct from trastuzumab, which has demonstrated activity in combination agents including capecitabine and docetaxel.[86,87] Ertumaxomab is a trifunctional bispecific monoclonal antibody with three binding sites; HER2, CD3-positive T cells, and the accessory cells of the immune system.[88] It therefore has the potential for cytotoxicity via direct HER2 binding and by initiating an immune response. Trastuzumab-MCC-DM1 is a novel agent composed of the cytotoxic DM1 (a maytansinoid antimicrotubule agent) conjugated to trastuzumab. This agent has a novel mechanism of action, as the monoclonal antibody (trastuzumab) binds HER2-positive cells, theoretically allowing preferential delivery of the cytotoxic agent to malignant cells.[89,90] As yet, no dominant compound from this group has emerged.

Heat shock protein (HSP) 90 is a molecular chaperone that is required for the refolding, activation, and assembly of many proteins, including HER2, under conditions of environmental stress.[91] Several HSP90 inhibitors, such as geldanamycin and its derivatives, alvespimycin and tanespimycin, are being investigated with trastuzumab and as monotherapy in HER2-positive MBC with promising early results in heavily pretreated patients.[91,92] Again, these agents are relatively early in development but broadly represent an exciting avenue for investigation, particularly for patients with HER2-positive MBC resistant to trastuzumab.

VEGF

Angiogenesis plays an important role in the progression of MBC; therefore, drugs that interfere with the angiogenic pathway have been actively investigated. The humanized monoclonal antibody bevacizumab targets vascular endothelial growth factor (VEGF) and has been investigated in a variety of solid-organ tumors. In heavily pretreated patients with MBC the addition of bevacizumab to capecitabine did not improve progression-free or overall survival.[93] In contrast, two large studies have demonstrated that the addition of bevacizumab to taxane-based chemotherapy in the first-line setting is associated with a significant improvement in progression-free survival, although no overall survival benefit has been seen.[94,95] Almost all the patients in these studies did not overexpress HER2. Bevacizumab is now sometimes recommended in combination with chemotherapy for newly diagnosed MBC. There is currently no evidence that it should be continued beyond disease progression with successive chemotherapy regimens or continued as monotherapy, although studies addressing these issues are ongoing.

Attempts to identify biomarkers that accurately identify patients likely to derive benefit from bevacizumab have been largely unsuccessful to date. Tumor expression of VEGF does not appear to correlate with response to bevacizumab in other tumors, nor does expression of putative biomarkers thrombospondin-2, k-ras, b-raf, and p53.[96,97] In breast cancer, recent

evidence has suggested that patients whose tumors expressed the VEGF-2578 AA genotype or the VEGF-1154AA genotype were more likely to derive benefit from the combination of paclitaxel and bevacizumab.[98] However, these are data from one randomized trial, in which tumor blocks were available from only about half the patients. It is also unclear whether the expression of these single nucleotide polymorphisms in primary tumors, as measured in this study, correlates with expression in the metastatic lesions. Further validation of these biomarkers is needed before they can be used to direct treatment decisions. A related issue will be the potential for such biomarkers to direct the use of oral VEGF receptor tyrosine kinase inhibitors (sunitinib, sorafenib, others) should they prove clinically effective.

Androgen Receptor

Another nuclear hormone receptor, the androgen receptor (AR), is expressed in 60 percent to 80 percent of breast cancers, often in conjunction with ER, and is involved in the initiation and progression of breast cancer.[99,100] High levels of androgens have also been associated with an increased risk of developing breast cancer.[101] In addition, expression of the AR has been identified in a small subset of triple-negative breast cancers.[99] In vitro studies have demonstrated that androgen blockade is associated with a reduction in growth of triple-negative cell lines. This represents a possible future avenue for development, and clinical studies targeting AR using antiandrogens are ongoing.

Src

Src is a widely studied nonreceptor protein tyrosine kinase; elevated Src expression has been seen in multiple tumors, including breast cancer.[102] Src has been shown to be important for cellular growth proliferation, as well as angiogenesis, invasion, and metastasis. Elevated Src activity may be caused by overexpression of upstream growth factor receptors, such as EGFR, HER2, and VEGF.[102] Recent data also suggest that inhibition of Src is effective in vitro at preventing the growth of the basal subgroup of breast cancer.[103] Hence there is a strong rationale for developing agents that interfere with Src. Several drugs are being clinically tested in breast cancer, including dasatinib, which inhibits multiple tyrosine kinase inhibitors and is already widely used for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumor (GIST).

PARP

The poly(ADP-ribose) polymerase (PARP) family of proteins plays a critical regulatory role in DNA repair and other cellular processes. In particular, PARP plays an important role in the repair of single-strand breaks (SSBs) following DNA damage (Figure 39.5).[104] In brief, following DNA damage, PARP is activated and binds to the exposed SSB. It then catalyzes the successive transfer of ADP-ribose units from the substrate nicotinamide adenine dinucleotide (NAD+) to acceptor nuclear proteins. This produces polymers of poly(ADP-ribose) (PAR) and creates a negatively charged target at the SSB, which recruits the enzymes required for successful base excision repair, such as X-ray repair cross-complementing 1 (XRCC1), DNA ligase III, and the DNA polymerase pol β (Figure 39.5). Studies in vitro and using animal models have shown that tumors with deletions of the tumor suppressor genes BRCA1 and BRCA2 are particularly sensitive to drugs that inhibit PARP. (Inherited autosomal dominant mutations in BRCA1 and BRCA2 are associated with an increased lifetime risk of ovarian and breast cancer, with a disproportionately higher risk of triple-negative breast cancer.) Inhibitors of PARP are therefore of great interest for the treatment of MBC, particularly that associated with BRCA1 and BRCA2, and could be beneficial for patients with triple-negative breast cancer. Several oral PARP inhibitors are in early clinical trials.[104]

Bone Metastases

It is estimated that up to 85 percent of patients with metastatic breast cancer will develop bone metastases during the course of their disease.[105] Bone metastases can be associated with significant pain and life-threatening skeletal complications. Therefore, patients with MBC to bone are frequently treated with bisphosphonates to improve quality of life and to decrease the risk of skeletal-related events. Bisphosphonates are pyrophosphate analogs and are widely used for the treatment of osteoporosis. Because they inhibit osteoclast-mediated bone resorption, they have also been extensively studied for the treatment of bone metastases from breast cancer. Intravenous bisphosphonates significantly decrease the risk of skeletal-related events, including the need for radiation or surgery to bone, pathological fractures, and spinal cord compression.[106–108] One of the most recently developed bisphosphonates is zoledronic acid, which has demonstrated markedly higher potency in preclinical models than older bisphosphonates such as pamidronate.[109]

In recent years, rare cases of osteonecrosis of the jaw, characterized by painful bone exposure in the mandible, maxilla, or both, have been reported with intravenous bisphosphonate therapy.[110] Because the benefits of intravenous bisphosphonates beyond two years are unknown and the risk of osteonecrosis of the jaw may increase with increasing duration of therapy, many clinicians now administer bisphosphonates less

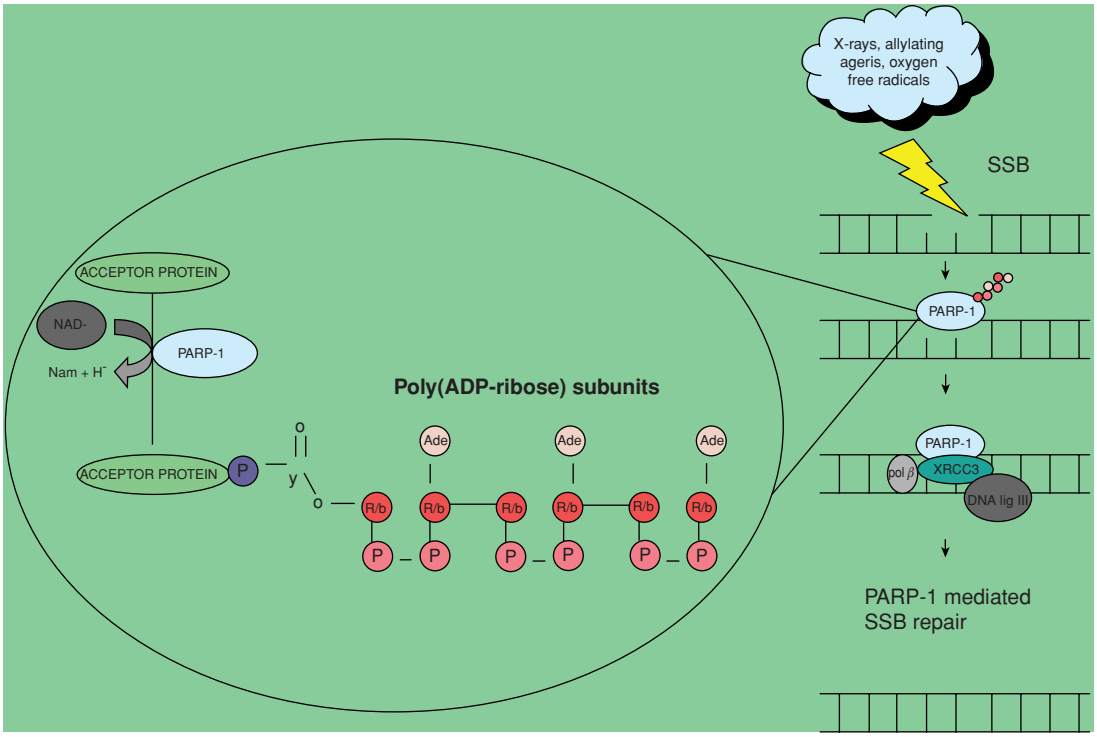

Figure 39.5. The role of PARP in single-strand break repair (from Drew and Calvert 2008).[104]

frequently after two years of monthly therapy, although the optimum frequency to maintain efficacy and minimize the risk of osteonecrosis is unknown. Given the proven activity of intravenous bisphosphonates in the treatment of established bone metastases, there has been significant interest in incorporating these agents into adjuvant regimens. Several clinical studies are ongoing, although as yet no consistent benefit has been demonstrated for this approach.

FUTURE DIRECTIONS

Breast cancer expression of ER/PR and HER2 both has prognostic significance and is useful in predicting response to agents that target these receptors. There are currently limitations to our understanding of even these subtypes of MBC. In particular, there is a lack of biomarkers that predict response to endocrine therapies and HER2-targeting agents. More striking is the lack of understanding about the mechanisms of resistance to these agents and, hence, a lack of therapeutic strategies to overcome these mechanisms of resistance. Beyond these subgroups, there is a need to understand the expression of other targets and active pathways, which could form the basis for future therapeutic advances.

Currently in hormone-responsive MBC, clinical factors remain the only proven method of selecting patients for endocrine therapy. The development of biomarkers could help determine which patients benefit from serial hormonal manipulations, which patients should be treated with a combination therapy including hormone therapy, and which patients should be treated with chemotherapy. For patients who do benefit from endocrine therapy, an optimal schedule of agents has not been elucidated; again, there is a need to develop biomarkers to choose appropriate agents in sequence. A further challenge is to determine if and how endocrine therapy can be combined with therapies directed against HER2, VEGF, or other agents, such as inhibitors of Src and PARP.

Following a large growth in the number of active cytotoxic agents for breast cancer around the turn of the century, in recent years there have been fewer developments. Epothilones represent a novel class of drugs that act as microtubule stabilizing agents. To date, the only epothilone with proven activity in breast cancer is ixabepilone.[111] In the pivotal Phase III study, this agent was associated with significant neurotoxicity in patients previously treated with taxanes. There is therefore a need to improve on currently available clinical parameters to select patients for such novel cytotoxic agents; any possible biomarkers to assist this would be welcome. In addition, there is a need to develop agents and strategies to overcome the resistance mechanisms of drugs such as taxanes, including the expression of the P-glycoprotein drug efflux pump, which are beginning to be understood.

In terms of targeted agents, why some patients respond and some do not remains unclear. In the case

of HER2-targeted therapies, for example, it is likely that the inherent limitations of currently available techniques for determining HER2 status may result in suboptimal patient selection. However, a more fundamental issue is the significant gap in our understanding of the pathophysiology of HER2-positive breast cancer. It is hoped that future insights will ultimately lead to the development of improved drugs and, in turn, survival benefits for affected patients.

To meet these significant challenges, three important strategies are needed. First, there must be increased participation in clinical trials. Overall, fewer than 5 percent of patients with breast cancer participate in clinical trials.[112] To make more rapid progress, this must improve radically. Second, clinical trials must be designed rationally, to incorporate tissue acquisition. This will allow an in-depth study of tumor biology and response to therapy. The acquisition of serial tissue samples can be difficult to justify in MBC outside a research setting but offers a potentially rich resource to investigators. Third, there must be greater emphasis on collaboration among investigators. In particular, a greater partnership between clinical and laboratory investigators could provide significant opportunities. The mechanisms of resistance to currently available drugs can be understood only by investigating gene expression in serial tumor samples. An understanding of intrinsic and acquired drug resistance will improve our patient selection and identify appropriate subgroups of patient who will gain most from individual treatments. Biomarkers of response and resistance can then be tested and validated. Finally, gaining an understanding of tumor resistance pathways will lead to a significant opportunity for the rational development of novel agents for specific targets, allowing a far more individualized approach to treatment in the future.

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40 Gynecologic Malignancies

Sarah M. Temkin and S. Diane Yamada

The three major classes of gynecologic malignancies include ovarian, endometrial, and cervical cancer. Although most women with ovarian cancer will present with metastatic disease (75%–80% of patients), a relatively small percentage of women with endometrial cancer have metastatic disease at diagnosis (20% of patients). For women with cervical cancer, 60 percent will have metastatic disease when they are initially diagnosed.[1] Ovarian cancer is the most lethal of the gynecologic malignancies, as its pattern of spread is intraperitoneal and often causes the patient few readily identifiable symptoms. Endometrial cancer has the best prognosis overall, as it is usually detected at an early stage when it can be treated with surgery alone. Cervical cancer, despite its prevalence worldwide, is uncommon in the developed world and is treatable and often curable, given its sensitivity to radiation, even after locally advanced spread.

Each of the gynecologic malignancies presents the practitioner with unique clinical and treatment challenges. Advances in imaging techniques, surgical techniques, chemotherapy administration, and the development of targeted therapies have led to improved treatment options for patients diagnosed with gynecologic malignancies. This chapter highlights common patterns of metastasis for each of the major tumor types, diagnostic modalities, and the standard and emerging treatments for ovarian, endometrial, and cervical cancer.

EPITHELIAL OVARIAN CANCER

An estimated 21,550 new cases of ovarian cancer were diagnosed in 2009, with 14,600 estimated deaths, making ovarian cancer the most lethal of the gynecologic malignancies.[2] The majority (95%) of cancers arising from the ovary are epithelial in origin. The most common histologic types are serous, mucinous, endometrioid, clear-cell, and undifferentiated tumors.[3] Stage I disease is cancer limited to one or both ovaries; stage II is disease spread only within the pelvis; stage III encompasses disease spread to the upper abdomen; and stage IV consists of disease spread to the parenchyma of the liver, the pleural cavity, or distant organs. The poor prognosis associated with the diagnosis of epithelial ovarian cancer is a result of the advanced stage of disease present at the time of diagnosis and the eventual development of chemotherapy-resistant disease.

Clinical Presentation of Metastatic Ovarian Cancer

For the 20 percent of patients fortunate enough to be diagnosed with stage I or II disease, survival is approximately 85 percent. For the 75 percent to 80 percent of patients diagnosed with widely metastatic ovarian cancer, survival decreases to 10 percent to 25 percent,[4] despite aggressive surgery and adjuvant chemotherapy. From a clinical standpoint, ovarian cancer has been labeled the "silent killer" because of its insidious nature and the vague symptoms associated with it. These symptoms include abdominal pain, bloating, distension, and a change in bowel habits.[5]

These symptoms coexist with a well-characterized pattern of metastatic spread that has usually occurred by the time the patient's symptoms are recognized as pathologic. Ovarian cancer and fallopian tube cancer cells are believed to exfoliate from their primary organs and spread by extension to adjacent areas. There is a clear predilection of these cells, carried by the flow of peritoneal fluid, to implant on the bowel mesentery, the diaphragmatic peritoneum, and the omentum, eventually becoming peritoneal implants and an omental "cake" (Figures 40.1 and 40.2).[6,7] This "seeding" of the peritoneal cavity is frequently associated with ascites formation and an elevation of CA-125 in 85 percent of advanced-stage patients. CA-125 is a mucinous glycoprotein and the product of the MUC16 gene. Although it is elevated in 85 percent of women with

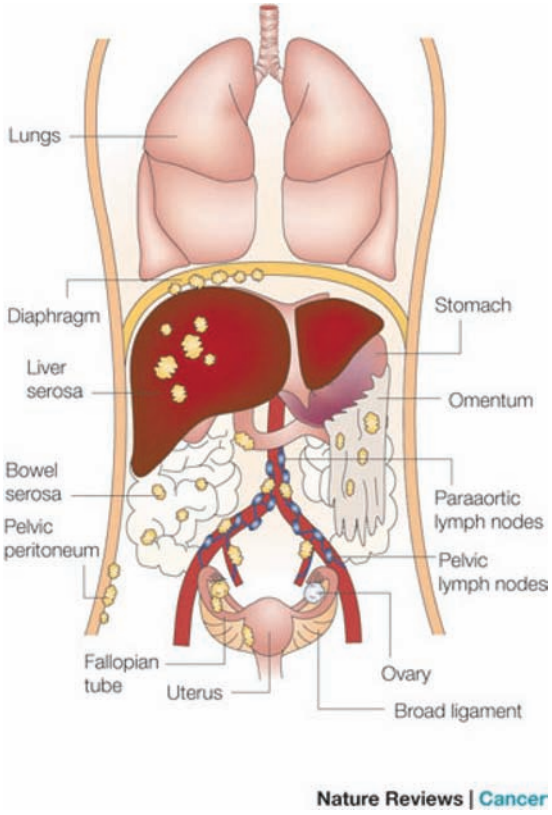


Figure 40.1. Common sites of metastasis in epithelial ovarian cancer.[153]

advanced ovarian cancer, only 50 percent of women with stage I disease have an elevated CA-125, making it an ineffective screening tool.[4] However, for monitoring disease following diagnosis, the CA-125 remains the most reliable and widely used blood test in the management of ovarian cancer today, because preoperative CA-125 levels do correlate with tumor stage, grade, histologic type, the presence of ascites, and response to chemotherapy. CA-125 has also been shown to be

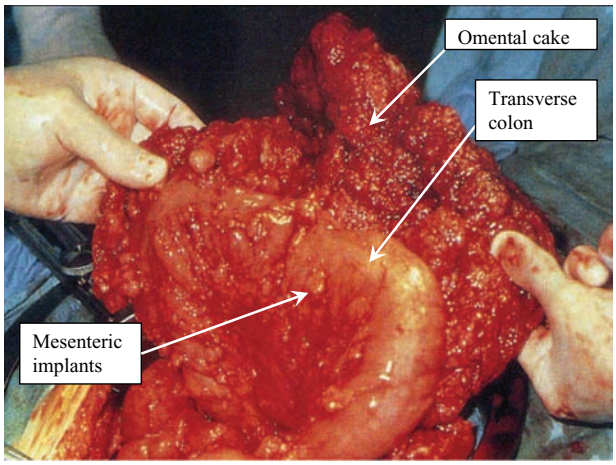


Figure 40.2. Omental caking with mesenteric implants.

TABLE 40.1. Ability of preoperative CA-125 to predict advanced stage (III–IV) ovarian cancer

CA-125 (U/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
≤60	94	27	83	53
160	88	47	86	50
290	79	60	88	44
400	71	77	92	41
500	62	80	92	36
600	58	80	92	34
925	46	83	91	29
1,400	36	90	93	27
2,380	25	97	97	26
4,450	13	100	100	24

an independent prognostic factor in epithelial ovarian cancer (Table 40.1).[8–10] Additional screening tools, such as ultrasound and other biomarkers, have, to date, not been found to be cost-effective or sufficiently specific to be implemented in the general population of patients.

Although intraperitoneal metastases are the hallmark of ovarian cancer, vascular and lymphatic metastases occur as well. The incidence of lymph node metastasis has been reported to be as high as 74 percent in women with advanced stage disease.[11] One-fourth of apparent stage I and half of stage II tumors will be upstaged owing to lymph node involvement following systematic lymphadenectomy.[11] Parenchymal liver or lung metastases characteristic of stage IV disease occur in 16 percent of patients. Hematogenous spread rarely occurs at the time of presentation and is more commonly associated with recurrent disease.[1,12]

Metastatic recurrences that involve a reaccumulation of ascites fluid or involvement of the peritoneal or bowel surfaces can lead to specific complications. Massive ascites leads to abdominal distension and discomfort, anorexia, nausea, vomiting, and respiratory compromise. The development of ascites is thought to result from a combination of several factors, including (1) increased peritoneal fluid production owing to damaged peritoneal membranes; (2) decreased absorption caused by obstructed lymphatics; (3) oncotic abnormalities, resulting in loss of fluid into the peritoneal cavity and "third spaces"; and (4) aberrant expression of genes for permeability-inducing factors, including vascular endothelial growth factor (VEGF), interleukin (IL)-6, or tumor necrosis factor (TNF).[13–15]

Another complication specific to advanced and metastatic ovarian cancer is bowel obstruction. Up to half of women with ovarian cancer will experience a bowel obstruction at some point during their treatment,

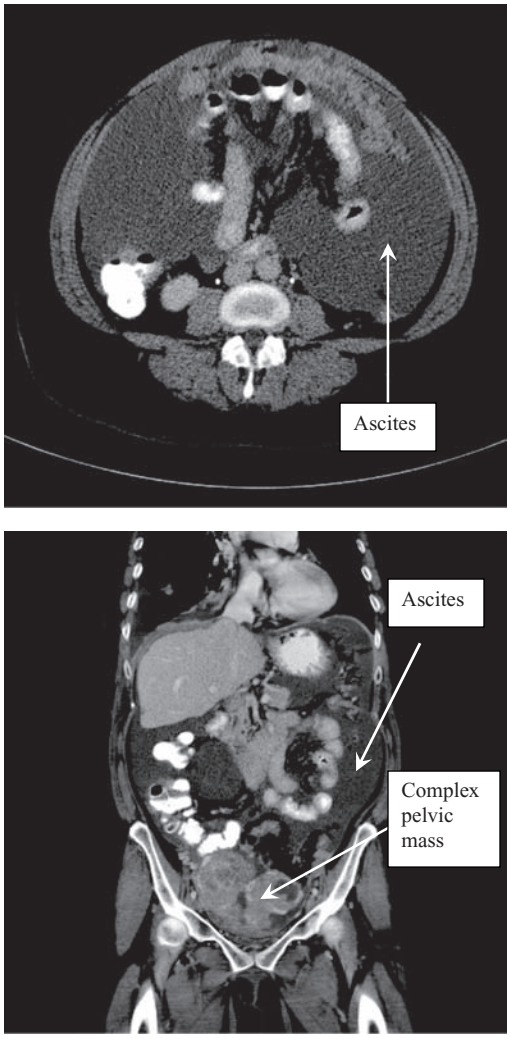


Figure 40.3. Typical CT scan findings in advanced ovarian cancer.

with 26 percent requiring hospitalization and reoperation within their life span.[16,17] Even in women without overt obstruction requiring surgical intervention, peritoneal disease will lead to more subtle gastrointestinal symptoms, such as poor appetite, nausea, abdominal pressure, malnutrition, and failure to thrive.

Diagnosis, Imaging, and Staging

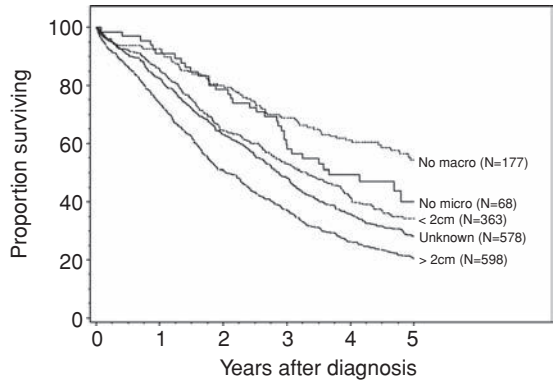
Currently, CT scan is the imaging modality of choice in ovarian cancer (Figure 40.3). The accuracy of CT in the detection of stage in ovarian cancer ranges between 53 percent and 92 percent.[18–21] High-resolution multidetector CT imaging can provide information on the presence of a complex adnexal mass; ascites; the presence, size, and location of peritoneal implants; and lymph node enlargement. The presence of presacral disease, lymph node metastases above the renal hilum, invasion of cancer into the abdominal wall, parenchymal liver metastases, and implants of >2 cm in sites that present surgical challenges such as the porta hepatic and small bowel mesentery all suggest that optimal debulking may not be feasible.[22,23] The most important limitation of current CT scans, however, is detection of sub-centimeter peritoneal metastases.

The use of fluorodeoxyglucose (FDG)-PET in other malignancies has led to recent interest in the use of this imaging technique in ovarian cancer. FDG-PET, which makes use of a radiolabeled glucose molecule to gauge metabolic activity, may be useful in providing information regarding response to treatment with chemotherapy.[24] Some preliminary studies suggest that FDG-PET may be useful in the early detection of recurrences, particularly retroperitoneal recurrences or miliary intraperitoneal disease, which is difficult to detect by CT scan.[24–27] Combining FDG-PET reading with CA-125 appears to improve the sensitivity for detecting recurrent disease.[25]

In select cases when recurrence is suspected but not identifiable by CT scan, MRI may be useful. However, MRI generally provides no additional information over that which is gleaned from CT scan. Diffusion-weighted MRI has been used in preliminary studies to detect peritoneal implants in women suspected to have ovarian cancer, with sensitivity and specificity rates of 90 percent and 95.5 percent.[28]

Standard Treatment

During the past few decades, advances in surgical treatment and chemotherapy regimens have improved overall survival to the extent that 45 percent of patients with advanced disease survive at least five years.[29] Surgery is the mainstay of the initial treatment of ovarian cancer. Accurate surgical staging is of the utmost importance in providing accurate prognostic information and tailoring adjuvant treatment. A complete staging procedure includes a hysterectomy with bilateral salpingo-oophorectomy, omentectomy, pelvic and paraaortic lymph node dissection and intraperitoneal washings. A survival advantage has been consistently demonstrated with optimal reduction in tumor burden (tumor debulking) performed during a surgical staging procedure (Figure 40.4). Optimal debulking has been generally defined as leaving residual tumor less than 1 cm in largest diameter, but the patients who experience the longest overall survival are those left with no visible disease at the end of surgery.[30–32] Postoperatively, optimal primary cytoreduction is important in lengthening patients' time to progression and has been shown to improve the initial chemotherapy response.[33] In patients with serious medical comorbidities or when the possibility of optimal tumor debulking is slim, it is preferable to treat patients with neoadjuvant chemotherapy prior to surgery (interval debulking).



Residual disease	Patients (*n*)	Mean age (yr)	Overall survival (%) at 1 year	2 years	3 years	4 years	5 years	Hazards ratio[a] (95% CI)
No micro residual	66	57.5	90.8	78.5	59.7	49.0	41.3	Reference
No macro residual	177	58.2	92.6	80.0	68.7	61.0	55.1	0.6 (0.4–0.9)
<2 cm	363	57.8	85.3	64.7	53.0	41.4	34.0	1.1 (0.8–1.7)
>2 cm	598	61.2	73.9	50.7	36.9	26.2	20.7	1.8 (1.2–2.5)
Unknown	578	60.3	82.4	63.1	48.4	35.6	28.0	1.2 (0.9–1.7)

[a]Hazards ratio and 95% Confidence Intervals obtained from a Cox model adjusted for age, stage and country.

Figure 40.4. Survival versus residual disease in ovarian cancer.[154]

Chemotherapy is almost universally recommended following surgical staging and debulking procedures. Intravenous carboplatin plus paclitaxel is generally considered the standard regimen for ovarian cancer based on a number of randomized clinical trials.[34–37] Because of the predominant peritoneal surface involvement of metastatic ovarian cancer, delivering active chemotherapy agents by an intraperitoneal (IP) route has been investigated since the 1970s. The benefit of IP chemotherapy as part of the initial up front approach in patients with stage III optimally debulked ovarian cancer is supported by the results of three randomized clinical trials that tested the role of IP drugs against the standard IV iv regimen. Superior progression-free survival (PFS) and overall survival (OS) favoring the IP arm were documented in all three trials. Specifically, the most recent study, GOG-172, resulted in a median survival rate of sixty-six months for patients on the IP arm versus fifty months for patients who received IV administration of cisplatin and paclitaxel ($P = 0.03$).[38–41] Patients in GOG-172 who had no gross residual disease at the end of their initial surgery have not yet reached their median survival. A benefit to intraperitoneal chemotherapy has been confirmed in a Cochrane review.[42]

Following surgery and chemotherapy, the majority of women with advanced disease will achieve clinical remission. Despite additional interventions, such as incorporating a third agent to up-front carboplatin and paclitaxel.[43] There is a consistent time course and pattern of recurrence (Figure 40.5). Ultimately, 80 percent to 90 percent of patients with advanced-stage disease will have recurrences. Consolidation chemotherapy[44] has been studied in the setting of advanced-stage disease and has been shown to prolong PFS by seven months, although without changing overall survival.[44a] CA-125 trends often provide the earliest clue to treatment efficacy during chemotherapy and also may provide the earliest evidence of disease recurrence when a patient is being monitored during remission.[9,10,45] Imaging studies such as CT or PET scans are frequently ordered in the face of symptoms suggestive of a recurrence or a rising CA-125. The results of a European randomized clinical trial (EORTC OV05/55955) analyzing whether routine CA-125 monitoring after completion of chemotherapy improves survival or quality of life have recently been reported. The results show that earlier detection of a recurrence with an increasing CA-125 does not improve survival and diminishes quality of life.[46]

The most important prognostic factor in women with ovarian cancer is sensitivity to platinum-based chemotherapy, also known as platinum sensitivity. The degree of platinum sensitivity correlates directly with overall survival. Clinical recurrences that take place within six months of completion of a platinum-containing regimen are considered platinum-refractory or platinum-resistant recurrences. Despite the overall sensitivity of ovarian cancer to chemotherapy, patients with this short an interval between treatment and recurrence have an exceedingly poor prognosis; the goals of treatment are to improve the symptom-free and progression-free survival as well as to improve quality of life. Chemotherapy drugs most commonly used to treat this patient population include liposomal doxorubicin and topotecan.[47–49] Response rates to chemotherapy are only 10 percent to 15 percent, with median overall survivals of approximately one year. These patients are encouraged to participate in clinical trials.

As a patient's platinum-free interval after initial chemotherapy increases, her likelihood of response to subsequent chemotherapy improves, translating into better overall prognosis. Recurrences diagnosed more than twelve months after the completion of initial therapy are best retreated with a platinum-based agent. Several studies have suggested that retreatment with chemotherapy doublets (e.g., carboplatin and paclitaxel or carboplatin and gemcitabine) will prolong overall survival when compared to a platinum-based agent alone.[47,50–53] Secondary surgical cytoreduction may be considered in patients with platinum-sensitive disease, especially if there is a long disease-free interval and an isolated site of recurrence.[54]

Novel, Emerging, and Targeted Therapies

There is a growing number of active chemotherapy agents that are frequently used sequentially in ovarian

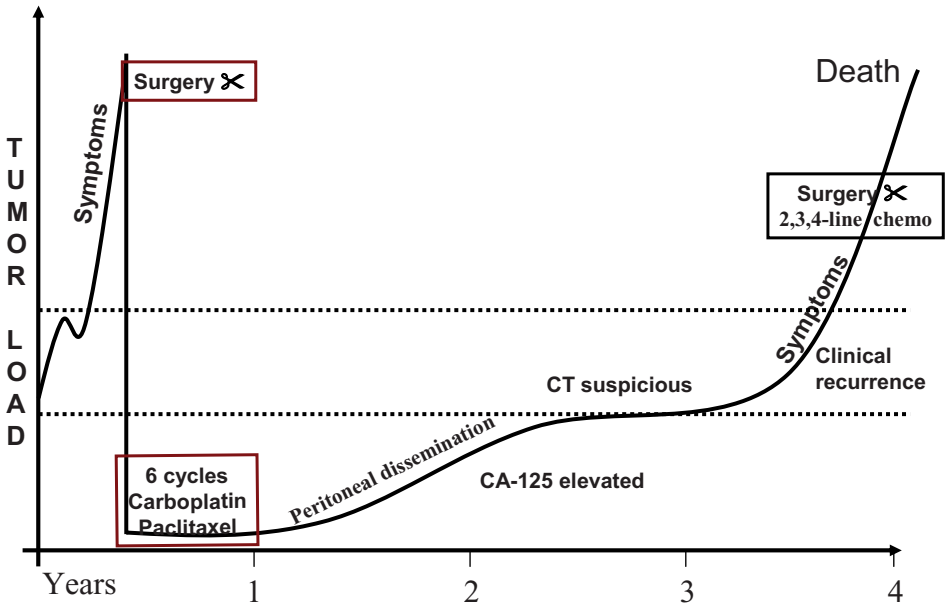


Figure 40.5. Typical time course for the progression of ovarian cancer.

cancer; this has led to incremental improvements in survival. A burgeoning area in ovarian cancer research is the discovery of molecularly targeted agents that are active in recurrent disease.

VEGF, a multifunctional cytokine that increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis, is of particular interest in the pathogenesis of ascites in ovarian cancer. VEGF is present in patients with ovarian cancer and is associated with poor prognosis and decreased overall survival.[55–57] It may function by directly promoting angiogenesis and by increasing vascular permeability, potentially creating an avenue for tumor metastasis. VEGF receptors are overexpressed on ovarian cancer cells, and VEGF itself is abundant in malignant ascites fluid. Levels of VEGF are higher in ascites associated with ovarian cancer than in ascites associated with other tumor types (Figure 40.6). Recently, VEGF inhibition has been shown to significantly decrease ascites formation and improve patients' symptoms.[58]

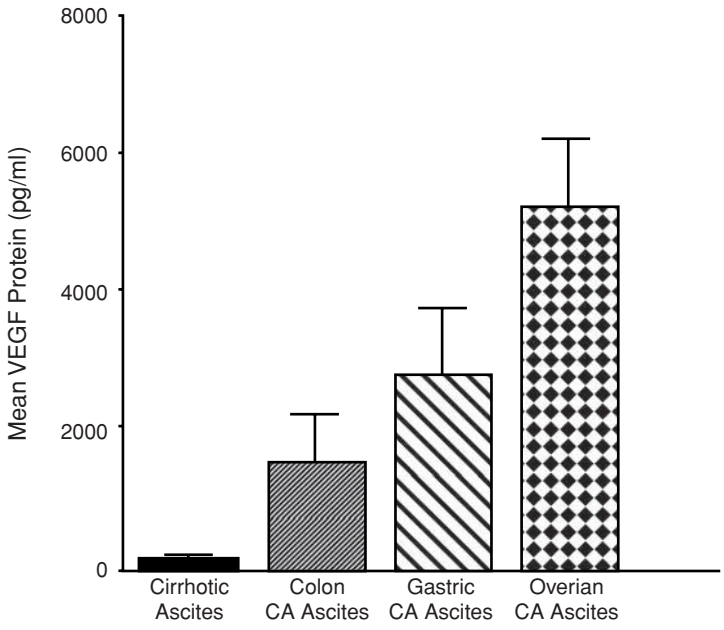


Figure 40.6. Mean VEGF protein levels in malignant ascites from patients with colon, gastric, and ovarian cancers. Samples of cirrhotic ascites were used as the nonmalignant control. VEGF protein levels in malignant ascites were markedly elevated compared with levels in nonmalignant cirrhotic ascites.[155]

A burgeoning area in the treatment of metastatic ovarian cancer involves the use of antiangiogenesis agents. VEGF receptors are expressed on ovarian cancer cells and VEGF itself is abundant in malignant ascites fluid.[55] Phase II studies using bevacizumab, a monoclonal antibody to VEGF, have demonstrated response rates of 16 percent to 21 percent, with the percentage of patients achieving PFS at six months at 27 percent to 56 percent when bevacizumab is used as a single agent or in combination with other therapies.[58–62]

The most common side effects associated with the antiangiogenesis agents are hypertension and fatigue. Problems with wound healing and spontaneous gastrointestinal perforation are also a significant concern and appear to occur with greater incidence in patients with bowel wall thickening and bowel obstructions.[59] Recent studies have sought to combine agents such as bevacizumab with other

targeted pathway inhibitors, such as sorafenib, a Raf kinase and VEGF receptor inhibitor[63] approved for renal cell cancer, or erlotinib, an EGFR inhibitor.[62] Although in some cases promising activity has been demonstrated, some patients have experienced significant toxicity, raising questions of whether the combination of such drugs will be tolerable.

Other molecular signaling pathways that are currently being targeted include the PI3K pathway, the mTOR pathway, the EGFR pathway, and the Ras-Raf kinase pathway via such agents as sorafenib, lapatinib, and erlotinib. It is becoming increasingly clear that the various epithelial histologic subtypes of ovarian cancer have different molecular expression patterns; this may, in fact, translate into the use of different targeted therapies (e.g., BRCA mutation carriers and PARP inhibitors).

Genetic Profile and Metastatic Progression

Differences in the gene expression profile and behavior of the various epithelial ovarian cancer subtypes have recently been described. Advanced serous and endometrioid ovarian cancers have a distinct expression profile when compared with clear-cell cancers arising from the ovary, uterus, and kidney, which have overlapping expression profiles.[64] Similarly, borderline malignant potential and low-grade serous ovarian cancers have distinct gene expression profiles that correlate with better prognosis when compared with their high-grade counterparts.[65] Activating BRAF and KRAS mutations have been identified in more than 60 percent of low malignant potential (serous borderline) tumors and low-grade serous tumors in conjunction with an activated downstream mitogen-activated protein kinase (MAPK).[66,67] A distinctive molecular profile associated with low-grade serous carcinomas has provided the justification for implementation of a clinical trial currently being conducted by the Gynecologic Oncology Group (GOG) using AZD 6244, an oral small molecule inhibitor of MAPK, MEK-1/2, which plays a central role in a number of processes important to the metastatic process, such as proliferation and regulation of apoptosis.

Patients who inherit a mutation in the *BRCA1* or *BRCA2* genes are at significantly increased risk of developing ovarian and fallopian tube cancer, as both genes are important in the regulation of DNA double-strand break repair by homologous recombination. The cumulative ovarian cancer risk by age 70 with an inherited *BRCA1* mutation is 39 percent (95% CI 22%–51%), whereas the lifetime risk with an inherited *BRCA2* mutation is 11 percent (95% CI 4.1%–18%).[68] Although the prognosis for patients who develop ovarian cancer in the face of an inherited mutation may be better, a significant proportion of patients still develop recurrent disease. Poly(ADP ribose) polymerase (PARP) is an enzyme that plays a central role in the repair of single-strand DNA breaks. *BRCA1* or *BRCA2* dysfunction sensitizes cells to PARP inhibition, resulting in increased chromosomal instability and apoptosis.[69] This differential effect in *BRCA* hereditary tumors has provided the rationale for the use of PARP inhibitors in Phase I and II clinical trials specifically designed for *BRCA* mutation carriers.[70] Gene expression profiling and a deeper understanding of the molecular differences in the epithelial cancers will continue to yield new targeted therapies; the future challenge will be in determining how to gauge "activity" of these agents, how to determine that they are, in fact, "hitting" their targets, and how to incorporate them into the increasing armamentarium of currently active chemotherapeutic agents.

There has not been a more exciting time in ovarian cancer research than the present. Incremental improvements have been made in survival, and there are a number of promising agents with nonoverlapping toxicities whose place in the sequential treatment of metastatic and recurrent ovarian cancer should be elucidated.

ENDOMETRIAL CANCER

Endometrial cancer is the most common gynecologic malignancy in the United States, with 40,100 new cases and 7,470 deaths estimated in 2008.[29] Endometrial cancer is predicted to be a continuing problem in the future owing to an aging population and an alarming obesity epidemic. Obesity is the greatest risk factor for the development of endometrial cancer, as it results in the production of excess endogenous estrogen.

Clinical Presentation of Endometrial Cancer

Patients commonly present with postmenopausal bleeding; following an endometrial biopsy they are typically diagnosed with cancer relatively early in their course of the disease. Patients with stage I endometrial cancer generally have an excellent prognosis, and the five-year relative survival rate is 97.4 percent.[71] The probability of lymph node metastasis increases with increasing tumor grade. The likelihood of pelvic node metastases in clinical stage I grades 1, 2, and 3 endometrial cancer is 2.8 percent, 8.7 percent, and 18.3 percent, respectively, and the reported risk for aortic node metastases is 1.6 percent, 4.9 percent, and 11.1 percent, respectively.[72] Other risk factors for metastases include the presence of deep myometrial invasion, the presence of lymph vascular space invasion, age over 60, cervical extension of disease, and high-risk cellular histologic types, such as papillary serous or clear cell carcinoma[72–74] (Table 40.2). In the GOG-33, patients with clinical stage I endometrial cancer were

TABLE 40.2. Risk of lymph node metastasis with endometrial cancer[152]

Risk Factor	No. patients	Pelvic no. (%)	Aortic no. (%)
Histology			
Endometrioid	599	56 (9)	30 (9)
Others	22	2 (9)	4 (18)
Grade			
1	180	5 (3)	3 (2)
2	288	25 (9)	14 (5)
3	153	28 (18)	17 (11)
Myometrial invasion			
endometrial	87	1 (1)	1 (1)
superficial	279	15 (5)	8 (3)
middle	116	7 (6)	1 (1)
deep	139	35 (25)	24 (17)

surgically staged and were stratified into three risk categories. High-risk patients were defined as having either deep myometrial invasion or intraperitoneal disease. Patients with deep myometrial invasion had an 18 percent risk of pelvic node and a 15 percent risk of paraaortic lymph node metastasis. Intermediate-risk patients, defined as grade 2 or 3 histology and/or inner to mid-myometrial invasion, had a 2 percent to 6 percent risk of nodal metastasis. Low-risk patients with grade 1 histology and endometrial involvement only had no lymph node metastases in this study.[72]

Although serous and clear-cell carcinomas of the uterus account for fewer than 10 percent of the cases of endometrial cancer diagnosed, these tumors are clinically aggressive and usually present at a higher stage of disease than their endometrioid-type counterparts. Despite the low prevalence of this histologic type, serous tumors account for approximately 25 percent of patients in GOG trials of advanced/recurrent disease.[75] Unlike cancers of endometrioid histology, serous carcinomas frequently spread intraabdominally even in the presence of minimal or absent myometrial invasion. These tumors are more common in African American women than in non-Hispanic white women, which may account for some of the racial disparities in outcomes in endometrial cancer survival. African American women tend to have poorer overall survival from endometrial cancer than their white counterparts.[76] In contrast to the more common endometrioid tumors, the serous papillary tumors tend to lack estrogen and progesterone receptor expression and stain positively for p53. They rarely exhibit microsatellite instability or *PTEN* mutations.[77–79]

Uterine carcinosarcomas, previously referred to as mixed malignant Müllerian tumors, are an even less common yet similarly aggressive subset of endometrial tumors. They commonly present at an advanced stage (only 50% of patients present with stage I disease when appropriately surgically staged), and the five-year relative survival rate for patients with stage I disease is only 74 percent.[80] As with other poor-prognosis endometrial cancers, African American women are disproportionately affected.[81] Although carcinosarcomas were originally classified as sarcomas for the purpose of treatment and clinical trials, it has become clear that their behavior is different from the mesenchymally derived sarcomas, and most pathologists now generally regard them as metaplastic carcinomas.[82]

Diagnosis, Imaging, and Staging

Since 1988, the International Federation of Gynecology and Obstetrics (FIGO) has recommended surgical staging for women diagnosed with endometrial cancer. Surgical staging involves hysterectomy with bilateral salpingo-oophorectomy, pelvic and paraaortic lymph node sampling, and omentectomy for patients with serous histology on initial biopsy. Stage I disease includes disease confined to the uterus; stage II includes extension to the cervix; stage III includes spread to pelvic organs and/or lymph nodes; and stage IV includes metastases to the upper abdomen or distant parenchymal organs. Surgical staging of endometrial cancer allows for identification of patients who might benefit from adjuvant therapy, including allowing well-staged patients to avoid the toxicity of radiation. The information gleaned from lymph node dissection changes postoperative management in up to 30 percent of patients with endometrial cancer.[83] Several retrospective studies have demonstrated improved survival in patients undergoing lymphadenectomy with increasing numbers of lymph nodes retrieved. This raises the question of whether lymphadenectomy may remove occult sites of metastatic disease that may give rise to future recurrences.[84–87] A large randomized trial has not shown an improvement in survival with lymphadenectomy but did not control for subsequent treatment after metastatic disease was found.[152]

As imaging modalities become more sophisticated, there is hope that a noninvasive radiographic test will provide information concerning the status of the lymph nodes. However, to date, results have been disappointing. CT scans have poor sensitivity and specificity in detecting the depth of myometrial invasion, cervical and parametrial involvement, and lymph node metastases.[88] MRI is more accurate than CT scans, with an overall staging accuracy of 85 percent.[89] However, MRI was only 54 percent sensitive in determining deep myometrial invasion and had limited specificity for detecting pelvic and paraaortic node metastases.[89] In a more recent study comparing MRI with PET/CT, Park et al. demonstrated that PET/CT had a sensitivity of 69.2 percent, specificity of 90.3 percent, positive predictive value of 42.9 percent, and negative predictive

value of 96.6 percent. Although these results were statistically comparable to MRI, it does not appear that PET/CT can replace surgical staging.[90]

Treatment of Metastatic Disease

Prognosis is poor in the subgroup of patients with metastatic endometrial cancer. The median survival of women enrolled in clinical trials for recurrent or metastatic endometrial cancer is approximately twelve months.[91] Standardized treatment protocols for advanced metastatic endometrial carcinoma have been difficult to develop, in part because of the limited therapeutic efficacy of radiation therapy, chemotherapy, and hormonal therapy on large-volume disease. The mainstay of treatment of recurrent and metastatic endometrial cancer remains systemic therapy in the form of cytotoxic chemotherapy or hormones. Patients with low-grade disease with estrogen and progesterone receptor-positive carcinoma tend to respond as well to hormonal therapy as to cytotoxic chemotherapy, but with fewer side effects. The use of hormones may also be preferable in patients with poor performance status and/or multiple medical comorbidities. Cytotoxic chemotherapy may be more appropriate for patients with high-grade disease and fewer comorbidities. For patients who have undergone an initial surgery and are left with low-volume disease but with a high risk of recurrence, adjuvant combination chemotherapy in the form of adriamycin and cisplatin has been shown to improve progression-free and overall survival when compared to whole-abdomen radiation. The stage-adjusted death hazard ratio was 0.68 (95% CI, 0.52–0.89; $P < 0.01$) favoring chemotherapy.[92]

Doxorubicin has historically been the most active single-agent chemotherapy available in the treatment of metastatic endometrial cancer, with reported response rates of 17 percent to 37 percent.[75,93,94] However, multiple other single agents are active in endometrial cancer, including the platinum compounds and paclitaxel. Combination chemotherapy has consistently been shown to have higher response rates than single-agent regimens. The most active regimen described to date in a randomized controlled trial is doxorubicin, cisplatin, and paclitaxel with G-CSF support. The three-drug arm produced more objective responses than the two-drug arm of doxorubicin plus cisplatin (57% versus 34%, $p < 0.01$). PFS was extended to 8.3 months, compared with 5.3 months in the control arm ($p < 0.01$), and overall survival reached a median of 15.3 months, compared with 12.3 months ($p < 0.037$). As seen in previous trials, increasing efficacy with more chemotherapy also led to more toxicity. Patients receiving the three-drug combination were more likely to suffer grade 3 and 4 hematologic toxicity and neurotoxicity.[95]

A Cochrane review recently attempted to address the issue of whether more chemotherapy leads to better outcome in treating advanced, recurrent, or metastatic endometrial cancer. Eleven randomized clinical trials were identified that included 2288 patients. A meta-analysis of six trials showed improved PFS with more intensive chemotherapy as compared with less intensive chemotherapy (HR = 0.80, 95% CI 0.71–0.90; $p = 0.004$) but also showed a comparable overall survival (HR = 0.90, 95% CI 0.80–1.03; $p = 0.12$). Grade 3 and 4 toxicity, particularly in the form of myelosuppresion and gastrointestinal toxicity, was higher in patients receiving more intense chemotherapy regimens.[96]

Because serous tumors can metastasize at an early stage, it has become common practice to treat even early-stage patients with chemotherapy. Despite the biologic differences, the response rates to and survival benefits from cytotoxic chemotherapy for women with advanced-stage serous tumors do not seem to be any different from those for women with advanced-stage tumors of endometrioid histology. There is no survival difference between node-positive women with serous or clear-cell carcinoma compared with women with endometrioid endometrial cancer.[97]

Patients diagnosed with carcinosarcoma have a particularly poor prognosis. There are limited drugs with documented single-agent activity; these include ifosfamide, cisplatin, and paclitaxel. Prospective randomized combination therapy trials have used ifosfamide-based regimens. As with carcinomas, combination therapy has been somewhat more active, yet concurrently more toxic. Ifosfamide in combination with cisplatin was shown to be superior to ifosfamide alone, with response rates of 54 percent versus 36 percent and overall survival of 9.4 months versus 7.6 months.[98] Ifosfamide combined with paclitaxel has also recently shown increased response rates compared with ifosfamide alone.[99]

In select patients with metastatic disease, localized therapy may be useful. A small minority of women who have recurrences will present with solitary lesions that are amenable to radiation with or without surgical resection. Women with vaginal recurrences who have not received radiation may be treated with radiation, with complete response rates between 40 percent and 81 percent.[100–102] Small central pelvic recurrences within a radiated field may be cured with pelvic exenteration.[103] Multiple reports of women with isolated recurrences to the lung parenchyma, brain, or liver who achieve long-term survival following an excisional surgical procedure have been published.[104,105]

The role of debulking surgery for patients with metastatic disease is less clear. Surgical stage IV disease represents less than 10 percent of all endometrial cancer. Maximum surgical cytoreduction similar to that with ovarian cancer has been shown to improve

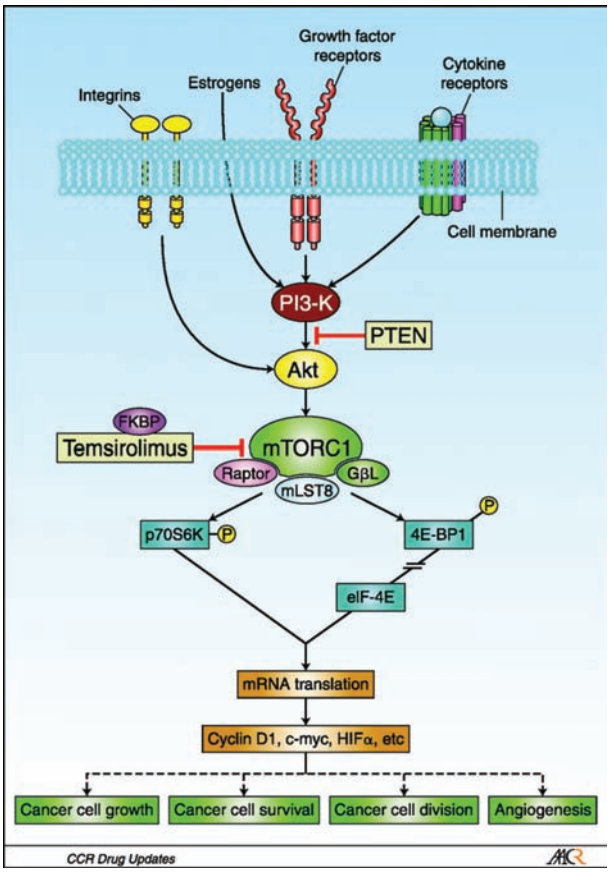


Figure 40.7. Molecular targets in endometrial cancer.[157]

survival in several studies.[106–108] However, these studies have all been retrospective in nature. The prognosis for patients who have recurrences of endometrial cancer is poor because of the paucity of active chemotherapy agents and other effective targeted modalities.

Investigational, Emerging, and Targeted Therapies

As measured by immunohistochemistry (IHC), a loss or decrease of phosphatidylinositol-3 kinase (PTEN) expression is seen predominantly in endometrioid-type cancers.[109–113] PTEN knockout mice develop atypical endometrial hyperplasia and endometrial cancer.[114] PTEN-deficient cells are sensitive to mammalian target of rapamycin (mTOR) inhibitors in vitro, as loss of PTEN leads to constitutive activation of Akt, which in turn upregulates mTOR activity (Figure 40.7). mTOR inhibition in in vivo models has decreased progression of PTEN-derived endometrial hyperplasia to endometrial cancer, leading to interest in using mTOR inhibitors to treat endometrial cancer.

The National Cancer Institute of Canada reported a preliminary response rate of 26 percent in chemotherapy-naïve endometrial cancer patients treated with the mTOR inhibitor temsirolimus. Response in this group of patients was not correlated to PTEN status, as evaluated by IHC.[115] Preliminary studies of other mTOR inhibitors, everolimus and AP2357, have shown clinical responses mainly in the form of stable disease (8 of 15 and 7 of 19 women, respectively).[116,117] Combinations of mTOR inhibitors with hormonal therapy, chemotherapy, and other targeted therapies, such as EGFR inhibitors and anatiangiogenic agents, have all been promising in the preclinical setting, and numerous trials developing and testing such combinations are under way.

The rationale for studying antiangiogenesis agents in endometrial cancer has included finding high VEGF expression levels in 56 percent of invasive endometrial cancers. Stage and grade of tumor, VEGF levels, and microvessel density have been associated with shorter disease-specific survival.[118] A small, retrospective review of the use of bevacizumab in recurrent uterine neoplasms showed two responses and three women with stable disease among ten evaluable patients.[119] A Phase II trial of single-agent bevacizumab in metastatic endometrial cancer has been completed within the GOG.[120] Additional Phase II studies using other agents with antiangiogenesis activity such as VEGF trap, sorafenib, and sunitinib, are ongoing.

HER-2 overexpression has been demonstrated and linked to prognosis in many cancer types. Amplification of the HER2 gene, resulting in overexpression of the receptor, is more commonly found in nonendometrioid endometrial cancers and is associated with an aggressive form of the disease with significantly shortened disease-free survival and OS.[113,121] A recent study of HER2 expression in banked tissue from patients with endometrial cancer revealed that 104 of 234 patients (44%) showed positive (2+/3+) cellular membrane HER2 expression on IHC staining. Uterine papillary serous carcinoma had the highest rate of HER2 overexpression by IHC and of gene amplification measured by fluorescent in situ hybridization (FISH; 43% and 29%). IHC and FISH positivity resulted in a hazard ratio of disease-specific death of 6.86 (95% CI 3.45–13.63).[122] Trastuzumab is a monoclonal antibody to the extracellular domain of the HER2 protein. Although the HER2 overexpression seen in serous carcinoma of the uterus provides a strong biologic rationale for the use of trastuzumab in the treatment of this malignancy, a GOG study examining the use of trastuzumab in women with HER2-positive endometrial cancer did not report any activity.

It is hoped that some of these newer therapies will be able to target specific known molecular defects in endometrial cancer, such as mismatch repair defects or PIK3CA mutations, and will make some meaningful improvements in the prognosis of women with metastatic disease. Unlike ovarian cancer, for which incremental improvements have been made in survival,

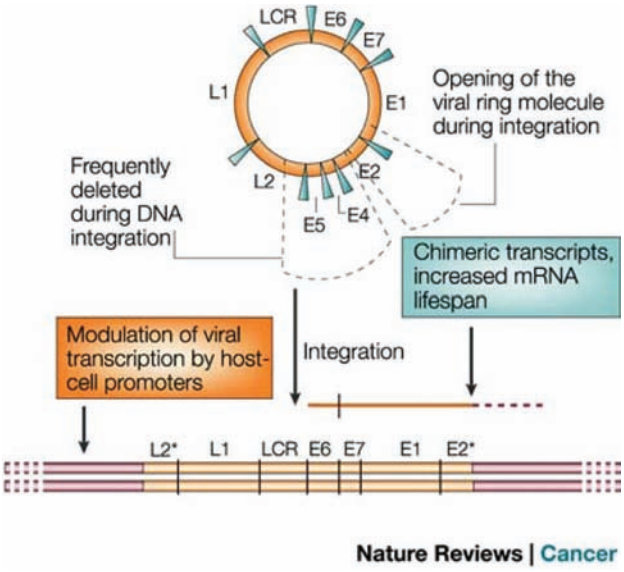


Figure 40.8. HPV genome.[158]

there are a limited number of active second-line treatments that can be used to prolong PFS and disease stabilization. These patients should continue to be encouraged to participate in clinical trials.

CERVICAL CANCER

Cervical cancer is the sequela to a long-term, unresolved infection by certain genotypes of the human papillomavirus (HPV).[123] Although cervical cancer remains a major cause of morbidity and mortality worldwide, in the United States fewer than 11,000 women per year will be affected with cervical cancer.[29]

Etiology of Cervical Cancer

The HPVs are DNA double-strand viruses of small size (approximately 8000 pairs of bases) that have cohabited with the human species for millennia. The cumulative prevalence of four types (HPVs 16, 18, 45, and 31) is associated with approximately 80 percent of the worldwide cases of cervical cancer. The HPV genome is divided into eight open reading frames – E6, E7, E1, E2, E4, E5, and L2 and L1 – coding for "early" (E) or "late" (L) functions (Figure 40.8). In the course of cancer development, the viral molecule frequently becomes integrated into host-cell DNA.[124] The HPV virus oncogenicity arises from the E6 and E7 viral genes. The products of these two genes bind respectively to the p53 and Rb tumor suppressor genes, spurring cellular immortalization and growth.[125,126]

The malignant transformation of the cervix typically occurs at the transformation zone of the cervix – which represents the squamocolumnar junction, the most mitotically active zone of the cervix. The majority of carcinomas of the cervix are squamous cell (80%) and adenocarcinoma (15%).[4]

Diagnosis, Imaging, and Staging

Metastatic disease in cervical cancer typically travels via the lymphatics in a predictable and stepwise fashion. From the cervix, metastases travel to the paracervical lymph nodes and then to the pelvic lymph nodes, to the paraaortic lymph nodes, and finally to the scalene lymph nodes. As stage increases, the likelihood of metastasis increases. For women with stage III and IV disease, pelvic and paraaortic nodes will be involved with tumor in approximately 50 percent and 30 percent of cases, respectively.[4,123,127–129] Direct extension of cancer into the bladder and rectum does occur at times, putting the patient at risk for fistula formation either directly from extension of disease or from tissue necrosis following the start of treatment. Direct extension of cancer to the pelvic sidewalls may lead to obstructive uropathy as a result of pressure placed on the ureters and, eventually, renal failure, uremia, and death.

Because of its prevalence outside the developed world, cervical cancer represents the only remaining gynecologic malignancy that is staged clinically through a combination of physical examination and basic radiologic studies. Clinical staging may leave lymph node metastases undiagnosed.[127,130] It is accepted commonly that patients with paraaortic lymph node (PALN) metastases have high rates of recurrence. Controversy exists over surgical staging for cervical cancer. Proponents cite the ability to debulk grossly positive lymph nodes[131,132] as well as to modify patients' radiation fields.[127,129,133,134] A survival advantage to surgical staging has been demonstrated in some studies.[129,134,135] However, as these studies are retrospective and biased by patient selection, critics of surgical staging cite an increase in morbidity to surgery and an increase in time to treatment with minimal improvement in survival when compared with that gained by the addition of chemotherapy to the treatment of locally advanced cervical cancer with radiation since the 1990s.[136]

A variety of imaging modalities have been used to detect lymph node disease in women with cervical carcinoma. Historically, lymphangiography (LAG) was one of the first procedures used to evaluate the pelvic and paraaortic lymph nodes. More recently, CT, MRI, and ultrasonography have been used for the evaluation of the regional lymph nodes. Although LAG depends on the presence of lymph node filling defects for the detection of metastatic lymph node involvement, MRI and CT rely on lymph node enlargement. A meta-analysis of seventeen studies comparing LAG, MRI, and CT revealed that all three tests are equally inaccurate for the identification of metastatic disease.[137]

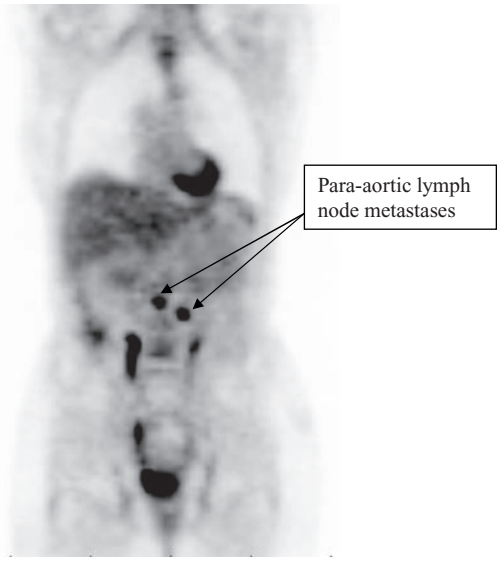


Figure 40.9 FDG-PET scan in a patient with metastatic cervical cancer to the lymph nodes.

The sensitivity and specificity of PET scan has improved and can be used to identify metastases (Figure 40.9).[27,138–141] PET scanning had a sensitivity of 75 percent, a specificity of 92 percent, a positive predictive value of 75 percent, and a negative predictive value of 92 percent in women with locally advanced cervical cancer, when PET was subsequently verified by surgical removal of lymph nodes.[142] FDG-PET has been shown to be useful in cervical cancer for initial staging and treatment planning, as well as posttreatment surveillance and follow-up.

Treatment Options

The primary treatment options for early cervical cancer (stage IIA or less advanced) are surgery and chemoradiotherapy, which have similar survival rates.[4] Locally advanced cervical cancer (Stage IIB and greater) is, however, treated with whole-pelvic radiation combined with brachytherapy. In 1999, two randomized controlled clinical trials showed the benefits of adding chemotherapy to radiation as a radiation sensitizer to improve overall survival.[143–145] Concurrent chemotherapy is now given in the form of weekly cisplatin or cisplatin plus 5-fluorouracil every three weeks along with radiation for the treatment of locally advanced cervical cancer.

The prognosis for recurrent, metastatic cervical cancer is poor, with overall survival of only six to nine months. Standard therapy involves platinum-based chemotherapy. Typical response rates to single-agent cisplatin are only 20 percent, and these responses tend to be short-lived. Combining cisplatin with other agents, such as ifosfamide, paclitaxel, and topotecan, have increased response rates to 27 percent to 36 percent.[146–149] Unfortunately, these improvements in response rate translate to minimal improvements in overall survival.

Few targeted therapies have shown benefit in this patient population. The most promising targeted agent to date is cetuximab, a monoclonal antibody directed at the extracellular domain of EGFR.[150] Cetuximab has shown activity in other advanced squamous cell malignancies, such as head and neck cancer. Overexpression of this target has been correlated to poor prognosis in cervical cancer patients.[150,151] However, the role of targeted agents in the treatment of metastatic cervical cancer is not defined at this time.

CONCLUSION

Gynecologic malignancies present unique oncologic and clinical challenges to clinicians, scientists, and patients. Recent advances in the molecular biology and genetic basis of cancer have led to an improved understanding of the nature of these diseases. The treatment options for patients have grown exponentially over the last few years, and we look forward to these advances leading to improved patient outcomes.

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41 Prostate Cancer Metastasis: Thoughts on Biology and Therapeutics

Kosuke Mizutani, Russell S. Taichman, and Kenneth J. Pienta

Prostate cancer accounts for 25 percent of all cancer diagnoses in American men (186,320 in 2008) as well as 10 percent of cancer deaths in men (28,660 in 2008) [1]. Although mortality has fallen by 25 percent over the past decade and the five-year survival is approaching 100 percent, several challenges to decreasing the morbidity and mortality from metastatic prostate cancer remain.

The end result of the process of metastasis in prostate cancer is clear; nearly 100 percent of men suffer from osteoblastic bone metastases at the time of death (Figure 41.1) [2–5]. The mechanisms by which prostate cancer metastasizes to bone sites remain obscure, but there is no doubt that forces that drive preferential seeding are at play. In 1889, Stephen Paget proposed the "seed-and-soil" theory to explain the nonrandom pattern of cancer metastasis [6]. His theory suggested that factors within the metastatic site promote growth, which is analogous to the tendency of seeds to grow in fertile soil (i.e., factors in the environment of the metastatic site could contribute to the proliferation of cancer cells). In 1928, James Ewing proposed that cancer cells grow at a particular site because they are directed to that site by the direction of blood flow and lymphatics [7]. It appears that both these theories have correct elements. Isaiah Fidler defined the modern "seed-and-soil" hypothesis as consisting of three principles [8]. First, cancerous tissues contain heterogeneous subpopulations of cells with different angiogenic, invasive, and metastatic properties. Second, the metastatic process is selective for cells that have survived the journey to the distal site. Finally, the success of the metastatic cells depends on their ability to interact and use the "soil" provided in their new microenvironment [8, 9].

Studies of metastasis have revealed it to be a very inefficient process, with experimental models demonstrating an inefficiency on the order of 10^{-6} to 10^{-7} [10]. Preclinical studies of luciferase-labeled PC-3 human prostate cancer cells delivered into immunocompromised mice via intracardiac injection led to localization of cancer cells within fifteen minutes postinjection in the lungs, kidneys, and long bones; however, no viable cells were visualized after twenty-four hours, indicating that most of the injected cells were either dead or metabolically inactive (dormant) [11, 12]. The process by which cells spread to bone sites and remain viable remains undefined. Previous studies have demonstrated the frequency of organ involvement by prostate cancer metastases at the time of death [13]. By combining these data with data on blood flow rates to various organs, it can be demonstrated that prostate cancer cells metastasize to bone at a much more efficient rate than can be simply explained by blood flow to those organs (Table 41.1) [9, 13–15]. In Table 41.1, metastatic efficiency is calculated at the percent involvement of organs at autopsy/the blood perfusion rate in mL/kg/min. Comparing percent organ involvement in advanced prostate cancer patients and corresponding blood perfusion rates demonstrates that the rate of metastasis does not correlate with blood flow. These data strongly support the seed-and-soil hypothesis and suggest that the frequency of bone metastases in prostate cancer patients is not a simple function of number of cancer cells delivered to the bone microenvironment, but rather of mechanisms that lead to increased numbers of cells potentially residing in the bone marrow and increased cell viability through periods of cell dormancy and growth.

Recent data suggest that a major reason prostate cancer cells migrate and flourish in bone is secondary to the unique properties of the bone marrow microenvironment to maintain adult hematopoiesis. Blood formation occurs in unique environments or "niches" in the marrow. Niches facilitate the maintenance of hematopoietic stem cells (HSCs) as progenitor cells, while also facilitating their differentiation. Prostate cancer cells act as molecular parasites to harvest

TABLE 41.1. Relative efficiency of successful prostate cancer metastases in different organs

Organ	Percent involvement at autopsy	Blood perfusion rate mL/kg/min	Met efficiency
Bone	90	30	3
Liver	65	1,000	0.065
Lymph	59	500	0.118
Lung	38	400	0.095
Adrenal	24	2,000	0.012
Brain	10	560	0.017
Spleen	5	1,200	0.004
Thyroid	3	5,000	0.0006
Kidney	3	4,000	0.00075

resources from the niche environment [16, 17]. This parasitism represents a process that precedes the cascade of events regulated by osteoblast–osteoclast interactions commonly referred to as the "vicious cycle." The metastatic process is functionally akin to the migrational or "homing" behavior of HSCs to the bone marrow. Numerous molecules have been implicated in regulating HSC homing, participating as both chemoattractants and regulators of cell growth. Previous studies have drawn heavily on the parallels between HSC homing and the homing of PCa cells to the bone marrow. For example, prostate cancer cells use the CXC chemokine stromal-derived factor-1 (SDF-1 or CXCL12) and its receptors (CXCR4 and RDC1 / CXCR7) as key elements in metastasis and growth in bone, whereas CXCR4 signaling leads to an angiogenic switch [18–25].

Identification of the HSC niche in the marrow remains an active area of investigation and is critical to our understanding of the way in which prostate cancer metastasizes (Figure 41.2) [26–29]. Evidence suggests that HSCs may exist in endosteal and/or endothelial niches. Recent work has demonstrated that annexin II (Anxa2), expressed by osteoblasts and endothelial cells, plays a critical role in niche selection and homing of not only HSCs but also prostate cancer cells to the bone microenvironment [26]. The preponderance of data suggests that the metastatic process is akin to the "homing" behavior of HSCs to the bone marrow and that prostate cancer cells act as molecular parasites to establish themselves in the bones of men with prostate cancer.

DETECTION OF METASTATIC PROSTATE CANCER TO BONE

Imaging

The technecium-99m radionuclide bone scan remains the most sensitive test for detecting skeletal prostate cancer metastases (Figure 41.1) [30]. The rate of positive bone scanning in patients showing PSA levels less than 10 ng/mL, 10–50 mg/mL, and more then 50 mg/mL are less than 1 percent, approximately 10 percent, and 50 percent, respectively [31–33]. Although bone scans are functional tests that reveal nonspecific bone injury, they can be correlated with structural imaging through standard radiography or computed tomography. Several other potential modalities for detection, as well as assessment of treatment response, are under active development [34–36]. Although the use of 18F-fluoride PET scanning has proven to have limited value, the use of 11C or 18F-choline PET/CT, as well as whole-body MRI, to detect prostate cancer metastases both appears promising [36–38].

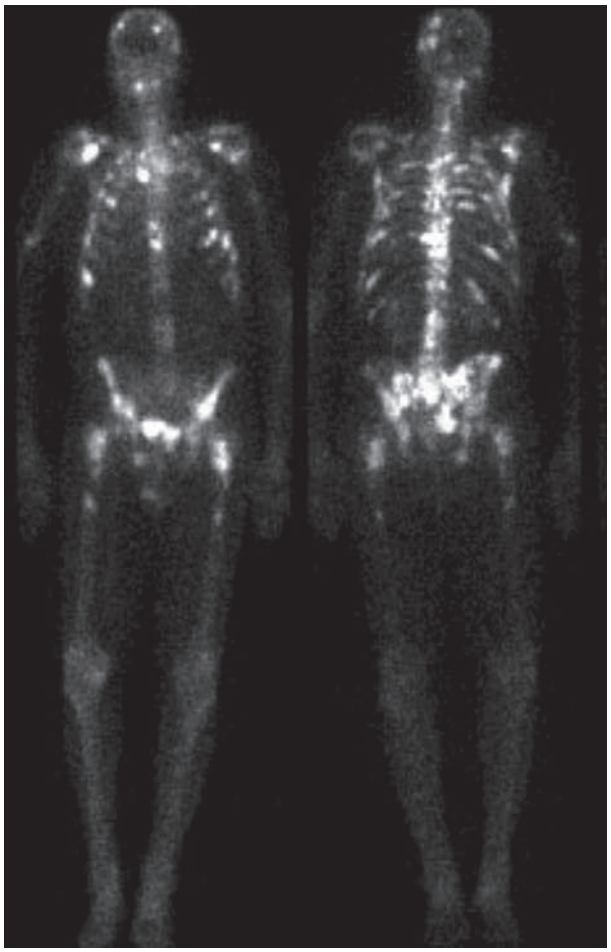

Figure 41.1. Radionuclide bone scan of a man with metastatic prostate cancer. The bone scan is currently the most sensitive widely available test to identify metastases to the bone. The technetium-99m radioisotope localizes to areas of bone damage [5].

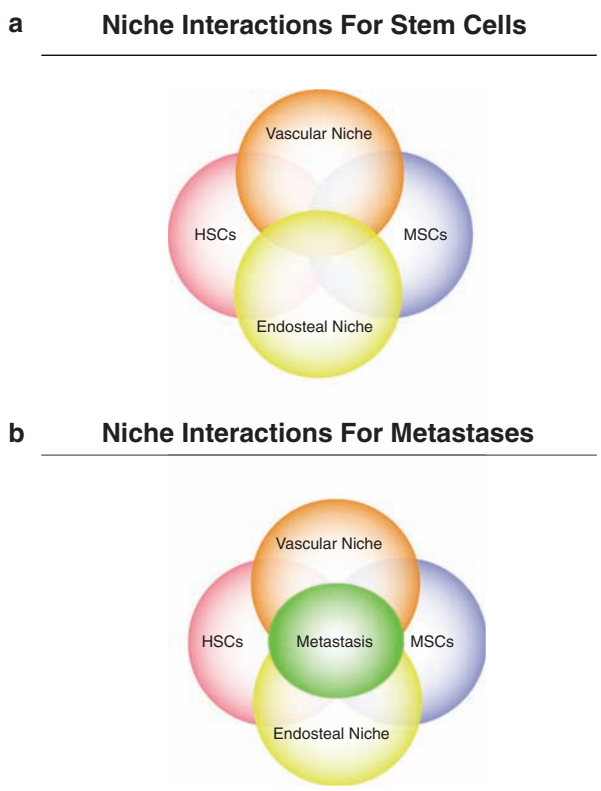

Figure 41.2. A model of bone marrow niches. (**A**) In the marrow, osteoblasts and endothelial cells constitute the major cellular components contributing to the endosteal and vascular niches that serve as the microenvironment for maintaining hematopoietic stem cells (HSCs; "HSC niche"). Osteoblasts and endothelial cells are derived from mesenchymal stem cells (MSCs) and hemangioblasts, respectively. Recent data suggest that MSCs themselves may reside in niches that are in close proximity to the HSC niche. In addition, there is growing evidence that HSCs and MSC coregulate activities of each other. In the model presented, overlap of HSC, MSC, vascular, and endosteal niche function occurs and is required for the coordinated function of the marrow. (**B**) A model of the neoplastic niche. Increasing evidence suggests that disseminated tumor stem cells reside in niches that facilitate the metastasis and survival of tumors in distant tissues. Much like HSCs and MSCs, the residence of metastatic cells in the niche provides signals that regulate dormancy and escape from chemotherapy and radiotherapy. As such, the residence of metastatic cells in the niche constitutes a molecular parasite of the normal host regulatory functions that exist to supply a constant flux of HSC and MSC progeny throughout the lifetime of the individual. Reprinted with permission [16].

Although steps are being made in the detection of bone metastases, assessment of disease response in these men remains problematic. Bone scans do not change fast enough to assess the response to therapy, and no other test has been demonstrated to be effective at assessing whether a therapy is killing cancer cells within bone lesions. Assessments of tumor response in bone using criteria defined by the International Union Against Cancer, the World Health Organization, and the Response Evaluation Criteria in Solid Tumors (RECIST) group do not meet the needs of oncologists in clinical practice. In fact, the RECIST system considers bone disease to be unmeasurable [39, 40]. Therefore, development and validation of an imaging technology that would be capable of reliably and accurately measuring antitumor effect in metastatic bone disease would provide a significant advance and aide in the timely investigation of new therapeutic agents not only for Pca, but also for other malignancies common to the bone.

The use of functional diffusion MRI for assessing response to cytotoxic therapy is based on its ability to quantify the random or Brownian motion of water. Diffusion of water within a tumor is reduced in the presence of cellular membranes that act to impede the random motion of water molecules. During the course of successful treatment, loss of tumor cells and/or tumor cell membrane integrity occurs, resulting in a reduction in the barriers that impede mobility of water molecules. Diffusion MRI can be used to assess the treatment effect through quantification of the amount of increased apparent diffusion coefficient (ADC) values in tumor regions experiencing a loss of cellular density [41]. Functional diffusion MRI has been demonstrated to quantify cancer cell death in a variety of tumors, including prostate cancer bone lesions (Figure 41.3) [41]). It is hoped that this type of technique will allow therapeutic testing to be accelerated by eventually serving as surrogates for overall survival in clinical trials.

Biochemical Markers of Bone Metabolism

Biochemical markers from bone resorption and bone formation can also be used for assessing the extent and activity of bone metastasis. An increase in bone turnover markers in prostate cancer patients may reflect bone metastases but also may be the result of androgen deprivation on bone metabolism. The major structural protein in bone is type I collagen. In normal adult bone, type I collagen is synthesized by osteoblasts and accounts for 90 percent of the organic matrix. The metabolic products of type I collagen synthesis and degradation reflect the activity of bone formation and resorption [42]. Bone resorption markers include alkaline phosphatase, calcium, hydroxyproline, collagen pyridinium crosslinks (Pyr and D-Pyr), tartrate-resistant acid phosphatase type 5b (TRACP5b), and type I collagen telopeptide fragments (NTX and CTX) that can be measured in the serum and/or urine of patients with bone metastasis, however, these factors have been not been put into widespread use because of lack of sensitivity and specificity [42–46]. Amino terminal and carboxy terminal telopeptides of collagen I (P1NP and ICTP) may provide more sensitive and specific measures of bone metastases, but further studies will need to be performed. The bone markers of resorption do appear to be an effective method to monitor the

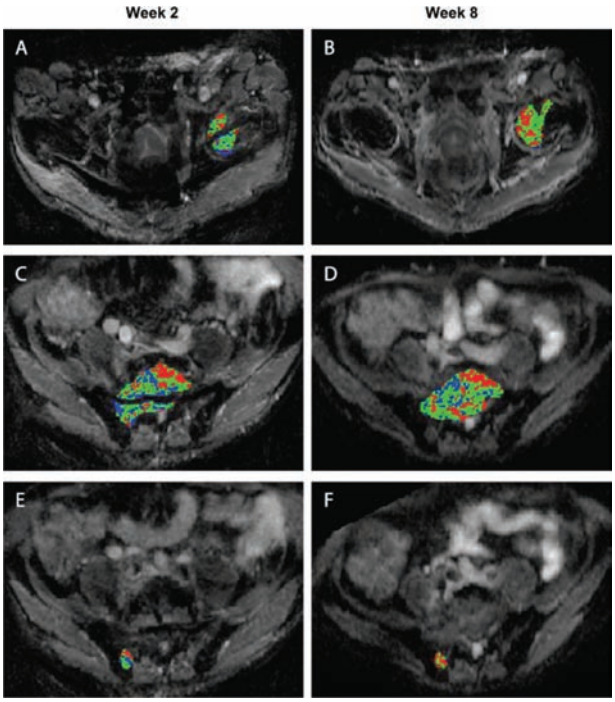

Figure 41.3. Functional diffusion maps in a patient treated for prostate cancer. Regional changes of ADC are plotted on the image to provide a visual representation of areas with increased ADC (red voxels), decreased ADC (blue voxels), and areas in which ADC did not change significantly (green voxels). fDM analysis of the femoral head lesion (yellow arrows) at (A) 2 and (B) 8 weeks after treatment initiation revealed distinct regions of red voxels signifying areas with significant increases in ADC ($>26 \times 10^{-6}$ mm^2/s). fDM analysis of the sacral lesion (red arrows) at (C) 2 and (D) 8 weeks after treatment revealed significant regions of increased ADC as depicted by the red voxels. fDM analysis of the ilium lesion (green arrows) at (E) 2 and (F) 8 weeks after treatment show large regions of increased ADC values (red voxels). Reprinted with permission [41].

inhibition of bone resorption by the bisphosphonates [46]. The importance of these measures in clinical practice remains to be defined.

CURRENT STANDARDS FOR TREATING BONE METASTASES IN MEN WITH ADVANCED PROSTATE CANCER

Systemic Hormonal Therapies and Chemotherapies

The interaction of prostate cancer cells with the bone microenvironment has been described as a vicious cycle in which prostate cancer cells interact with cells of the normal bone microenvironment in a complex interplay of deregulation and stimulation, resulting in osteoblastic metastases [47–49]. The first steps in treating metastatic prostate cancer are directed toward decreasing tumor burden through hormonal therapy and chemotherapy [50–52].

Androgens are primary regulators of normal prostate as well as primary and metastatic prostate cancer cell growth and proliferation. Initially, almost all metastatic prostate cancers require testosterone for growth, and the role of androgen deprivation as a first-line therapy for metastatic prostate cancer has been recognized for more than half of a century [53, 54]. Androgen deprivation is accomplished by surgical (orchiectomy) or medical (luteinizing hormone-releasing hormone agonists, antiandrogens) castration and leads to remissions lasting two to five years. Eventually, prostate cancer cells develop a variety of cellular pathways to survive and flourish in an androgen-depleted environment, including androgen receptor (*AR*) gene amplification, *AR* gene mutations, involvement of coregulators, ligand-independent activation of the androgen receptor, and replenishment of the tumor by cancer stem cells [50–52].

Two chemotherapeutic regimens are currently widely used to treat prostate cancer. The taxane docetaxel was the first chemotherapeutic agent to demonstrate a survival benefit for patients with androgen-independent prostate cancer [55]. In a trial involving 1006 men with hormone refractory prostate cancer, 75 mg/m^2 of docetaxel given every three weeks plus daily prednisone led to a median survival rate of 18.9 months, compared with a survival rate of 16.4 months in patients who received 12 mg/m^2 of mitoxantrone given every three weeks plus daily prednisone ($P = 0.009$). Docetaxel every three weeks plus prednisone therapy also resulted in improvement in pain, a decrease in prostate-specific antigen, and improved quality of life compared with treatment with mitoxantrone plus prednisone. Mitoxantrone, however, continues to be used to treat men with symptomatic metastatic prostate cancer as an effective palliative therapy [56].

Radiation Therapy

Radiation therapy has been used for patients with bone metastasis as an effective palliative measure for many years [57–59]. Local radiation (external X-ray or gamma ray beam) is effective at preventing spinal cord compression and obtaining pain relief in 80 percent to 90 percent of patients. Multiple sites of painful bone metastases can be treated effectively with the systemically administered radioisotopes [60–62]. In the United States, the two radioisotopes most commonly used are strontium-89 and samarium-153. Their biological behaviors are calciumlike and they bind to hydroxyapatite in damaged bone; therefore, they are taken up in areas of osteoclastic activity. The radioisotopes effectively deliver a radiation dose to the bone and bone microenvironment, including the cancer cells as well as the bone stromal cells, osteoblasts, and osteoclasts, via their ability to penetrate tissues (strontium, 2.4 nm and samarium, 0.6 nm). Currently, samarium is the most popular systemic radioisotope with clinicians because

TABLE 41.2. Agents that target the bone metastasis microenvironment

Cell type	Target	Agents
Osteoblast	Endothelin-1 receptor	Atrasentan, ZD-4054
	Proteosome	Bortezomib
Osteoclast	Pyrophosphate	Bisphosphonates, samarium, strontium
	RANKL	Denosumab
	Src	Dasatinib
Endothelial cell	VEGF	Bevacizumab, lenalidomide
	VEGFR	Sunitinib, vatalinib, sorafenib
	$\alpha v\beta 3/5$ integrin	Cilengitide, CNTO95, Abegrin
Immune cell	Macrophages	CNTO888, CNTO328
	Dendritic cells	GVAX, sipuleucel-T, lapaleucel-T
	T cells	Anti-CTLA-4

of its short half-life compared with strontium (1 month vs. approximately 2 months), allowing for more flexible treatment schedules around chemotherapy administration [60–62].

Bisphosphonates

Bisphosphonates are analogs of pyrophosphates that bind to the mineralized bone matrix, thereby inhibiting osteoclast-mediated osteolysis. They are useful in treating androgen deprivation–induced osteopenia and are used for management of prostate cancer metastasis [46, 63–66]. Zoledronate acid has been demonstrated to reduce skeletal-related events in patients with androgen-independent prostate cancer and is an approved agent for men with bone metastases in this disease state [64–66].

EVOLVING THERAPIES FOR BONE METASTASES IN MEN WITH ADVANCED PROSTATE CANCER

The recognition that metastatic lesions are complex systems involving a supporting framework of multiple host cells has allowed the development of several strategies that target these complex tumor cell–microenvironment interactions as well as the signal transduction pathways of other cells important to the development of metastases (Table 41.2, Figure 41.4). As noted earlier, an example of this strategy has been the use of the bisphosphonates in patients with metastatic prostate and other cancers that involve bone [63–66]. Bisphosphonates, as analogs of pyrophosphate, inhibit osteoclast maturation and function, interrupting tumor cell–host interactions. Radionucleotides also bind to pyrophosphate, exposing all cells in the bone microenvironment to radiation [60–62].

Another approach to interrupt the osteoblast–osteoclast axis involves inhibition of the osteoprotegerin (OPG) receptor–receptor activator of NF-κB ligand (RANKL) axis using the antibody denosumab [67]. Denosumab is a high-affinity, highly-specific, fully human monoclonal antibody to RANKL. Data from multiple studies have demonstrated that treatment with denosumab increases bone mineral density by inhibiting bone resorption. It is under active investigation to determine whether treatment will prevent bone metastases [67–69]. Other investigational agents interrupt the vicious cycle by targeting the osteoblast [70–73]. Endothelins are a family of peptides that have been demonstrated to be involved in multiple cellular processes including signal transduction pathways for growth and survival [70]. A Phase III trial of atrasentan, an endothelin antagonist, demonstrated an inhibition of PSA progression, decreases in markers of bone turnover, and a reduction of pain from bone metastases [72]. It is currently in clinical trials in combination with cytotoxic agents [74]. ZD-4054 is another endothelin inhibitor that is currently under clinical investigation [70, 71].

Another major target of the bone microenvironment are endothelial cells and their main stimulant for growth, vascular endothelial growth factor (VEGF) [75–77]. Antiangiogenic strategies are being actively pursued using several different paradigms of inhibition. The anti-VEGF antibody bevacizumab, in combination with docetaxel, is the subject of a current Phase III trial targeting men with advanced prostate cancer [75–77]. Thalidomide and its analogs represent potent immunomodulators that have multiple effects on the tumor microenvironment, including inhibition of blood vessel growth by modulating the effect of cytokines and growth factors on endothelial cells [78, 79]. The activation of VEGF receptors can be blocked by tyrosine kinase inhibitors and/or antibodies that bind to the VEGF receptors (Table 41.2) [80]. The growth of new blood vessels can also be blocked by

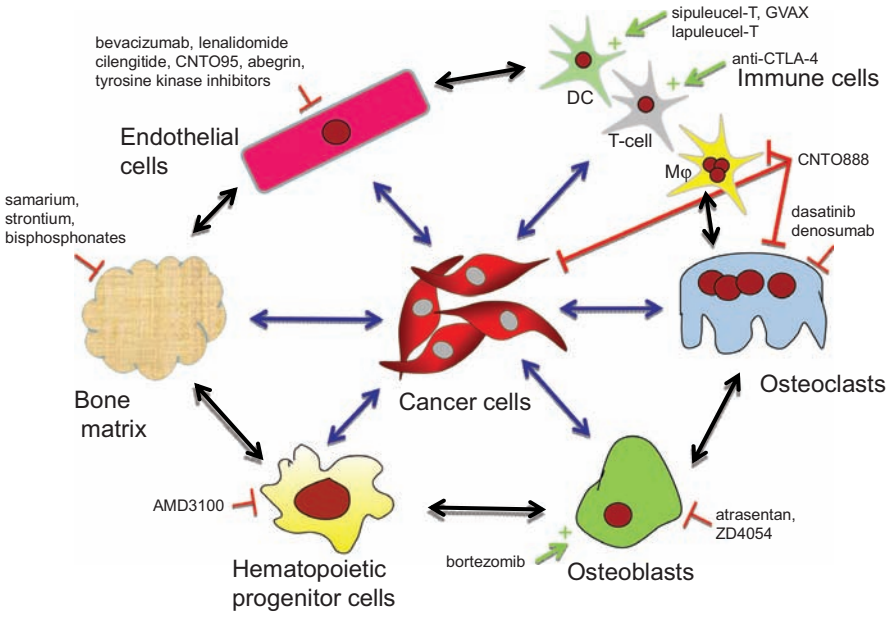

Figure 41.4. Targeting prostate cancer and host cell interactions in bone metastases.Tumor cells alter the bone microenvironment by interacting with osteoclasts, osteoblasts, endothelial cells, other stromal cells, the extracellular matrix, the cells of hematopoiesis, and cells of the immune system. Multiple agents are approved or in clinical trial to inhibit these interactions (see Table 41.2 and text). DC = dendritic cell. Mφ = macrophage.

inhibiting alpha$_v$beta$_{3/5}$ integrin function. The ability of new blood vessels to establish themselves is dependent on the ability of the proliferating endothelial cells to interact with diverse glycoprotein components of the extracellular matrix. This interaction is mediated by the alpha$_v$beta$_{3/5}$ integrins, which bind to extracellular matrix components containing the amino acid sequence Arg-Gly-Asp (RGD). This interaction can be inhibited by peptides that compete with RGD binding or by antibodies to alpha$_v$beta$_3$ integrin [81–83].

Another major cell type in the bone microenvironment are the many different cells that make up the immune system. One approach being tested in advanced prostate cancer is to stimulate the host immune system to recognize the prostate cancer cells as foreign and mount a response against them. GVAX is an immunotherapy comprised of two irradiated prostate cancer cell lines that have been genetically modified to secrete granulocyte macrophage colony-stimulating factor (GM-CSF) [84, 85]. A Phase III trial of GVAX versus a combination of docetaxel and in androgen-independent prostate cancer is under way. Sipuleucel-T (APC8015, Provenge) is a vaccine being tested in multiple clinical trials that uses autologous APCs loaded with a recombinant fusion protein of prostatic acid phosphatase [85, 86]. Clinical trial data suggest that sipuleucel-T may prolong survival by a median of 4.5 months compared with placebo. The drug is still being evaluated in Phase III trials.

Tumor-associated macrophages (TAMs) represent a new target for prostate cancer therapy [87–90]. TAMs have been demonstrated to facilitate tumor growth by stimulating angiogenesis as well as dissolution of the extracellular matrix. Chemokines and chemokine receptors that modulate TAMs offer attractive targets for treatment of bone metastases of prostate cancer patients. For example, monocyte chemoattractant protein 1 (MCP-1; CCL2) is a regulator of prostate cancer growth and metastasis. Treatment with neutralizing antibodies to CCL2 decreased prostate tumor burden in preclinical prostate cancer models [91, 92]. In addition, CCL2 stimulates TAMs to produce interleukin (IL)-6, an important survival factor for prostate cancer cells. CNTO328 is an antibody to IL-6 that is currently in clinical trials for metastatic prostate cancer [93]. The interaction between TAMs and tumor cells represents a prime example of the development of innovative therapeutic strategies based on inhibiting tumor–microenvironment interactions.

FUTURE DIRECTIONS

The combination of novel cytotoxics and new targeted therapies combining to inhibit multiple components that make up metastatic bone lesions offers an exciting avenue that will continue to be explored. Although prostate cancer remains a leading killer of men, these advances provide hope that major strides will continue

to made in decreasing the morbidity and mortality of prostate cancer.

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42 The Biology and Treatment of Metastatic Testicular Cancer

M. Houman Fekrazad, Robert Hromas, and Richard Lauer

Despite remarkable advances in the past three decades, metastatic testicular cancer is still a leading cause of cancer death in young men. It is not clear why some patients are cured and others are not, and new treatments are still needed for this disease. Testicular cancer is not one disease but rather has multiple histologic subtypes. A better understanding of the biology of these different histologic subtypes of testicular cancer, and the use of a multidisciplinary approach, have significantly contributed to improving how we currently approach a young patient with testicular cancer. In addition, DNA microarray analysis has provided insight into the molecular pathobiology of the types of testicular cancer. Genetic and epigenetic alterations are two other important factors that could play a role in the behavior of the types of testicular cancer. Because of the multipotential nature of these tumors, patients with metastatic testicular cancer can present with different clinical scenarios, requiring different treatments. In this chapter, we discuss the biology and the treatment options for patients with metastatic testicular cancer based on tumor type and prognostic categories. Future directions and unmet needs for patients suffering from this disease are also discussed.

EPIDEMIOLOGY OF TESTICULAR CANCER

Human testicular cancers result from malignant transformation of premeiotic or early meiotic testicular germ cells. These cancers can demonstrate differentiation of all three germinal layers. Testicular cancers are the most common malignant neoplasm of males 15 to 34 years of age and a major cause of death from cancer in this age group. They have an annual incidence of 4 per 100,000 [1]. Approximately 8000 new cases of testicular cancer were diagnosed in the United States in 2008 [2]. Although the incidence of testicular cancer continues to plateau in the United States, racial variance exists. The rate is highest in white Americans and is lowest in African Americans. African American males, however, present with higher-grade testicular cancer and therefore have a poor prognosis. In addition, African American males have the greatest increase in annual percentage change, indicating that their incidence of testicular cancer is rising [3]. Worldwide, testicular cancer has the highest incidence in Norway, Denmark, Germany, and Switzerland; the Far East has the lowest [4].

Prior history of contralateral testicular cancer can be found in 1 percent to 2 percent of newly diagnosed patients, in which it accounts for a 500-fold increase in incidence over that in the normal male population [5]. One of the most significant risk factors for testicular cancer is cryptorchidism. Based on modern analysis, the rate of prior cryptorchidism in men with testicular cancer is approximately 5 percent to 10 percent [6, 7]. Patients with undescended testes have a higher risk for developing seminoma than nonseminomatous germ cell tumors (NSGCTs). However, for those patients undergoing early orchiopexy, the most common malignancy is NSGCTs. Therefore, many urologists recommend that orchiectomy be considered in healthy patients with cryptorchidism who are between the ages of 12 and 50 years [8]. However, it is recommended that orchidopexy be performed before puberty to reduce the likelihood of testicular cancer later in life. The risk of developing testicular cancer among those treated with orchidopexy at 13 years of age or older is twice as high as in younger patients (9).

Klinefelter syndrome (47XXY) is also associated with extragonadal germ cell tumors (GCTs), particularly in the mediastinum [10]. The risk of developing testicular cancer, most commonly seminoma, is higher in patients with Down syndrome than that in the normal male population (0.5% vs. 0.09%) [11]. Other risk factors may include gonadal dysgenesis, prenatal

exposure to high estradiol levels, exposure to chemical carcinogens, orchitis, trauma, smoking, and childhood inguinal hernias [1].

PATHOBIOLOGY OF TESTICULAR CANCER

Testicular cancer is not one homogenous malignancy, but many arising from this single organ, each with differing clinical behaviors, and each from distinct cells within the testis. These tumor types are generally separated into seminomatous and NSGCTs. The earliest recognizable malignancy of the testis is intratubular germ cell neoplasia of unclassified origin, which histologically resembles seminoma. Seminomas have sheets of uniform cells resembling undifferentiated spermatogonia separated by septa of lymphocytes [12]. They have low mitotic and apoptotic indices and low metastatic potential. Patients with seminomas may be rendered disease-free with orchiectomy and radiation therapy (13). NSGCTs can be divided into two main types, embryonic and extraembryonic, depending on their cell of origin [12].

There are two types of testicular cancer of embryonal origin: embryonal carcinoma and teratoma. Embryonal carcinoma resembles early pluripotent zygotic tissue with sheets of glands, and papillary structures of primitive epithelial cells. Embryonal carcinomas have the highest mitotic and apoptotic indices of all testicular cancers and are probably the precursors for the second type of testicular cancer of embryonal origin, teratomas. Teratomas are more differentiated than the embryonal carcinomas, occasionally demonstrating somatic differentiation normally seen in the developing three germ layers of the early embryo. They usually have low proliferative indices. Teratomas and embryonal carcinomas can be intermixed in a single tumor, making definitive pathologic diagnosis difficult [12]. Rarely, mature teratomas can transform into a more aggressive and more primitive tumor arising from one of its components. An example of this is primitive neuroectodermal tumor (PNET), which has a high mitotic index and is difficult to treat. Teratomas usually are resistant to chemotherapy or radiation, whereas embryonal carcinomas are more treatable, as discussed in detail in later sections [13].

Yolk sac tumors and choriocarcinomas are two types of testicular cancer derived from extra-embryonic tissue. Yolk sac tumors express alpha-fetoprotein, and choriocarcinomas express human chorionic gonadotropin (HCG) and morphologically resemble normal extraembryonic tissue. Both of these have lower mitotic and apoptotic indices than embryonal carcinomas but higher indices than teratoma, and may often be cured with a combination of surgery and chemotherapy. Interestingly, some testicular cancers can burn out, demonstrating much slower proliferative indices and more localized disease. These "burnt-out" testicular cancers have foci of residual neoplasia and a well-defined zone of scarring with a lymphocyte infiltrate and calcification.

Microarray analysis has revealed insight into the molecular pathobiology of testicular cancer. It has been reported that in intratubular germ cell neoplasia of unclassified origin, genes that mediate spermatogenesis are downregulated, whereas genes that are expressed in the primitive embryonic stem cells, such as *Oct-4(POU5F1), NANOG*, *XBP1*, *XIST*, *LIN28*, *TFAP2C*, *PDPN*, *PRDM1* (human homolog of *Blimp1*), *SOX17*, and *KIT*, are upregulated [14]. There are also several known oncogenes that are also usually expressed, including *TCL1A*, *MYCN*, and *PIM* [14]. Gene expression studies in overt testicular cancers also found that genes usually expressed in embryonic stem cells, such as *Oct-4(POU5F1)*, *NANOG*, *DPPA3* (human homolog of *stella*), and *GDF3,* are highly expressed [15]. These data indicate that testicular cancers probably represent a dedifferentiation into a more primitive embryonic stem cell from more mature germ cells.

Within the teratoma group, there is strong evidence that ovarian and prepubertal testicular teratomas are derived from benign germ cells as opposed to the postpubertal testicular teratomas, which are derived from malignant germ cells, specifically representing differentiation within a pre-existent nonteratomatous cancer. These prepubertal teratomas are often manifested in undescended testes. Thus, teratomas in young boys are most often benign, whereas in postpubertal males they are malignant.

Several other immunohistochemical markers that can assist with the diagnosis of testicular cancer have been determined [12], including CD117 (c-Kit), which is responsible for the migration of the primordial germ cell from the neural crest to the testis during embryogenesis [15]. In addition, the Oct-4 protein can be expressed specifically in embryonal carcinoma [15]. Immunohistology of Oct-4 can not only help distinguish embryonal carcinomas from other testicular cancer types in the testis, but it can also demonstrate a testicular origin for carcinoma of unknown primary ([16] and Figure 42.1).

GENETICS OF TESTICULAR CANCER

Testicular cancer has an eight-fold increase in relative risk in brothers of patients and a four-fold increase in risk in fathers or sons of patients. However, families with multiple cases of testicular cancer are rare, and the great majority of those reported cases have only two affected members [17]. There is also an increase in

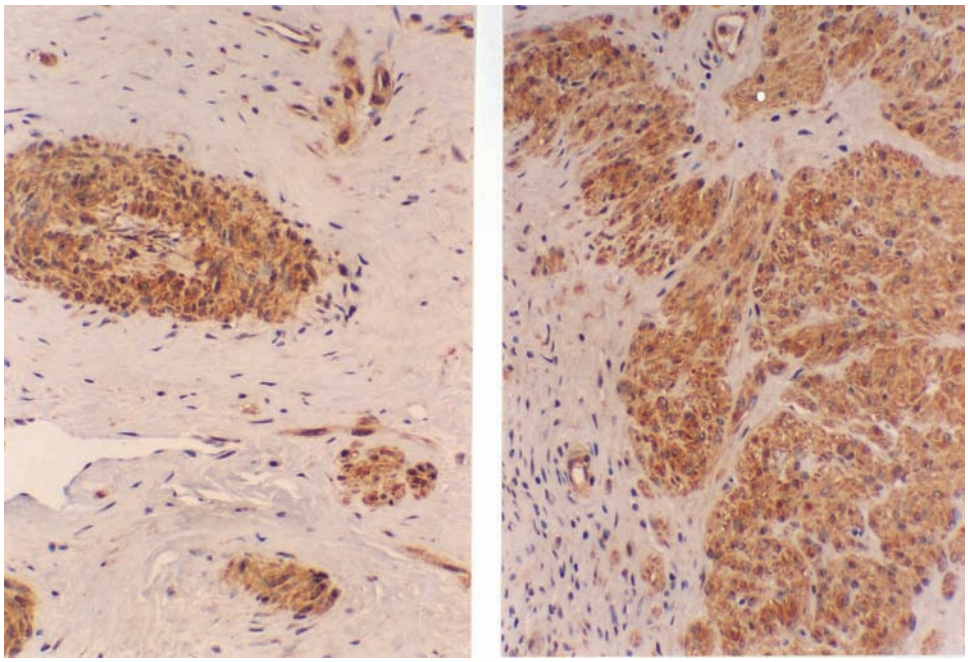

Figure 42.1. Oct-4 is an embryonic stem cell specific transcription factor that is also present in embryonal carcinoma. Its presence on immunohistochemical staining not only can differentiate embryonal carcinoma from teratoma and other types of testicular cancer but also can identify embryonal carcinoma in patients with carcinoma of unknown primary [15, 16]. This identification is critical, as metastatic embryonal carcinoma can be cured with appropriate chemotherapy. In both panels, the brown immunohistochemical stain identifies Oct-4 in the embryonal carcinoma, whereas the unstained cells are teratoma. The right panel shows a higher power magnification.

testicular cancer in the contralateral testicle in patients with one involved testicle, with 5 percent of patients having a contralateral tumor [18]. These initial reports strongly imply that there is a genetic component to testicular cancer.

The seminal studies of Atkin and Baker demonstrated conclusively that testicular cancer has a genetic component. In cytogenetic studies, they found that isochromosome of 12p was a recurring event in testicular cancer [19]. Bosl and Chaganti's group used fluorescence in situ hybridization to demonstrate that even in testicular cancer without isochromosome 12p, there was an amplification of 12p, implying that there is a testicular cancer oncogene on 12p [20]. This group defined the minimally amplified region, but despite the fact that there are a number of good candidates for this oncogene on 12p, including cyclin D2, Nanog, GDF3, and STELLAR, definitive isolation of the 12p oncogene remains elusive [13, 21, 22].

Another chromosomal alteration in some testicular cancers is a deletion on the Y chromosome, termed gr/gr, that somewhat increases the susceptibility of carriers to seminomas. However, the gene in this deletion has not yet been identified [23].

Genetics not only play an important role in the origins of testicular cancer but also play a role in its response to chemotherapy and radiation. In general, these tumors are very responsive to chemotherapy. The exquisite sensitivity of these tumors to chemotherapy probably is derived from their overexpression of normal p53 protein and their lack of p53 gene mutations commonly seen in other tumors [24]. In addition, the expression of Bax and Bcl2 can also predict response to therapy [25].

Several mutations have been discovered in genes thought to play a role in testicular cancer. For example, there are activating mutations in c-kit in approximately 10 percent of testicular cancers. Tian et al. identified an asp816-to-his mutation in the KIT gene in primary tissue samples from patients with germ cell tumors [26]. Given the importance of signaling in the development of primordial germ cells, it is not surprising that there are several signaling proteins implicated in the origination of testicular cancer [27]. Somatic mutations have been found in the SMAD4 gene in testicular cancer, and VEGF is overexpressed in a number of seminomas. This overexpression predicts metastatic behavior in these tumors. Finally, there is a murine model of seminoma that occurs when the cytokine GDNF is expressed [28]. The GDNF receptor is overexpressed in human seminoma, but the exact mechanism it plays in human seminoma is not clear.

There are also epigenetic alterations that could play a role in testicular cancers. The gene for SUZ12, needed for H3K27 methylation seen in undifferentiated embryonic stem cells, maps to a region of 17q amplified in both testicular cancer and embryonic stem cells. Overexpression of this protein could contribute to maintenance of pluripotency in germ cells [29].

PATTERN OF METASTASES

The clinical presentation of GCTs can be either as localized disease or as a widely metastatic malignancy. The classic presentation of local disease is a painless scrotal mass in an otherwise healthy young man. Other patients with localized GCTs may present with a swollen painful testicular mass, hydrocele, low back pain caused by retroperitoneal adenopathy from metastatic tumor, or gynecomastia from elevated human chorionic gonadotropin. Patients with disseminated disease usually display the signs and symptoms of lymphatic or hematogenous spread. Testicular cancer has a propensity to spread via the lymphatic system; initially, it travels to the retroperitoneal lymph nodes below the renal vessels. Supraclavicular adenopathy and pulmonary nodules may occur with or without retroperitoneal disease. Other clinical manifestations may include chest pain, cough, or shortness of breath due to mediastinal adenopathy. Hematogenous spread to the lungs, liver, bone, and central nervous system (CNS) can occur, and associated clinical symptoms may be dyspnea, cough, hemoptysis, abdominal pain, jaundice, bone pain, seizure, or other CNS manifestations respectively. A high index of suspicion is important if patients develop any of these signs.

STAGING AND PROGNOSTIC FACTORS

Testicular cancer is staged upon presentation by a classic tumor/node/metastasis (TNM) system. The TNM staging systems for testicular cancer is outlined in Table 42.1. It has three stages, and these stages have clear prognostic value. Five-year survival according to the TNM staging was 81 percent for stage I, 44 percent for stage II, and 10 percent for stage III [30].

Prognosis is also assessed according to the International Germ Cell Tumor Consensus Classification (IGCCC) scheme. Patients are assigned to one of three prognostic groups (good-risk, intermediate-risk, and poor-risk categories) on the basis of whether or not the tumor is seminomatous or NSGCTs, where the primary site of disease (gonadal or retroperitoneal versus mediastinal) lies, whether there is a presence or absence of extrapulmonary visceral metastases, and based on the serum tumor marker elevation (Table 42.2) [31].

Employing the IGCCC database, 5202 patients with NSGCTs and 660 patients with seminoma were assessed. The good-risk category was composed of 60 percent of these patients with a five-year survival rate of 91 percent and a five-year progression-free survival (PFS) of 88 percent. The intermediate-risk group included 26 percent of all patients with a five-year survival of 79 percent and a five-year PFS of 75 percent. Only 14 percent of these patients fell into the poor-risk category, with a 41 percent five-year disease-free survival and overall 48 percent five-year survival [31].

All extragonadal primary sites were also identified as an adverse prognostic factor, especially for primary mediastinal NSGCTs, which seem to have distinct clinical characteristics and usually respond poorly to treatment [32, 33]. For these patients a different prognostic outcome model has been developed [34]; however, it is not routinely used. In patients with clinical stage I seminoma, the size of the primary tumor and the infiltration of the rete testis are independent prognostic factors for occult metastases [35]. However, in patients with clinical stage I NSGCTs, the presence of lymphovascular invasion is the most important prognostic indicator for occult metastases [36–38].

Elevation of serum AFP, HCG, and LDH may help in the diagnosis of testicular cancer in otherwise healthy young men with malignancies of uncertain origin. AFP and HCG analyses play decisive roles in the classification, staging, choice of therapy, and surveillance of patients with testicular cancers. Although HCG and LDH are important tools for the management of advanced seminoma, there remains a need for more reliable markers to target these tumors. The degree of serum tumor marker elevation correlates with the patient's risk category [31].

TREATMENT OF METASTATIC TESTICULAR CANCER

Treatment of metastatic testicular cancer has changed significantly over the past few decades, with a substantial increase in cure rates from around 25 percent in the mid-1970s to approximately 80 percent today [31]. We discuss the treatment options for these patients based on prognostic categories below. Two chemotherapy regimens are effective for patients with seminomatous and NSGCTs in the good-risk testicular cancer category. These are four cycles of etoposide and cisplatin (EP) or three cycles of bleomycin, etoposide, and cisplatin (BEP). BEP chemotherapy is a combination of bleomycin 30 units per week (intravenous push administration) on days 1, 8, and 15, etoposide 100 mg/m^2 per day on days 1 through 5, and cisplatin 20 mg/m^2 per day on days 1 through 5. Each cycle of treatment is repeated at twenty-one-day intervals [39]. Both regimens produce durable response rates ranging from 81 percent to 92 percent with favorable toxicity profiles.

TABLE 42.1. TNM staging system for testicular cancer

Stage 0	Stage I	Stage II	Stage III
pTis, N0, M0, S0	pT1–4, N0, M0, SX	Any pT/Tx, N1–3, M0, SX	Any pT/Tx, Any N, M1, SX
	Stage IA	**Stage IIA**	**Stage IIIA**
	pT1, N0, M0, S0	Any pT/Tx, N1, M0, S0	Any pT/Tx, Any N, M1a, S0
		Any pT/Tx, N1, M0, S1	Any pT/Tx, Any N, M1a, S1
	Stage IB	**Stage IIB**	**Stage IIIB**
	pT2, N0, M0, S0		
	pT3, N0, M0, S0	Any pT/Tx, N2, M0, S0	Any pT/Tx, N1–3, M0, S2
	pT4, N0, M0, S0	Any pT/Tx, N2, M0, S1	Any pT/Tx, Any N, M1a, S2
	Stage IS	**Stage IIC**	**Stage IIIC**
	Any pT/Tx, N0, M0, S1–3	Any pT/Tx, N3, M0, S0	Any pT/Tx, N1–3, M0, S3
		Any pT/Tx, N3, M0, S1	Any pT/Tx, Any N, M1a, S3
			Any pT/Tx, Any N, M1b, Any S

Primary tumor (T) – The extent of primary tumor is classified after radical orchiectomy.
- pTX: Primary tumor cannot be assessed (if no radical orchiectomy has been performed, TX is used.)
- pT0: No evidence of primary tumor (e.g., histologic scar in testis)
- pTis: Intratubular germ cell neoplasia (carcinoma in situ)
- pT1: Tumor limited to testis and epididymis without lymphatic/vascular invasion
- pT2: Tumor limited to testis and epididymis with vascular/lymphatic invasion, or tumor extending through the tunica albuginea with involvement of the tunica vaginalis
- pT3: Tumor invades the spermatic cord with or without vascular/lymphatic invasion
- pT4: Tumor invades the scrotum with or without vascular/lymphatic invasion

Regional lymph nodes (N)
- NX: Regional lymph nodes cannot be assessed.
- N0: No regional lymph node metastasis
- N1: Metastasis in a single lymph node, 2 cm or less in greatest dimension
- N2: Metastasis in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension; or multiple lymph nodes, none more than 5 cm in greatest dimension
- N3: Metastasis in a lymph node more than 5 cm in greatest dimension

Distant metastasis (M)
- MX: Presence of distant metastasis cannot be assessed
- M0: No distant metastasis
- M1: Distant metastasis
- M1a: Nonregional nodal or pulmonary metastasis
- M1b: Distant metastasis other than to nonregional nodes and lungs

Serum tumor markers (S)
- SX: Tumor marker studies not available or not performed
- S0: Tumor marker levels within normal limits
- S1: LDH $< 1.5 \times$ Normal and HCG (mIu/mL) < 5000 and AFP (ng/mL) < 1000
- S2: LDH 1.5–10 $\times$ Normal or HCG (mIu/ml) 5000–50,000 or AFP (ng/mL) 1000–10,000
- S3: LDH $> 10 \times$ Normal or HCG (mIu/mL) $> 50{,}000$ or AFP (ng/mL) $> 10{,}000$

INTERMEDIATE- AND POOR-RISK TESTICULAR CANCER

The standard regimen for these patients is four cycles of BEP; however, the complete response rate is lower as compared with the rate for patients with good-risk testicular cancer. Several studies have been done to improve outcomes in these subgroups, including high-dose chemotherapy followed by autologous stem cell transplant; however, they have failed to demonstrate any advantage over four cycles of BEP [40]. Relapsed patients may still potentially be cured with second- and even third-line regimens. These include three-drug combinations with ifosfamide and cisplatin based or alternatively high-dose chemotherapy followed by autologous stem cell rescue in selected patients. In one study, the combination of paclitaxel, ifosfamide, and cisplatin (TIP regimen) showed a durable complete response in twenty-nine of forty-six patients (63%) with a median follow-up of sixty-nine months [41].

Several studies have indicated the value of resecting residual masses following first-line or salvage chemotherapy for NSGCTs [42]. Of note, normalization of tumor markers prior to surgical intervention is an important factor because elevated markers may define residual systemic disease and predict a higher likelihood of incomplete resection or recurrence [43, 44]. All metastatic sites, such as retroperitoneal lymph

TABLE 42.2. Risk classification

Risk	Seminomatous tumors	Nonseminomatous tumors
Good	Any primary site and No nonpulmonary visceral metastasis and Good markers (S1)	Testicular or retroperitoneal primary tumor and No nonpulmonary visceral metastasis and Good markers (S1)
Intermediate	Any primary site and Nonpulmonary visceral metastasis and Good markers (S1)	Testicular or retroperitoneal primary tumor and No nonpulmonary visceral metastasis and Intermediate markers (S2)
Poor	None	Mediastinal primary tumor or Nonpulmonary visceral metastasis (brain, liver, bone, etc.) or Poor markers (S3)

Adapted from International Germ Cell Cancer Collaborative Group [16].

nodes, liver, lung, or brain lesions, should be removed if accessible [45–48]. Full surgical resection and histology of resected masses (such as viable testicular cancer or differentiated teratoma) are strong predictors of long-term outcome [49]. Surgical resection of residual masses after chemotherapy in patients with seminomatous testicular cancer is not usually recommended. PET scan can be used to guide surgical decisions in these cases [50].

FUTURE DIRECTIONS AND UNMET NEEDS

Despite the fact that testicular carcinoma has been one of the great success stories in oncology, advances are still required in the treatment of patients afflicted with this disease. When considering future directions and unmet needs, one can divide testicular cancer into several subgroups: (1) clinical stage 1 NSGCTs (defined as testicular cancer without clinical evidence of spread), (2) good-prognosis disease, (3) intermediate- and poor-prognosis disease (as defined by the IGCCC; Table 42.2), (4) recurrent and refractory disease, and (5) long-term survivors. Each of these situations has its unique problems, approaches, and unmet needs.

Clinical stage 1 NSGCT is diagnosed through a negative AFP, HCG, chest, abdomen, or pelvic CT scan [51] and is treated by inguinal orchiectomy. Treatment options after orchiectomy include surveillance, retroperitoneal lymphadenectomy, and adjuvant chemotherapy. Patients under surveillance will have recurrences with metastatic disease approximately 30 percent of the time. These relapsed patients will require a longer course of chemotherapy and may require surgery. Further standard follow-up requires frequent CT scans, involving radiation exposure that may prove to be detrimental [52]. To date, attempts to select patients who have a high likelihood of recurrence based on their orchiectomy specimen have been less than optimal [53, 54]. Retroperitoneal lymphadenectomy has a higher predictive value for recurrence but is associated with significant morbidity and is unnecessary in approximately 70 percent of patients [55].

More recently, several clinical trials have examined the use of short-course chemotherapy as a therapeutic modality [56, 57]. There has been concern that this may be associated with unanticipated late toxicities and the fact that 70 percent of patients will have received chemotherapy that they do not require. In addition, patients may suffer recurrences in their abdomen, necessitating long-term follow up with serial CT scans. Clearly, a diagnostic study is needed that can target those patients who are destined to develop recurrent disease (i.e., patients with occult metastasis). A possible approach to this problem would be to identify molecular patterns of gene expression that would identify patients destined to have metastatic disease. The use of gene expression arrays in GCTs is still in an early stage. This is an area in which future studies are required. Another possible approach would be to determine whether patients have circulating tumor cells to see whether this has prognostic significance. Circulating tumors cells are currently being investigated in prostate cancer, breast cancer, colon cancer, and other cancers. Whether they would have prognostic significance in a setting of early-stage testicular cancer should be investigated.

Patients with good-prognosis germ cell tumors have a five-year survival rate of approximately 91 percent [31]. It will be difficult to improve on this cure rate with existing agents and modalities. Consequently, our clinical goal in the next few years is to decrease the toxicity of treatment. At present, standard treatment in

this setting is either three cycles of BEP chemotherapy [58] or four cycles of EP [59]. To date, reductions in dose intensity have resulted in decreased efficacy and increased recurrence. What is needed are new drugs that will not have the toxicities associated with BEP.

Patients with intermediate- and poor-prognosis disease have five-year survival rates of 79 percent and 48 percent, respectively [31]. Standard treatment for these patients is four cycles of chemotherapy with BEP followed, if appropriate, by surgery. Attempts at dose escalation [60], sequential chemotherapy [61, 62], and alternating chemotherapy [63] have been disappointing. Motzer et al. reported a large cooperative group study of conventional-dose chemotherapy with or without high-dose chemotherapy and autologous stem cell rescue as first-line treatment for patients with poor-prognosis metastatic GCTs [40]. Unfortunately, the addition of high-dose chemotherapy did not improve treatment outcomes in poor-prognosis patients. A question raised by the study was whether the rate of marker decline during the first two cycles of chemotherapy could be used to predict treatment outcome and to target those patients with chemotherapy-resistant disease. A slower rate of decline of a serum marker such as HCG or AFP might predict a greater likelihood of relapse. Other markers that predict not only chemotherapy resistance but also future relapse are needed. It has been known for some time that platinum resistance, defined as progressive disease within one month of cisplatin exposure, carries a poor prognosis. Recently, absence of the DNA repair component XPA (also termed ERCC1) has been associated with improved survival in patients with metastatic germ cell tumors of the testis treated with cisplatin. In contrast, patients carrying the ERCC1 C8092A polymorphic allele have increased resistance to cisplatin [64]. Alternatively, evidence has been presented that promoter hypermethylation of RASSF1A and HIC1 genes, which would reduce their expression, play a role in germ cell tumor cisplatin resistance, whereas inactivation of MGMT by epigenetic alterations confers sensitivity to cisplatin therapy [58]. It is clear that better treatments with new agents are needed for the front-line treatment of poor-prognosis patients because a large fraction of these still relapse, despite the success seen in patients with a better prognosis. Agents found to have activity in platinum-resistant patients will need to be added to their current regimes. It is tempting to speculate that the new PARP1 inhibitors might play a role in enhancing response to cisplatin in platinum-resistant patients.

Recurrent disease is defined as disease in patients who have relapsed after primary treatment. Patients whose primary treatment is surveillance are treated based on whether they have good-, intermediate-, or poor-prognosis disease. Patients who have recurrences within two years of standard chemotherapy can be treated with salvage chemotherapy. Although this does represent a heterogeneous group, two regimes that have demonstrated curative activity are etoposide or vinblastine, ifosfamide, and cisplatin (VIP) and paclitaxil, ifosfamide, and cisplatin (TIP) [59, 66]. These regimes result in failure-free survival rates of approximately 35 percent.

Another approach has been the use of high-dose chemotherapy (HDCT) with stem cell rescue. Einhorn et al. reported on 173 patients who received carboplatin and etoposide, each for three consecutive days, followed by autologous stem cell rescue, for two total stem cell transplantation courses [67]. Of the 135 patients who received treatment as second-line therapy, 94 were disease-free during follow-up, whereas 22 of 49 patients who received treatment as third-line or later were disease-free. The German Testicular Cancer Study Group examined one cycle of standard VIP followed by three cycles of high-dose etoposide and carboplatin and stem cell rescue versus three cycles of VIP followed by one dosage of HDCT [68]. There was no difference in survival between the groups; in addition, the group receiving three courses of high-dose chemotherapy had significantly more toxicity. Further, the survival rate seemed to be worse in this trial than that in the Indiana trial. Clearly, these studies had different ethnic populations and this may have accounted for some of the variations noted. Beyer et al. have proposed a prognostic model that might help to optimize the use of HDCT in germ cell tumors [69]. Multivariate analysis identified progressive disease before HDCT, mediastinal nonseminomatous primary tumor, refractory disease to cisplatin, and HCG levels greater than 1000 U/L as adverse prognostic variables. This model awaits validation. In addition, the timing of HDSCT and the optimum regimens have yet to be determined.

Patients who suffer recurrences within one month of initial platinum-based chemotherapy, as well as patients who recur after HDCT, have a particularly poor prognosis. New agents are needed for this group, and to date several chemotherapy drugs are being evaluated. Gemcitabine, epirubicin, and oxaliplatin all have activity in refractory disease and are being examined in combination [70–72]. In addition, targeted treatments such as sunitinib, bevacizumab, thalidomide, and imatinib are currently being investigated [73]. A better understanding is needed of the biology of germ cell malignancies and agents that are directed toward pathologic mechanisms.

One of the characteristics of GCTs is that they frequently occur in a young population. Given the curable nature of this neoplasm, there is an ever-increasing population of long-term survivors. Clearly, this is another area that requires more research. It is known that long-term survivors of testicular cancer have an increased risk of depression and anxiety disorders [74], along

with psychosocial problems. Fertility is also affected by platinum-based chemotherapy. Oligospermia and azoospermia will occur in the majority of patients but seem to improve with time [75]. Evidence is beginning to accumulate as well that long-term testicular cancer survivors who have received chemotherapy are at increased risk for cardiovascular problems. including hypertension, cardiac arrhythmias, and coronary vascular disease [76, 77]. Not only is further research warranted to study the long-term toxicities of both the diagnosis and treatment of testicular cancer, but new approaches also are required to decrease these toxicities.

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43 Applications of Proteomics to Metastasis Diagnosis and Individualized Therapy

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APPLICATION OF PROTEOMICS AND NANOTECHNOLOGY TO CANCER BIOMARKER DISCOVERY: CLINICAL NEED VERSUS PHYSIOLOGIC ROADBLOCKS

Proteomics Has Potential to Address Need for Specific Cancer Biomarkers

Cancer is too often diagnosed and treated too late, when the tumor cells have already invaded and metastasized. At this stage, therapeutic modalities are limited in their success. Detecting cancers at their earliest stages, even in the premalignant state, means that current or future treatment modalities might have a higher likelihood of a true cure. For example, ovarian cancer is usually treated at an advanced stage. The resulting five-year survival rate is 35 percent to 40 percent for patients with late-stage disease who receive the best possible surgical and chemotherapeutic intervention. In contrast, if ovarian cancer is detected at an early stage, conventional therapy produces a high rate of five-year survival (95%) [1]. Thus, early detection, by itself, could have a profound effect on the successful treatment of this disease. A clinically useful biomarker for early cancer detection should be measurable in a readily accessible body fluid, such as serum [2], urine [3], or saliva [4]. Clinical proteomic methods are especially well suited to discovering such biomarkers [5]. Serum or plasma has been the preferred medium for discovery, because this fluid is a protein-rich information reservoir that contains the traces of what has been encountered by the blood during its constant perfusion and percolation throughout the tissues [6].

Despite decades of effort in the face of this urgent clinical need, single biomarkers have not been found that can reach an acceptable level of specificity and sensitivity required for routine clinical use for the early stage detection of the most common cancers. Most investigators believe that this is because of both the molecular heterogeneity of tumors and the epidemiologic heterogeneity of the patients. In response to the dry pipeline of clinically useful candidate biomarkers, biomarker discovery science is moving away from the idealized single cancer-specific biomarker [7, 8]. Taking a cue from gene arrays, the hope is that panels of tens to hundreds of protein and peptide markers may transcend the biologic heterogeneity to generate a higher level of diagnostic specificity. The blood proteome or peptidome offers a potential treasure trove of new biomarker panels that can address this need for higher specificity [8].

The blood peptidome is one of the most promising new categories of biomarkers, because it is thought to provide a real-time recording of the tissue microenviroment [9]. Cancer itself is a product of the tissue microenvironment (Figure 43.1). The tumor microecology, through the process of aberrant cell growth, angiogenesis, cellular invasion, inflammation, and crosstalk between the tumor cells and the host cells, represents a unique constellation of shed molecules, growth factors, and proteases.

An added benefit for cancer biomarkers is the leaky nature of newly formed blood vessels and the increased hydrostatic pressure within tumors. This pathologic physiology would tend to push molecules from the tumor interstitium into the circulation. As cells die within the microenvironment, they will shed their degraded contents. The mode of death, apoptosis versus necrosis, would be expected to generate different classes of degraded cellular constituents. As a consequence, the blood peptidome may reflect ongoing recordings of the molecular cascade of communication taking place in the tissue microenvironment. Combinations of peptidome markers representing the specific interactions of the tumor tissue microenvironment can achieve a higher specificity and a higher sensitivity for early-stage cancers [9]. This optimism is based in part on the concept that the biomarkers are derived from

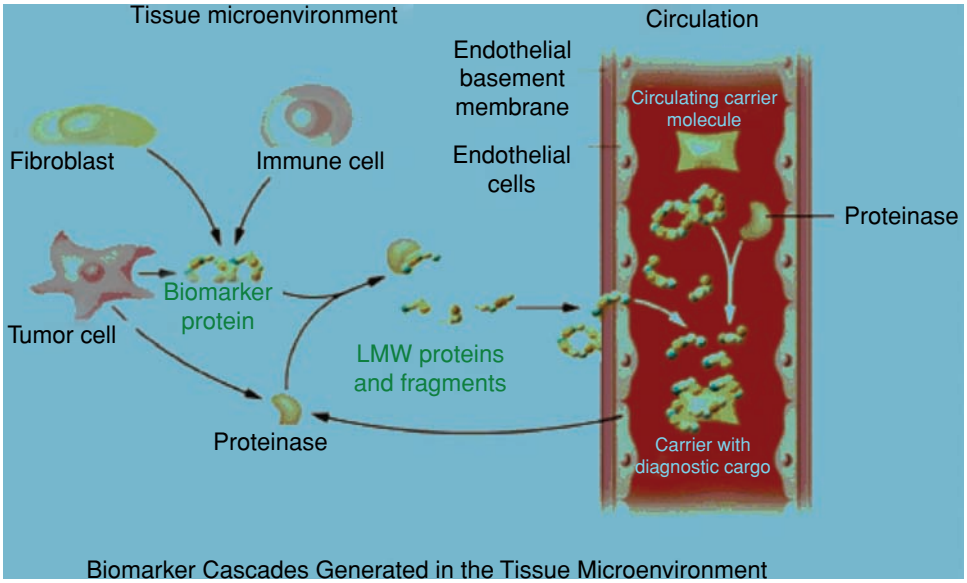

Figure 43.1. Under the peptidome hypothesis, circulating peptides and protein fragments are shed in circulation from all cell types in the tissue microenvironment. Proteolytic cascades within the tissue generate fragments that spread into the circulation. The identity and cleavage pattern of the peptides are useful diagnostic information. Adapted from Petricoin et al. [9].

a population of cells that comprise a volume that is greater that just the small precancerous lesion itself. In this way, the peptidome can potentially supersede individual single biomarkers and transcend the issues of tumor and population heterogeneity.

Biomarkers for Early Diagnosis of Cancer: Ultimate Goal Is Detection of Preinvasive Lesions

A large body of accumulating evidence supports the concept that the aggressive phenotype of human cancer is predetermined or preestablished very early in the course of the disease [10]. A case in point is breast cancer. It was previously assumed that ductal carcinoma in situ (DCIS) is a premalignant stage that exists along a spectrum of lesions that have progressed part of the way toward becoming fully invasive carcinomas. Additional genetic "hits" are thought to be required for DCIS cells to acquire the full capability of invasion and metastasis.

It has further been assumed that a large percentage of cancer progression from low to high aggressiveness is occurring after the malignant neoplasm emerges. We now know that the clinical aggressiveness is determined much earlier than we previously thought during the course of molecular carcinogenesis. Genetic transcript profiling of microdissected human breast cancer reveals that the profile of genes in DCIS is most similar to the invasive cancer in the same patient and supports the conclusion that the differentiated and metastatic potential of an individual patient's breast cancer is predetermined early at the level of DCIS [11]. Murine MMTv breast lesions with premaligant histologies are found to be enriched in markers thought to be associated with breast cancer stem cells [12]. Thus, to effect a positive clinical outcome, it would seem critical to detect and treat cancer as early as possible within the spectrum of progression from premalignant cancer to an overt malignant phenotype.

Fundamental Physiologic Roadblocks Prevent Proteomics from Realizing Its Promise to Yield Sensitive Biomarkers

There is a strong rationale that the main focus of cancer biomarker discovery should be the detection of very small cancers in the premalignant, preinvasive, or premetastatic stage. Unfortunately, the size of a stage I cancer may be less than 0.5 cm in maximum dimension. The levels of biomarkers emanating from the mass of a tumor less than 0.5 cm is extremely small, estimated to be in the range of picograms to femtograms per milliliter. This low concentration will require extremely sensitive biomarker discovery platforms and highly sensitive biomarker assays. Moving beyond the goal of stage I cancer detection to the early diagnosis of premalignant lesions will require even higher sensitivity, well below the femtogram-per-milliliter range. The challenge for biomarker discovery is daunting because mass spectrometry-based proteomics approaches, the most sensitive means to discover candidate diagnostic molecules, rarely achieves a sensitivity below tens of nanograms per milliliter [13–15].

Ovarian cancer is a specific example of a stage I tumor that may be very difficult to detect using conventional biomarker discovery methods [7]. Brown and Palmer developed models for the growth, progression, and detection of occult serous cancers on the basis of a comprehensive analysis of published data on serous cancers discovered by PBSO in BRCA1 mutation carriers [16]. They concluded that these cancers spend on average more than 4 years as in situ, stage I, or stage II cancers and approximately 1 year as stage III or IV cancers before they become clinically apparent [16]. For most of the occult period, serous cancers are less than 1 cm in diameter and not visible on gross examination of the ovaries and Fallopian tubes [16]. The median diameter of a serous ovarian cancer when it progresses to an advanced stage (stage III or IV) is about 3 cm. To achieve 50 percent sensitivity in detecting tumors before they advance to stage III, an annual screen would need to detect tumors of 1.3 cm in diameter; 80 percent detection sensitivity would require detecting tumors less than 0.4 cm in diameter [16]. This is a very small volume. Early-stage disease lesions, such as premalignant cancer, may arise within a tissue volume less than 0.10 mL. Assuming all the putative biomarkers emanating from this volume are uniformly dispersed within the entire blood volume of 5000 mL, then the dilution factor will be 50,000. Once again, based on the sensitivity of existing mass spectrometry technology, this size will be well below the threshold for detection.

Because of the low abundance of the biomarkers, analytical sensitivity is a challenge for biomarker discovery as well as for routine measurement. During the discovery phase, it is likely that large plasma or serum volumes, including pooled samples, can be available for analysis. In contrast, once a candidate marker is taken forward to clinical testing, the volume of blood available for an individual patient's assay may be less than 1 mL. When one takes all these factors into consideration, the analytical platform used to measure the candidate marker must have a detection sensitivity sufficient to reliably detect marker concentrations in the subfemtomolar or attomolar concentration.

The physiologic challenges of cancer biomarker discovery are not just limited to the low abundance of biomarkers. Candidate biomarkers have the potential to be rapidly excreted and must be separated from high-abundance blood proteins such as albumin and immunoglobulins, which exist in a billionfold excess. We can reasonably hypothesize that the most physiologically relevant proteins specific for the disease constitute a minor subpopulation of the cellular proteome. Consequently, one of the greatest challenges to biomarker discovery is the isolation of very rare candidate proteins away from a highly concentrated complex mixture of blood proteins.

A final major challenge is the instability of biomarkers. Biomarkers in the blood may be highly labile and subjected to degradation during blood collection, transportation, and storage. Biomarker instability is a significant source of bias for validation and can prohibit practical routine clinical use.

Nanotechnology to Overcome Fundamental Roadblocks to Biomarker Discovery

As outlined previously, there is a great need for a technology to increase the sensitivity of biomarker discovery and measurement without increasing the background nonspecific signal. We have discovered a novel core shell/affinity bait nanotechnology that overcomes the three major hurdles to disease associated biomarker discovery and measurement: (1) **very low abundance (<1 ng/mL)**: biomarkers of early stage and small volume malignant lesions cannot measured by conventional biomarker technology; (2) **masking by serum proteins:** biomarkers must be separated from the billionfold excess resident blood proteins; and (3) **degradation:** biomarkers are highly labile and perishable following blood collection [17–20]. The harvesting nanoparticles (0.5 μm) rapidly conduct, in one step, in minutes, in solution, (1) affinity capture, (2) 100-fold concentration of target analytes, (3) complete exclusion of serum immunoglobulins and albumin, and (4) complete protection of captured analytes from degradation (Figure 43.2).

We have synthesized hydrogel core-shell nanoparticles that are constituted by poly(N-isopropylacrylamide; NIPAm) and methylenebisacrylamide (BIS) as a crosslinker. The nanoparticles incorporate a variety of very high-affinity baits that target the following major categories of body fluid cancer biomarkers: basic and acidic proteins and peptides (e.g., membrane proteins, nuclear proteins, cytoplasmic proteins, and secreted soluble proteins), metabolites, and nucleic acids [20]. The bait chemistries recognize the three-dimensional conformation of target proteins with very high affinity (greater than most antibodies) such that virtually the entire solution phase content of the target biomarker can be sequestered in less than five minutes [18, 19]. The captured analytes can be readily eluted for analysis. For example, core shell particles can raise the concentration of an extremely dilute undetectable biomarker such as PDGF-BB in serum and human growth hormone in urine into the detection range of a commercial clinical-grade immunoassay platform [18]. A panel of dry lyophilized, submicron-sized harvesting particles that carry specific affinity baits for known classes of biomarkers can be introduced into the test sera; the respective particle populations remove all their target molecules, in one step, in solution, from the entire volume of the sample and concentrate the sequestered

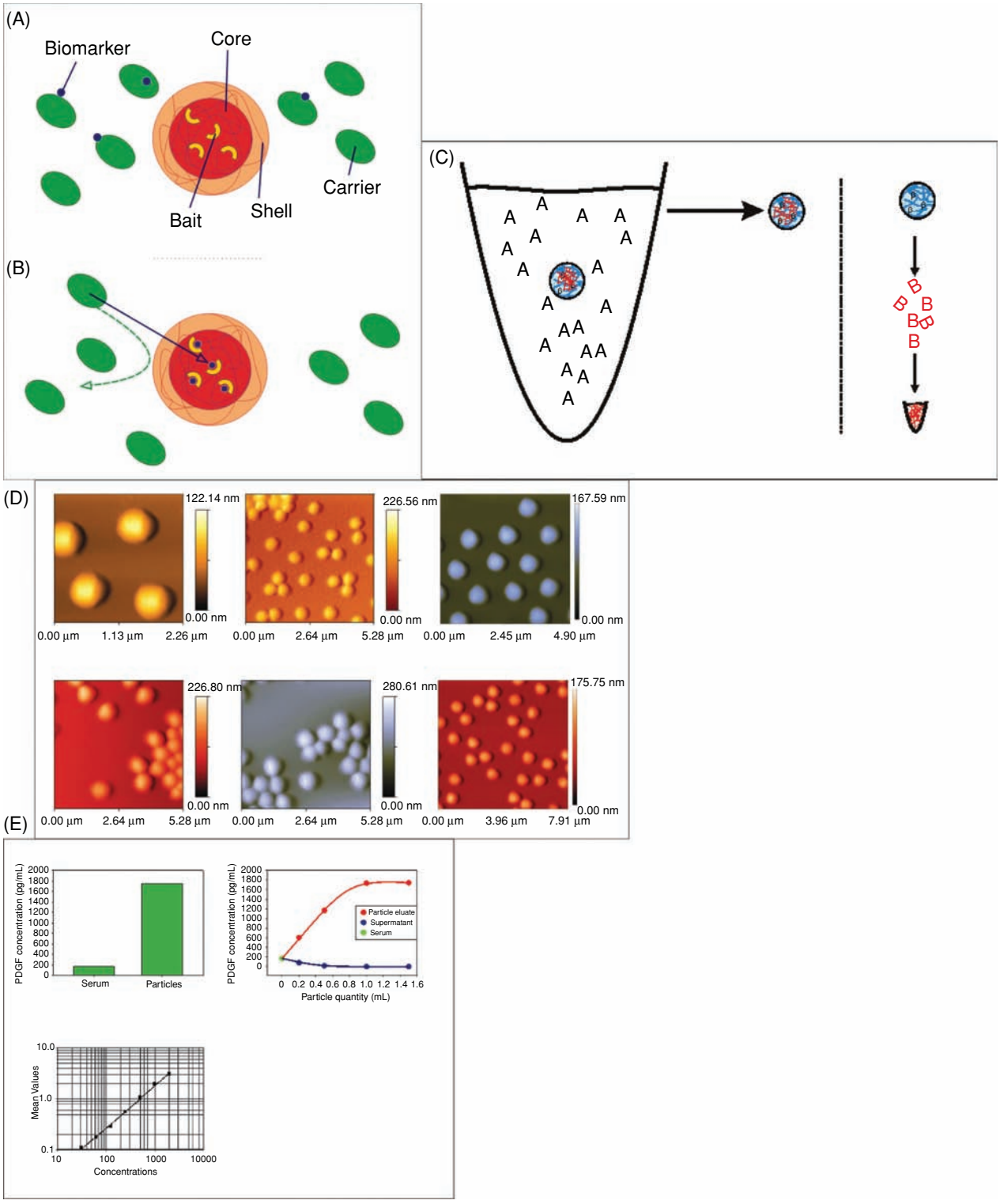

Figure 43.2. Schematic representation of the functioning of the nanoparticles. (A) Nanoparticles are constituted by a bait-containing core and a sieving shell. Core-shell nanoparticles are introduced in complex biological fluids (e.g., blood) in which biomarkers are present in exceedingly low concentration and non-covalently bound to carrier proteins, such as albumin. (B) Core-shell nanoparticles sequester the biomarker with total exclusion of the carrier; adapted from [20]. (C) Concentration mechanism; adapted from [18]. (D) Atomic force microscopy images of nanoparticles show homogeneous size distribution; adapted from [20]. (E) ELISA results show that native platelet-derived growth factor (PDGF) in serum is greatly concentrated by the action of the nanoparticles; adapted from [18].

analytes inside the particles. Analytes can then be eluted from the particles in a small volume to yield a much higher concentration and purification compared with the starting sample. With a starting volume of 500 μL, this technology can concentrate a biomarker many hundredfold and fully prevent biomarker degradation, within minutes.

Smart nanoparticles that bind specific tumor markers existing at very low concentrations in serum have the potential to be used as serum harvesting agents in vivo [21]. In the future, patients can potentially be injected with such nanoparticles that seek out, bind, and concentrate rare tumor or disease markers of interest. After the nanoparticles have bound their targets, they can be "harvested" from the serum to enable diagnosis or to monitor disease progression.

Reduction of Bias in Discovery Phase of Cancer Biomarkers

A basic tenet of the biomarker field is that the molecular composition of the circulation is a true mirror of ongoing cellular and organ system function [22]. Although this means that subsets of the blood peptidome can potentially reflect subtle disease events in small tissue volume, it also means that the peptidome is constantly fluctuating because of ongoing daily physiologic events. Epidemiologists and clinical chemists fear that the level of individual bloodborne biomarkers can be greatly influenced by a variety of non–disease-related epidemiologic factors and normal physiologic conditions. Thus, the promise of the specificity of the peptidome is counterbalanced by the sensitivity to interfering factors. Consequently, as with any biomarker discovery (single, or as a panel), great care is needed to reduce sample bias during the discovery and validation phase of peptidome biomarker translational research [23].

The starting point for rigorous discovery is the development of a carefully controlled study set consisting of a population of serum or plasma samples from (1) patients who have histologically verified cancer, (2) patients with benign or inflammatory nonneoplastic disease, and (3) unaffected, apparently healthy controls or hospital controls, depending on the intended use [24]. The issue of including specimens for initial discovery from patients with no evidence of cancer but with inflammatory conditions, reactive disease, and benign disorders is of critical importance to ensure that specific markers are enriched for from the outset. This issue is critical for cancer research, especially as the disease almost always occurs in the background of inflammatory processes that are part of the disease pathogenesis itself. The proteome may be especially sensitive to these nonneoplastic processes, which is why care must be taken to at least minimize the chance that nonspecific markers are selected. In fact, the reduction of the potential up-front bias is critical prior to undertaking the discovery phase of the research [24]. The next stage is peptidome fractionation, separation, isolation and enrichment, concentration, and mass spectrometry (MS)-based identification [15, 25].

It is recommended that iterative and repetitive MS-based analysis and MS/MS sequencing be conducted on each sample used for discovery [26]. Candidate peptides identified repetitively over much iteration within a sample and within a study set have a higher likelihood of being correct. The researcher ends up with a list of candidate diagnostic markers that are judged to be differentially abundant in the cancer versus the control populations. The next step is to find or make specific antibodies or other ligands for each candidate peptide marker [23]. After each antibody is validated for specificity using a reference analyte, the antibody can then be used to validate the existence of the predicted peptide marker in the disease and nondiseased discovery set samples [27].

An emerging generation of MS technology, multiple reaction monitoring (MRM), offers the potential to quantify and identify peptides with such high confidence that antibody validation may not be required [28]. Despite the methods, the goal is to develop a panel of candidate peptide biomarkers along with measurement reagents that are independent of the analytical technology that will ultimately be used in the clinical lab. Clinical validation of the candidate biomarkers starts with ensuring the sensitivity and precision of the measurement platform. After the measurement platform is proven to be reliable and reproducible, the clinical validation can proceed.

The final, and most critical, stage of research clinical validation is blinded testing of the biomarker panel using independent (not used in discovery), large clinical study sets that are ideally drawn from at least three geographically separate locations [24]. The required size of these test sets for adequate statistical powering depends on both the performance of the peptide analyte panel in the platform validation phase and the intended use of the analyte in the clinic. It is important to emphasize that sensitivity and specificity in an experimental test population does not translate to the positive predictive value that would be seen if the putative test is used routinely in the clinic. The true positive predictive value is a function of the indicated use and the prevalence of the cancer (or other disease condition within the target population). The percentage of expected cancer cases in a population of patients at high genetic risk for cancer is higher than the general population. Consequently, the probability of false positives in the latter population would be much higher. For this reason the ultimate adoption of a peptidome- or proteome-based

test will be strongly dependent on the clinical context of its use.

Finally, the information content of the peptidome will never be fully realized unless blood collection protocols and reference sets are standardized [23], new instrumentation for measuring panels of specific fragments is proved to be reproducible and sensitive [29], and extensive clinical trial validation is conducted under full CAP/CLIA regulatory guidelines.

UNDERSTANDING AND TREATING HUMAN METASTASIS IN CONTEXT OF TISSUE MICROENVIRONMENT

Microdissection Technology Permits Microscopic Molecular Analysis of Cancer Invasion and Metastasis

Molecular analysis of pure cell populations in their native tissue environment is necessary to understand the microecology of the disease process [30]. Accomplishing this goal is much more difficult than just grinding up a piece of tissue and applying the extracted molecules to a panel of assays. Tissues are complicated three-dimensional structures composed of large numbers of different types of interacting cell populations, and the cell subpopulation of interest may constitute a tiny fraction of the total tissue volume. After the computer adage "garbage in, garbage out," if the extract of a complex tissue is analyzed using a sophisticated technology, the output will be severely compromised if the input material is contaminated by the wrong cells. Culturing cell populations from fresh tissue is one approach to reducing contamination. However, cultured cells may not accurately represent the molecular events taking place in the actual tissue microenvironment from which they were derived. Thus, the problem of cellular heterogeneity has been a significant barrier to the molecular analysis of normal and diseased tissue.

In response to this need, we have originated laser capture microdissection (LCM) to provide scientists with a fast and dependable method of capturing and preserving specific cells from tissue, under direct microscopic visualization [31–33]. The mRNA from microdissected cancer lesions has been used as the starting material to produce cDNA libraries, microchip microarrays, differential display, and other techniques to find new genes or mutations [34]. The development of LCM allows investigators to determine specific protein expression patterns from tissues of individual patients [35]. Using multiplex analysis, investigators can correlate the pattern of expressed genes and posttranslationally modified proteins with the histopathology and response to treatment [35]. Microdissection can be used to study the interactions between cellular subtypes in the organ or tissue microenvironment. Efficient coupling of LCM of serial tissue sections with multiplex molecular analysis techniques is leading to sensitive and quantitative methods to visualize three-dimensional interactions between morphologic elements of the tissue. The goal is the integration of molecular systems biology with tissue morphogenesis and pathology.

LCM has provided two important contributions to the field of invasion and metastasis. First, it is now possible to study individual human cells and cell populations participating in the transition from in situ to invasive cancer in human tissue [36]. LCM can be used to procure premalignant cells from all stages of human cancer progression in any type of tissue. Tumor cells within the microscopic area of breast cancer, or prostate cancer microinvasion, can be separated directly from the body of the parent DCIS or prostatic intraepithelial neoplasia (PIN) lesion just a few microns away [11]. Second, LCM has provided the means to compare the tumor cells in the metastasis with the tumor cells in the primary parent mass [37, 38].

Beyond Functional Genomics to Cancer Proteomics

The application of proteomics to biomedical research has reached a high level of sophistication. The field has moved beyond merely cataloging proteins that change between benign and diseased cells. The current goals of proteomic research now include the synthesis of proteomic information into functional pathways and circuits in cells and tissues [39]. Such synthesis must take into account the dynamic state of protein post-translational modifications, protein–protein or protein–DNA/RNA interactions, crosstalk between signal pathways, and feedback regulation within cells, between cells, and between tissues. This higher level of functional understanding will be the basis for true rational therapeutic design that specifically targets the molecular lesions underlying human disease [40].

Whereas DNA is an information archive, proteins do all the work of the cell. The existence of a given DNA sequence does not guarantee the synthesis of a corresponding protein. Moreover, protein complexity and versatility stem from context-dependent post-translational processes. Nucleic acid profiling (including microRNA) does not provide information about how proteins link together into networks and functional machines in the cell. In fact, the activation of a protein signal pathway, causing a cell to migrate, die, or initiate division, can immediately take place before any changes occur in DNA/RNA gene expression. Consequently, the technology to drive the molecular medicine revolution from the correlation to the causality phase is emerging from protein analytic methods.

Although individualized treatments have been used in medicine for years, advances in cancer treatment have now generated a need to more precisely define and identify patients who will derive the most benefit from new-targeted agents. A molecular profiling using gene expression array has shown considerable potential for the classification of patient populations in all of these respects [41, 42]. Nevertheless, transcript profiling, by itself, provides an incomplete picture of the ongoing molecular network for a number of clinically important reasons. First, gene transcript levels have not been found to correlate significantly with protein expression or the functional (often phosphorylated) forms of the encoded proteins. RNA transcripts also provide little information about protein–protein interactions and the state of the cellular signaling pathways. Finally, most current therapeutics is directed at protein targets, and these targets are often protein kinases and/or their substrates [43]. The human kinome, or full complement of kinases encoded by the human genome, comprises the molecular networks and signaling pathways of the cell [44]. The activation state of these proteins and these networks fluctuates constantly depending on the cellular microenvironment. Consequently, the source material for molecular profiling studies needs to shift from in vitro models to the use of actual diseased human tissue. Technologies that can broadly profile and assess the activity of the human kinome in a real biological context will provide a rich source of new molecular information critical for the realization of patient-tailored therapy [5, 37, 38].

Protein Microarray Tools to Guide Patient-Tailored Therapy

Theoretically, the most efficient way to identify patients who will respond to a given therapy is to determine, prior to treatment initiation, which potential signaling pathways are truly activated in each patient [5]. Ideally, this would come from analysis of tissue material taken from the patient through biopsy procurement. In general, previous traditional proteomic technologies have significant limitations when they are applied to very small tissue samples, such as biopsy specimens, from which only a few thousand cells may be procured.

Protein microarrays represent an emerging technology that can address the limitations of previous measurement platforms and are quickly becoming powerful tools for drug discovery, biomarker identification, and signal transduction profiling of cellular material [45]. The advantage of protein microarrays lies in their ability to provide a map of known cellular signaling proteins that can reflect, in general, the state of information flow through protein networks in individual specimens [37]. Identification of critical nodes, or interactions, within the network is a potential starting point for drug development and/or the design of individual therapy regimens. Protein microarrays that examine protein recognition events (phosphorylation) in a global, high-throughput manner can be used to profile the working state of cellular signal pathways in a manner not possible with gene arrays [46].

Protein microarrays may be used to monitor changes in protein phosphorylation over time, before and after treatment, between disease and nondisease states and responders versus nonresponders, allowing one to infer the activity levels of the proteins in a particular pathway in real time to tailor treatment to each patient's cellular circuitry [39]. The application of this technology to clinical molecular diagnostics will be greatly enhanced by increasing numbers of high-quality antibodies that are specific for the modification or activation state of target proteins within key pathways. Antibody specificity is particularly critical, given the complex array of biologic proteins at vastly different concentrations contained in cell lysates. Very often, the final number of actual tumor cells microdissected or procured from biopsy tissues for analysis may be as low as a few thousand [47]. Assuming that the proteins of interest, and their phosphorylated counterparts, exist in low abundance, the total concentration of analyte proteins in the sample will be very low. Newer generations of protein microarrays with highly sensitive and specific antibodies are now able to achieve adequate levels of sensitivity for analysis of clinical specimens containing fewer than a few thousand cells.

At a basic level, protein microarrays are composed of a series of immobilized spots. Each spot contains a homogeneous or heterogeneous bait molecule. The array is queried with a probe (labeled antibody or ligand) or an unknown biologic sample (for instance, cell lysate) containing analytes of interest. When the query molecules are tagged directly or indirectly with a signal-generating moiety, a pattern of positive and negative spots is generated. For each spot, the intensity of the signal is proportional to the quantity of applied query molecules bound to the bait molecules. An image of the spot pattern is captured, analyzed, and interpreted. Protein microarray formats fall into two major classes, forward phase arrays (FPAs) [48] and reverse phase arrays (RPAs) [45] (originated by our lab), depending on whether the analyte(s) of interest is captured from solution phase or bound to the solid phase. In FPAs, capture molecules are immobilized onto the substratum and act as the bait molecule. In the FPA format, each array is incubated with one test sample (for instance, a cellular lysate), and multiple analytes are measured at once. Examples of their use in cancer research include the identification of changes in protein levels following treatment of cancer cells with ionizing radiation [49] and identification of serum protein biomarkers [46].

Despite their great potential, antibody array use is limited currently by the availability of well-characterized antibodies. A second obstacle to routine use of antibody arrays surrounds detection methods for bound analyte on the array. Current options include the use of specific antibodies recognizing distinct analyte epitopes from the capture antibodies (similar to a traditional sandwich-type ELISA), or the direct labeling of the analytes used for probing the array, both of which present distinct technical challenges. In contrast to the FPA format, the RPA format immobilizes an individual test sample in each array spot, such that an array is composed of hundreds of different patient samples or cellular lysates. Though not limited to clinical applications, the RPA format provides the opportunity to screen clinical samples that are available in very limited quantities, such as biopsy specimens. Because human tissues are composed of hundreds of interacting cell populations, RPAs coupled with LCM provide a unique opportunity for discovering changes in the cellular proteome that reflect the cellular microenvironment.

The RPA format is capable of extremely sensitive analyte detection with detection levels approaching attogram amounts of a given protein and variances of less than 10 percent [5]. The sensitivity of detection for the RPAs is such that low abundance phosphorylated protein isoforms can be measured from a spotted lysate representing fewer than ten cell equivalents. This level of sensitivity, combined with analytical robustness, is critical if the starting input material is only a few hundred cells from a biopsy specimen. Because the RPA technology requires only one antibody for each analyte, it provides a facile way for broad profiling of pathways in which hundreds of phospho-specific analytes can be measured concomitantly. Most important, the RPA has significantly higher sensitivity than bead arrays or ELISA such that broad screening of molecular networks can be achieved from tissue specimens routinely procured. A number of studies illustrate the utility of reverse phase protein microarrays for the analysis of human tissues and demonstrate the potential for the technology [5, 11, 37–39, 45, 46, 48–50].

RPA technology is well suited to assess the signaling differences between mutant and wild-type cells, and these studies are currently under way. Instead of single measurements of selected analytes, future pathology reports can be envisioned to include a phosphoproteomic portrait of the functional state of many relevant specific downstream endpoints and entire classes of signaling pathways as a guide for therapeutic decision making and prognosis. Molecular profiling of the proteins and signaling pathways produced by the tumor microenvironment, host, and peripheral circulation hold great promise in effective selection of therapeutic targets and patient stratification (Figure 43.3). For many of the more common sporadic cancers, there is significant heterogeneity in cell signaling, tissue behavior, and susceptibility to chemotherapy. Proteomic analysis is particularly useful in this area, given the ability to study multiple pathways simultaneously. Cataloging of abnormal signaling pathways for large numbers of specimens will provide the data necessary for a rationally based formulation of combination therapy that presumably would be more effective than monotherapy and would help to minimize the issues of tumor heterogeneity. The promise of proteomic-based profiling, different from gene transcript profiling alone, is that the resulting prognostic signatures are derived from drug targets (such as activated kinases), not genes, so the pathway analysis provides a direction for therapeutic mitigation. Thus, phosphoproteomic pathway analysis becomes both a diagnostic/prognostic signature and a guide to therapeutic intervention.

Protein Biomarkers' Stability in Tissue: A Critical Unmet Need

The promise of tissue protein biomarkers to provider evolutionary diagnostic and therapeutic information will never be realized unless the problem of tissue protein biomarker instability is recognized, studied, and solved [51]. There is a critical need to develop standardized protocols and novel technologies that can be used in the routine clinical setting for seamless collection and immediate preservation of tissue biomarker proteins, particularly those that have been posttranslationaly modified, such as phosphoproteins.

Although investigators have worried about the effects of vascular clamping and anesthesia prior to excision, a much more significant and underappreciated issue is the fact that excised tissue is alive and reacting to ex vivo stresses [51]. The instant a tissue biopsy is removed from a patient, the cells within the tissue react and adapt to the absence of vascular perfusion, ischemia, hypoxia, acidosis, accumulation of cellular waste, absence of electrolytes, and temperature changes. In as little as thirty minutes postexcision, drastic changes can occur in the protein signaling pathways of the biopsy tissue as the tissue remains in the operating room suite or on the pathologist's cutting board. It would be expected that a large surge of stress-related, hypoxia-related, and wound repair–related protein signal pathway proteins and transcription factors will be induced in the tissue. Over time the levels of candidate proteomic markers (or RNA species) would be expected to widely fluctuate upward and downward [51]. This will significantly distort the molecular signature of the tissue compared to the state of the markers in vivo. Moreover, the degree of ex vivo fluctuation could be quite different between tissue types and influenced by the pathologic microenvironment.

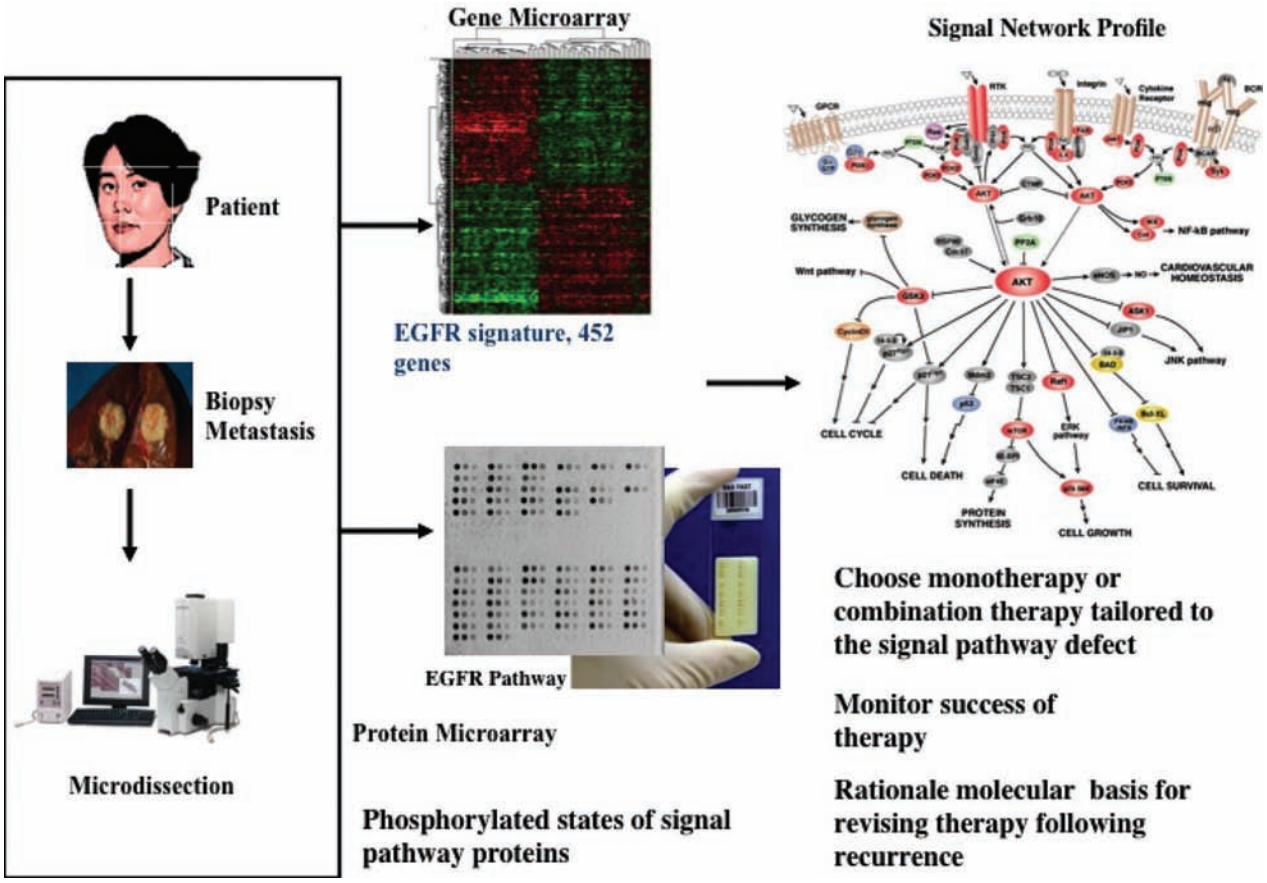

Figure 43.3. A roadmap for individualized metastatic cancer therapy includes biopsy or needle aspiration procurement of the sample, laser microdissection, and signaling pathway analysis, performed using protein microarrays for phosphoproteomic analysis and RNA transcript analysis. The specific signaling fingerprint is the basis of patient-tailored therapeutic intervention. Therapeutic evaluation is performed by follow-up biopsy and reassessing molecular fingerprint of signaling events. Adapted from [5].

Formalin Fixation May be Unsuitable: Advent of Molecular Preservation Chemistries

Although it is now possible to extract proteins from formalin-fixed tissue, because of the long period required for formalin tissue fixation, the procedure may be not optimal for phosphoprotein analysis. For tissue placed directly in formalin, the formalin penetration rate is 0.1 mm/hr, and so the cellular molecules in the depth of the tissue will have significantly degraded by the time formalin permeates the tissue [52]. Formalin crosslinking, the formation of methylene bridges between amide groups of protein, blocks analyte epitopes, as well as decreases the yield of proteins extracted from the tissue [52]. Because the dimensions of the tissue and the depth of the block that is sampled are unknown variables, formalin fixation would be expected to cause significant variability in protein and phosphoprotein stability for molecular diagnostics. Phosphorylation and dephosphorylation of structural and regulatory proteins are major intracellular control mechanisms. Protein kinases transfer a phosphate from ATP to a specific protein. Phosphatases remove the phosphoryl group and restore the protein to its original dephosphorylated state. Hence, the phosphorylation–dephosphorylation cycle can be regarded as a molecular on–off switch. At any point in time within the cellular microenvironment, the phosphorylated state of a protein is a function of the local stoichiometry of associated kinases and phosphatases specific for the phosphorylated residue. During the ex vivo time period, if the cell remains alive, it is conceivable that a phosphorylation state of certain proteins may transiently change [51].

A variety of chemical-based and protein-based inhibitors of kinase/phosphatases exist. Thus, there is adequate chemistry knowlege to design rational stabilizers for the preservation of phosphoprotein stability without freezing. In the near future preservative chemistries will be available in the OR and clinic so the molecular integrity of the tissue can be preserved immediately at the time of procurement. This future chemistry will preserve proteins, RNA, and

histomorphology. Moreover, this chemistry can be the starting point for processing into a standard paraffin block. In this way, molecular profiling can be seamlessly integrated into routine surgery and pathologic diagnosis.

Gathering molecular information at the cellular level will be critical for the individualized therapy of cancer metastasis, as the molecular pathways that drive the growth and survival of metastatic colonies are a product of the tissue context [53]. A therapy that targets a tumor cell in the primary mass may not be effective for treating a metastatic lesion derived from that same primary tumor. This is because the metastatic cells have adapted to survive and colonize the local target organ microenvironment, which can be quite different from the microenvironment of the primary tumor [54, 55]. For example, the microenvironment of the colonic mucosa, the site of the primary colorectal carcinoma, is quite different from the parenchyma of the liver, the common organ for metastasis [37].

Individualized Molecular Targeted Therapy of Cancer Metastasis

As summarized in Figure 43.3, new technology is now making it possible to map the activated state of the protein signal pathways with the neoplastic cells using only a needle biopsy. Two technologies, LCM [31–33, 56] and reverse phase protein microarrays (RPMA) [45], have solved the challenges of tissue cellular heterogeneity and the the need to map the activity of therapeutic drug targets in small numbers of cells. The availability of this technology now provides the opportunity to individualize molecular targeted therapy for metastasis [37].

Personalized cancer metastasis therapy is conducted in the following steps: (1) A biopsy of the metastasis is microdissected to yield a pure population of carcinoma cells. (2) The carcinoma cells are lysed and analyzed for the activated state of signal pathways containing the drug targets for one or more drugs in a panel of molecular targeted inhibitors. (3) The optimal drug from those in the panel is matched to the activated signal pathways in the patient's metastasis [37]. For example, if the EGF pathway is activated in the metastasis, as demonstrated by the phosphorylation of the EGF receptor as well as phosphorylation of downstream endpoint proteins such as Akt and Erk, then an EGF pathway inhibitor (tyrosine kinase inhibitor or monoclonal antibody) might be appropriate for the patient [37].

No longer a dream for the future, this concept is a reality today. Individualized molecular targeted therapy for metastasis is now undergoing evaluation in clinical research trials with the authors' own laboratory. This is a first-of-its-kind therapeutic trial, accepting qualified patients with stage IV metastatic colorectal cancer with metastasis to the liver. Biopsies of liver metastasis are obtained under stereotactic guidance. The procured tissue is subjected to LCM and analyzed by RPMA. The activated level of signaling pathways containing tyrosine kinase inhibitor targets is scored, and this score is used to stratify the patients to receive a molecular targeted inhibitor that knocks out this pathway in the metastasis. At the time this chapter is being written, the trial has reached one-tenth of the patient accrual goal.

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44 Critical Issues of Research on Circulating and Disseminated Tumor Cells in Cancer Patients

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Early spread of tumor cells is usually undetected even by high-resolution imaging technologies, preventing potentially effective early intervention. However, sensitive immunocytochemical and molecular assays now enable the specific detection of "occult" metastatic tumor cells even at the single-cell stage. These technologies provide the potential to track systemic tumor cell dissemination in the blood and the bone marrow (BM) as one of the first crucial steps in the metastatic cascade.

In colorectal cancer, approximately 50 percent of patients undergoing a curative resection (R0) die from metastatic disease within five years. Even among lymph node–negative (N0) patients, the relapse rate is 30 percent [1, 2]. In lung cancer, the prognosis is even worse, with 60 percent of R0 and 40 percent of N0 patients dying of the disease [3]. Whereas in breast and prostate cancer the overall survival today is relatively high (5 years, 80%–90%; 10 years, 70%–80%), a considerable fraction of node-negative patients still relapse (25%–30% and 15%–50%, respectively) and this can often take place many years (>10 years) after the removal of the primary tumor [4–6].

Various clinical studies have provided evidence for an association between the presence of disseminated tumor cells (DTCs) detected at the time of initial tumor resection and postoperative metastatic relapse in patients with cancers of the breast, prostate, lung, and gastrointestinal tract [7]. This work paved the way for the introduction of DTCs in international tumor staging systems [8, 9], and in 2007 DTCs and CTCs (circulating tumor cells) were mentioned for the first time in the American Society of Clinical Oncology (ASCO) recommendations on tumor markers [10].

DETECTION METHODS: POTENTIAL CHALLENGES AND LIMITATIONS

The detection of CTCs in peripheral blood of cancer patients holds great promise but remains a technical challenge. Identification and characterization of CTCs require extremely sensitive and specific analytical methods that are usually combined with prior enrichment procedures, including density gradient centrifugation, immunomagnetic procedures with antibodies against either tumor-associated antigens (positive selection) or the common leukocyte antigen CD45 (negative selection), as well as filtration. Positive selection is usually carried out with antibodies against the epithelial cell adhesion molecule (EpCAM), and subsequent immunocytological detection of CTCs is performed with antibodies to cytokeratins (CKs), the intermediate filaments of epithelial cells [11].

Among the current EpCAM/CK-based technologies, the FDA-approved CellSearch system has gained considerable attention over the past five years [12]. More recently, a new microfluid platform called the "CTC-chip," which consists of an array of anti-EpCAM antibody-coated microspots, was presented. The high incidence of patients with CTCs (>95%) and the high CTC counts, particularly in patients without overt metastases, warrant further investigations on the specificity of this assay [13]. Moreover, all EpCAM-based enrichment systems face the problem that EpCAM can be downregulated during epithelial–mesenchymal transition of disseminated cancer cells. Recent work indicates that this transition might, in particular, affect tumor cells with stem-cell–like properties [14].

Besides immunocytological detection methods, an alternative technique called EPISPOT (EPithelial ImmunoSPOT) was introduced for CTC analyses [11]. This technique detects specific marker proteins actively released by CTCs cultured for forty-eight hours, using an adaptation of the enzyme-linked immunospot technology. Because apoptotic cells are unable to secrete a sufficient amount of the marker protein, the observed immunospots are indicators of viable CTCs [15].

Reverse-transcriptase polymerase chain reaction (RT-PCR)–based assays targeting specific mRNAs are

the most widely used alternative to immunological assays. Many target transcripts (e.g., CK18, CK19, CK20, mucin-1, and carcinoembryonic antigen) are also expressed at low levels in normal blood and BM cells [11], and quantitative RT-PCR assays with validated cutoff values are required to overcome this problem. Moreover, target gene transcription might be downregulated in CTCs and DTCs (e.g., in the course of epithelial–mesenchymal transition [14]), which argues in favor of multimarker RT-PCR approaches.

In principle, detection of tumor-specific DNA aberrations would be the most specific approach to detect CTCs; however, the marked genetic heterogeneity of solid tumors poses a problem. Circulating cell-free DNA in the blood of cancer patients harbors tumor-specific aberrations; this DNA appears also to reflect the presence of disseminating tumor cells in blood and BM [16].

CLINICAL RELEVANCE OF DTC/CTC DETECTION

Relevance of DTC in BM

The reported significant correlations between the presence of DTCs in BM and metastatic relapse in various tumor types indicate that the founder cells of overt metastases might be among those DTCs.

Numerous studies have been published investigating the presence and clinical relevance of DTCs in BM of breast cancer patients [11, 17–19]. The size of the patient cohorts analyzed and the applied detection methods vary considerably among these studies (Table 44.1). Depending on the method used and the number of patients analyzed, the different studies have come to somewhat different conclusions regarding the clinical relevance of DTCs (Table 44.1). However, Braun et al. [17] published a meta-analysis consisting of 4703 breast cancer patients. In this pooled analysis, the presence of DTCs in BM was not only predictive of the development of skeletal metastases but was also predictive for the development of metastases in other organs [17].

Besides their presence at primary diagnosis and surgery, DTCs have been described to survive chemotherapy and hormonal therapy [20–23], and they can persist in BM over many years postsurgery. This persistence is also linked to an increased risk of late metastatic relapse [24–29]. For example, in high-risk breast cancer patients (>3 involved axillary lymph nodes or extensive invasion of cutaneous lymph vessels), the presence of tumor cells after therapy was associated with an extremely poor prognosis [20].

In colorectal cancer, a positive association between DTCs in BM and an increased recurrence rate [30, 31] or a reduced overall survival [32, 33] have so far been reported in four studies. In contrast, three other reports – mostly on smaller patient cohorts – could not detect any association between prognostic factors and the presence of DTCs [34–36]. The largest study, performed by Flatmark et al. [37], including 275 patients and 206 noncancer control patients, presented no clinical follow-up. Using RT-PCR analysis with CK20 mRNA as a marker transcript, two groups reported no association to survival [38, 39], whereas four groups found an association between the presence of CK20 transcripts and worse overall survival [40–43]. However, both negative reports were performed on metastatic patients only. Taken together, the clinical significance of DTC in colorectal cancer patients remains controversial (Table 44.2).

In non–small-cell lung carcinoma (NSCLC), several immunocytochemical studies investigated the prognostic relevance of DTCs using the monoclonal antibody CK2 against CK18 or different pan-cytokeratin antibodies [44]. The rate of CK-positive cells in the different studies ranged between 22 percent and 60 percent. Interestingly, a higher frequency of DTC was found not only in BM aspirations from the rib or sternum in lung cancer patients [45–47] but also among breast and esophageal cancer patients when compared with BM samples taken from the iliac crest [48, 49]. Irrespective of the localization of the BM puncture, several studies have shown a correlation between DTCs in BM and worse clinical outcome [45–47, 50–54]. However, the largest study conducted so far, on 196 patients, could not find an association; this can, however, be explained by a very short follow-up time (median, 8 months) [55]. Using RT-PCR–based assays, the follow-up data base is small thus far. Analyzing a small cohort of 50 patients free of overt distant metastases [56], Sienel et al. reported that the presence of MAGE-A was associated with poor prognosis. In conclusion, although there is some evidence that DTC might indicate an unfavorable outcome larger standardized studies are needed to confirm the prognostic value of DTCs in lung cancer.

In prostate cancer, BM is the most prominent metastatic site, and several research groups have focused on the detection of DTC in this organ over the past ten years. However, most of the studies included a relatively small number of patients and/or follow-up information, and data about the influence of hormonal treatment on prognosis are sparse or lacking. Using immunocytochemistry, DTC detection rates ranged between 10 percent and 90 percent [28, 57–60] and some evidence for a correlation of DTCs to clinically established risk factors, such as histological differentiation of the primary tumor, was found [61, 62]. Moreover, a correlation of DTC measurement to early prostate-specific antigen (PSA) recurrence was shown by two studies [60, 63]. Although a DTC-positive BM status was associated with grading and increased risk of metastasis, the study by Berg et al. in 2007 [62] on 266 patients did not find a correlation of DTC detection and survival. Most recently, Köllermann et al. [60] reported

TABLE 44.1. Detection of DTC in breast cancer patients

Method	No. of patients*	Stage	Detection markers (antibody)	Detection rate	Correlation to clinical/ pathologic variables	Prognostic/ predictive value	Reference
ICC	155	T1–4	CK18 (CK2)	18%	DM ($p < 0.001$)	ND	[125]
ICC	100	T1–4, M0	EMA, pan-CK	38%	NS	RFS ($p < 0.001$), OS ($p = 0.017$)	[126]
ICC	727	T1–4	Mucin TAG12 (2E11)	N0: 31%, N1:55%	TS ($p < 0.001$), G ($p = 0.002$), LN ($p = 0.001$)	DDFS ($p < 0.001$), OS ($p < 0.001$)	[127]
ICC	109	T1–4	Breast-associated antigens – MBr1, MBr8, MOV8, MOV16, MluCl	31%	NS	NS	[128]
ICC	350	T1–T4	EMA	25%	TS ($p = 0.008$), LN ($p = 0.005$), vascular invasion ($p = 0.001$)	RFS ($p < 0.001$), OS ($p < 0.001$)	[129]
ICC	552	T1–3	pan-CK (A45–B/B3)	36%	TS ($p < 0.001$), LNM no. ($p < 0.001$), tumor type ($p < 0.001$), G ($p = 0.017$), DM ($p < 0.001$)	DMSF ($p < 0.001$), OS ($p < 0.001$),	[117]
ICC	393	≥T1	CK+EMA	42%	DM no. ($p < 0.001$), bone metastases ($p = 0.028$), LN ($p = 0.001$)	TRD ($p = 0.01$), DSF (p = 0.0003), OS (p=0.022)	[130]
ICC	554	pT1–2, N0/1, M0	CK8, 18, 19 (clone 5D3)	N0: 31%, N1: 37%	TS ($p < 0.0001$), G ($p = 0.034$), lymph vessel invasion ($p = 0.005$), blood vessel invasion ($p = 0.047$), ER ($p = 0.026$), Ki-67 ($p < 0.0001$), uPA ($p = 0.016$), PAI-1 ($p = 0.03$)	DFS (p < 0.0001), OS (p < 0.0001)	[131]
ICC	920	T1–T4	pan-CK (AE1/AE3)	13%	TS ($p = 0.013$), LN (p < 0.0005), vascular invasion ($p = 0.045$), HER2 ($p = 0.024$)	ND	[132]
ICC	817	T1–4	pan-CK (AE1/AE3)	13%	TS ($p = 0.011$), LN ($p < 0.001$), ER/PR ($p = 0.051$)	DDFS ($p < 0.001$), BCSS ($p < 0.001$)	[133]
ICC	114	T1–4	pan-CK (A45–B/B3)	59%	menopausal status ($p = 0.024$), ER ($p = 0.026$), TS ($p = 0.026$)	OS ($p = 0.0004$), DSF (p = 0.051)	[134]
ICC	228	pT1–2, pN0–3, pM0	pan-CK (A45–B/B3)	Persistent DTC: 13%	NS	RFS ($p = 0.0003$), DFS ($p < 0.0001$), OS ($p = 0.002$), Persistent DTC: OS ($p = 0.0008$)	[24]
ICC	265	≥T1	pan-CK (A45–B/B3)	26%	NS	OS ($p = 0.03$)	[135]
ICC+RT-PCR	131	T1–3, M0	ICC: pan-CK (A45–B/B3), RT-PCR: CK19	ICC: 31%, Q–RT-PCR: 41%, Combined: 51%	NS	12 (48) months after surgery 35% (59%) of patients had positive ICC/Q–RT-PCR results	[25]
ICC	112	pT1–4	pan-CK (A45–B/B3)	83% before chemother., 24% after surgery and chemo/ endocrine ther.	DTC persistence: G ($p = 0.02$)	NS	[109]

(Continued)

TABLE 44.1 *(Continued)*

Method	No. of patients	Stage	Detection markers (antibody)	Detection rate	Correlation to clinical/ pathologic variables	Prognostic/ predictive value	Reference
RT-PCR	148	T1–4, M0, M1	CK19, MAM	CK19, M0: 23%, M1: 47%, MAM, M0: 16%, M1: 38%	PR (CK19: $p = 0.028$, MAM; $p = 0.026$)	OS (CK19: $p = 0.0045$, MAM; $p = 0.025$)	[77]
ICC	621	T1–4, M0	pan-CK (A45–B/B3)	15%	NS	OS ($p = 0.02$), DMSF ($p = 0.006$), LRFS ($p = 0.0009$)	[116]
RT-PCR	195	T1–4	CK19	12%	NS	DMSF ($p = 0.01$), OS ($p = 0.005$)	[136]
ICC+RT-PCR	177 (RT-PCR), 83 (ICC)	T1–3, M0	ICC: pan-CK (AE1/AE3) RT-PCR: MAM, CEA, PSE, PIP	ICC: 6%, RT-PCR comb. 11%, MAM: 4%, PIP: 2.8%, PSE: 2.8%, CEA: 1.1%	ND	ND	[137]

* Only studies including at least 100 patients are listed.
ICC: immunocytochemistry, RT-PCR: reverse-transcriptase polymerase chain reaction, MAM: mammaglobin, EMA: epithelial membrane antigen, CEA: carcinoembryonic antigen, PSE: prostate-specific Ets factor, PIP: prolactin-inducible protein, ER: estrogen receptor, PR: progesterone receptor, LN: lymph node, G: histological grade, TS: tumor size/stage, NS: not significant, ND: not determined, DFS: disease-free survival, OS: overall survival, DM/LM: distant/lymph node metastasis, DMSF: distant metastasis-free survival, LRSF: local relapse-free survival, DDFS: Distant disease-free survival, BCSS: breast cancer-specific survival, RFS: relapse-free survival, TRD: tumor-related death.

on the prognostic relevance of DTCs in BM found in 86 of 193 (44.6%) patients with clinically localized prostate cancer submitted to neoadjuvant hormonal therapy followed by radical prostatectomy and a median follow-up of forty-four months. There are also studies that used RT-PCR for DTC detection, mostly amplifying PSA- or MAGE-specific cDNAs as markers with strongly varying detection rates [44]. DTC detection correlated to PSA

TABLE 44.2. Detection of DTC in colorectal cancer patients

Method	No. of patients	Stage	Detection markers	Detection rate	Correlation to clinical/ pathologic variables	Prognostic/ predictive value	Reference
ICC	156*	Duke A–D	CK18 (CK2)	27%	LN and tumor stage	RR ($p < 0.05$)	[138]
ICC	109	I–IV	CEA (C1P83), mucin (Ra96), pan-CK (KL-1), CA 19–9, 17–1A†	49% (Ra96 and 17–1A and CEA: <10%, CA19–9: <15%, KI-1: <25%)	Tumor stage	NS	[35]
ICC	167	84 N0, 43 M1	pan-CK (A45–B/B3)	24%	NS	OS ($p = 0.006$), DFS ($p < 0.001$)	[33]
ICC	275	I–IV	EpCAM (MOC31)	17%	NS	ND	[37]
RT-PCR	295	I–IV, R0	CK 20	31%	Tumor stage	OS	[40]
RT-PCR	109	I–IV, 20 M1	CK20, GCC	CK20: 11% GCC: 6%	NS	ND	[139]
RT-PCR	127	I–IV	CK 20	33% without (total, $n = 103$) and 17% with (total, $n = 24$) neoadjuvant treatment	ND	OS ($p < 0.04$) and DFS ($p < 0.03$) for neoadjuvant chemoradiation patients	[140]

* only studies including at least 100 patients listed.
† CA19–9: Lewis blood group antigens, 17–1A: membrane antigen, CD54–0: membrane antigen, GCC: guanylylcyclase, CEA: carcinoembryonic antigen, LN: lymph node, NA: information not available, NS: not significant, ND: not determined, DFS: disease-free survival, OS: overall survival, RR: recurrence rate.

serum levels, and some evidence for the prognostic relevance of these findings have been reported [58, 64]. In conclusion, there is some evidence that the detection of DTCs in the BM of prostate cancer patients might represent a prognostic parameter, but larger multicenter studies, followed by nomogram testing against the established risk parameters, are required to introduce DTC detection into the future clinical management of prostate cancer patients.

Additional studies have been performed in patients with other epithelial tumor entities, such as gastric cancer [65], esophageal cancer [27, 66], pancreatic cancer [67, 68], ovarian cancer, and head and neck carcinomas [22, 69–75].

RELEVANCE OF CTCs IN BLOOD

Although the detection and monitoring of minimal residual disease by repeated BM sampling is standard of care in patients with leukemias or lymphomas, it appears to be difficult to introduce in the clinical management of patients with solid tumors. Sequential peripheral blood analyses are more acceptable, and many research groups are currently assessing the clinical utility of CTCs. Most of the work is again performed on patients with breast cancer (Table 44.3). All studies comparing BM and peripheral blood examinations performed at the same time points showed a higher frequency of BM-positive aspirates than blood-positive samples from the same patients [21, 76, 77], probably owing to the fact that BM might provide conditions for homing and survival of DTC, thus contributing to their accumulation in this compartment, whereas blood analyses allow only a "snapshot" of tumor cell dissemination.

Although the clinical significance of CTCs is still under active investigation, encouraging results on the association between CTC detection and metastatic relapse in breast and colorectal cancer patients at various disease stages have been recently published, using immunocytochemical and RT-PCR–based assays and various markers for the detection of CTCs in the peripheral blood (Tables 44.3 and 44.4). Thus far, few studies have directly compared BM and blood analyses in the same patients [21, 76–78]. The largest study on 341 stage I–IV breast cancer patients published by Wiedswang et al., showed that the detection of DTC in BM had superior prognostic significance over CTC measurements in stage M0-patients [76]. In contrast, Bidard et al. reported a superior significance of the CTC counts but they analyzed only thirty-seven metastatic (stage M1) breast cancer patients [78]. Currently, these findings do not support an exchange of DTCs in BM with CTCs from blood as a prognosticator in breast cancer, but future studies on larger cohorts of patients with improved CTC detection technologies (as discussed earlier) may help to clarify this important issue.

Many research groups are currently assessing the clinical value of CTC analyses for therapy monitoring, which has provided significant prognostic information in metastatic breast cancer [79, 80], and seems to be superior over conventional imaging methods for response evaluation [13, 81]. The clinical utility of these findings is now being prospectively addressed in a randomized trial, SWOG S0500, led by the Southwest Oncology group (www.cancer.gov/clinicaltrials/SWOG-S0500). The aim of this trial is to determine whether patients with elevated CTC levels after three weeks of first-line chemotherapy show an improved overall survival and progression-free survival when changing to an alternative chemotherapy regimen at the next course, rather than waiting for clinical evidence of progressive disease.

The real challenge of DTC/CTC technologies is to monitor minimal residual disease in patients without signs of overt metastasis. Pierga et al. monitored CTC counts in 118 patients before and after primary systemic chemotherapy in a Phase II trial (REMAGUS 02); they showed that the presence of CTC after a short follow-up time of eighteen months was an independent prognostic factor for shorter metastasis-free survival [82]. Interestingly, they did not find a significant correlation with the response of the primary tumor to chemotherapy, which is usually used as an indicator for treatment response. Pachmann et al. reported that a tenfold increase in CTC counts at the end of adjuvant chemotherapy was correlated with a significantly reduced relapse-free survival [83]. However, they detected two to three log units higher CTC counts than other groups, which has raised discussions about the specificity of their MAINTRAC assay [83].

The follow-up analyses of two other German trials using the CellSearch technology (i.e., the GEPARQuattro trial [www.germanbreastgroup.de/geparquattro] focusing on primary systemic chemotherapy [+/− trastuzumab] and the SUCCESS trial [www.success-studie.de] focusing on adjuvant chemotherapy) are still ongoing and will show whether the observed decreases in CTC rates [84, 85] will be associated with an improved survival rate of the cancer patients. In the GEPARQuattro trial, CTCs were detected in 22 percent of patients before primary systemic chemotherapy, and this rate decreased to 11 percent after chemotherapy [84]. In the SUCCESS trial, 1767 patients have been recruited; CTCs were evident in 10 percent of the patients before adjuvant chemotherapy and in 7 percent after completion of therapy [85].

Because most DTCs and CTCs are in a noncycling state, chemotherapy might have rather limited effects on these cells. Thus, the use of targeted therapies in

TABLE 44.3. Detection of CTC in breast cancer patients

Method	No. of patients	Stage	Detection markers (antibody)	Detection rate	Correlation to clinical/pathologic variables	Prognostic/ predictive value	Reference
RT-PCR	148*	T1–2, M0	CK19	30%	NS	DFS ($p < 0.0007$), OS ($p = 0.011$)	[141]
RT-PCR	94	M1	CK19, p1B, pS2, EGP2	31% (all 4 markers)	NS	DFS ($p = 0.015$), OS ($p = 0.0053$)	[142]
ICC	111	M1	CKs (mAbs 260F9, 520C9, 317G5), high-molecular weight mucin-like component of the human milk fat globule (mAb BrE-3)	16%†	Bone marrow involvement, disease status	RFS ($p = 0.04$) UV	[143]
ICC	177	M1	CK8,18,19	61% (2 or more CTCs)	Therapy (line: $p = 0.001$, type: $p = 0.02$), time to metastasis: $p = 0.03$	RFS ($p < 0.001$); OS ($p < 0.001$)	[80]
RT-PCR	100	T1–3, M0	CK19	33%	microvessel density ($p = 0.002$)	RFS ($p = 0.0003$)	[144]
RT-PCR	100 preoperative plus 100 postoperative	T1–3, N0, M0	CEA	30% preoperative; 14% postoperative	NS	DFS ($p = 0.001$)	[145]
ICC	177	M1	CK8,18,19	49% (≥5 CTC) at baseline; 54% (≥5 CTC) at any blood draw‡	ND	PFS ($p = 0.0001$–0.00134)§; OS ($p < 0.0001$–0.0013)§	[79]
ICC	341	T1–4, M0+M1, 40 months postsurgery	Pan-CK (AE1/AE3)	10%	ND	DFS ($p < 0.001$); BCSS ($p < 0.001$)	[76]
RT-PCR	119	T1–2, M0	CK19	18% after adjuvant tamoxifen treatment	NS	persistency during tamoxifen treatment: DFS ($p = 0.0001$); OS ($p = 0.0005$); Increased risk of relapse ($p = 0.00006$) and death ($p < 0.00001$)	[146]
ICC	151	M1	CK8,18,19	44% (>5CTC)	NS	OS ($p < 0.0001$); Cox model: HR (death) = 2.2 ($p = 0.003$)	[147]
RT-PCR	444	T1–3, M0	CK19	41%	NS	DFS ($p < 0.001$); OS ($p < 0.001$)	[148]
RT-PCR	185	T1–3, M0	CK19, HER2	34% (CK19), 18% (CK19+HER2)	NS	RFS; CK19+HER2 ($p = 0.029$); OS: CK19 ($p = 0.002$)	[149]
RT-PCR	175	T1–3, M0	CK19; mammaglobin A; HER2	41% (CK19); 8% (mammaglobin A); 29% (HER2)	Mammaglobin to tumor stage	DFS: CK19 ($p < 0.001$); mammaglobin ($p = 0.011$); HER2 ($p < 0.001$); OS: CK19 ($p = 0.044$), mammaglobin ($p = 0.034$)	[150]
ICC	118	T1–4, M0	Pan-CK (CK8, 18, 19)	27% (pre- and/or post-PST)	age (pre- and/or post-PST) ($p = 0.005$)	DMFS ($p = 0.017$)	[82]

* Only studies including at least 100 patients listed.
† CTC in peripheral blood progenitor cell apheresis products.
‡ Screening for CTC at different succeeding time points during systemic therapy (0, 3–5, 6–8, 9–14 and 15–20 weeks).
§ Significant difference between <5CTC and >or =5CTC at each blood draw during systemic therapy.
PST: primary systemic therapy, DFS: disease-free survival, DMFS: distant metastasis-free survival, OS: overall survival, RFS: relapse-free survival, PFS: progression-free survival, BCSS: breast cancer-specific survival, ER: estrogen receptor, CEA: carcinoembryonic antigen, ND: not determined, and NS: not significant.

TABLE 44.4. Detection of CTC in colorectal cancer patients

Method	No. of patients*	Stage	Detection markers (antibody)	Detection rate	Correlation to clinical/ pathol. variables	Prognostic/ predictive value	Reference
IMB+ICC	430	I–IV, 323 M1	EpCAM	26 % (≥3 CTC)	M1	PFS ($p = 0.0002$), OS ($p < 0.001$)	[151]
Array	194	All II, R0	hTERT, CK19, CK20, CEA	27%	Depth of invasion ($p < 0.001$), vascular invasion ($p < 0.001$), lymph node ($p = 0.031$)	MV RR ($p < 0.001$), OS ($p < 0.001$)	[152]
RT-PCR	200	I–III	CEA	22%	NS	PFS MV ($p = 0.007$), OS ($p = 0.04$)	[153]
RT-PCR	196	Duke A–C, all M0	CK20, CEA	pre-OP: 63%, 24h post-OP: 31%, 1 week post-OP: 41%	ND	24h post-OP MV, DFS ($p < 0.001$)	[154]
RT-PCR	167	Duke A–D	CK20, CEA	PB: 10.2%, MB: 34.1%	PB and MB: depth of invasion, stage, lymph node, liver metastasis	MB: MV DSF ($p < 0.001$), OS ($p < 0.001$), PB: UV DSF ($p < 0.001$), OS ($p < 0.001$)	[155]
RT-PCR	100	I–III	CEA	POB: 49%, PB: 39%	NS	POB: reduced RR ($p = 0.01$), DFS and OS: NS	[156]
RT-PCR	144	I–IV	CK20	No neoadjuvant: 56%, adjuvant treat. 40%	Stage ($p < 0.05$)	NS	[140]
RT-PCR	121	I–IV, 15 M1	CEA	POB: 51%, PB: 42%	Stage	NS	[157]

* Only studies including at least 100 patients listed.
RR: recurrence rate, R: recurrence, PFS: progression-free survival, OS: overall survival, POB: portal blood, PB: peripheral blood, and MB: blood from mesenteric vein draining the tumor.

addition to chemotherapy and radiotherapy has started a new era in clinical oncology [86]. Selection of cancer patients for targeted therapies, however, requires the identification of therapeutically relevant targets on tumor cells. The HER2 proto-oncogene is currently the most predominant biological target for systemic therapy, with remarkable results of clinical trials using a humanized monoclonal antibody (trastuzumab) in breast cancer [87]. Currently, all patients are stratified to trastuzumab therapy by primary tumor analysis only. The detection of HER2-positive DTCs/CTCs might enable a real-time assessment of the HER2 status during the clinical course of disease. Several groups reported a striking discrepancy between the detection of HER2-positive DTCs/CTCs and the HER2 score of the corresponding primary tumor [88–91], suggesting that a small subclone of HER2-overexpressing cancer cells easily missed by routine primary tumor analysis may have the potential to disseminate. The detection of HER2-positive DTCs and CTCs was correlated with an unfavorable clinical outcome in breast and esophageal cancer [88, 89, 92]; HER2 gene amplification can be acquired during tumor progression of the cancer [93, 94]. Thus, the assessment of the HER2 status on DTCs and CTCs might add important information for the clinical management of cancer patients.

BIOLOGY OF DTCs/CTCs

Further molecular analyses of DTCs and CTCs may help to reveal the biology of disseminating tumor cells. In particular, unraveling the puzzling phenomenon of "cancer dormancy" (i.e., the latency period between resection of the primary tumor and metastatic relapse, which can take more than ten years in breast cancer) and the identification of metastatic founder cells (stem cells) are of utmost importance.

Cancer Dormancy

Metastatic relapse in breast cancer patients can occur even more than ten years after the diagnosis and

resection of the primary tumor [95]. This latency period, referred to as *cancer dormancy*, is characterized by the presence of minimal residual disease over many years before overt metastases may eventually arise [24]. The group of Jonathan Uhr has shown that CTCs were detected in breast cancer patients free of overt metastases up to twenty-two years after primary tumor diagnosis [96]. Thus, it cannot be excluded that many "cured" cancer patients may harbor dormant tumor cells.

The steady-state regulating dormancy might be disturbed by changes in both the DTCs (e.g., additional mutations or epigenetic modifications in genes controlling cell proliferation and apoptosis) and the surrounding microenvironment (e.g., release of growth and angiogenic factors) [7]. Koebel et al. [97] have recently pointed to the importance of immune surveillance on tumor dormancy in an osteosarcoma mouse model, and Mahnke et al. reported that the BM microenvironment has special features important for the maintenance of tumor dormancy of DTC and immunological T-cell memory [98]. Moreover, Galon and coworkers demonstrated a strong positive correlation between T-cell activation and survival in colon cancer patients independent of the primary tumor size and nodal status [99]. However, the role of the immune system as a potentially important host component for controlling metastatic progression is still under debate. Certain subsets of macrophages can even support metastatic spread by facilitating angiogenesis and extracellular matrix breakdown and remodeling [100].

The ability to induce angiogenesis is thought to be important for the escape from cancer dormancy and the subsequent formation of metastases [101]. Although this hypothesis is clearly supported by numerous experimental studies, the information on the expression of angiogenic factors in DTCs/CTCs is sparse, with only one recent publication demonstrating frequent VEGF expression on CTCs (68% of total CTCs) in metastatic breast cancer patients [102]. Besides angiogenesis, other microenvironmental processes may also influence the dormant state of DTCs and micrometastases. It has been shown that during inflammation and wound healing, a plethora of cytokines is being released, and some of these factors can induce the migration and growth of epithelial tumor cells [103]. Interestingly, a gene expression signature specific for wound healing predicted metastatic relapse in breast cancer patients [104]. Thus, it cannot be excluded that, for instance, accidental bone fractures in cancer patients with minimal residual disease might facilitate the escape from dormancy.

Metastatic Stem Cells

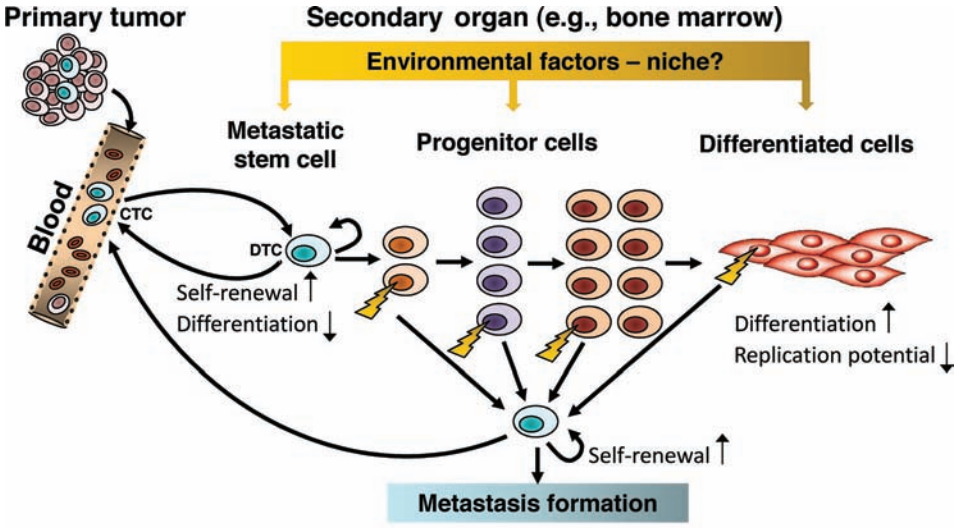
The cancer stem cell field has received great attention over the past five years, and new potential stem cell markers are being discovered for all types of solid tumors [105, 106]. With regard to metastasis, it is assumed that cancer stem cells can disseminate from the primary tumor to distant sites. This assumption is supported by the observation that primary tumor stem cells show an expression profile associated with metastatic relapse in breast cancer patients [107]. Moreover, expression of a new breast stem cell marker (ALDH1) was associated with poor clinical outcome, and only ALDH1-positive cells were able to form metastasis in mice [108]. Recently, the group of Robert Weinberg showed that cancer stem cells may have a particular capacity to undergo an epithelial–mesenchymal transition [14], which increases their mobility and invasiveness and allows them to survive their stressful passage through the bloodstream to distant organs.

There is also some evidence that the founder cells of overt metastases (i.e., metastatic stem cells) might be among the DTCs/CTCs detected by the current methods in cancer patients (Figure 44.1): (1) the presence of DTCs in BM is significantly correlated to metastatic relapse [17]; (2) most DTCs/CTCs are nonproliferating (i.e., Ki-67 negative) and resistant to chemotherapy [21, 109, 110] as postulated for cancer stem cells; and (3) subsets of DTCs/CTCs have a breast cancer stem cell phenotype (e.g., $CD44^{+}CD24^{-/low}$, $CK19^{+}MUC1^{-}$, $EpCAM^{+}$) [15, 111–113]. Functional studies using appropriate xenograft mouse models are now needed to demonstrate which subsets of DTCs/CTCs are the actual founder cells of overt metastasis.

Future research will show whether DTCs may use the same BM niches as normal stem cells [114] to persist over many years in cancer patients [24]. DTCs can be found in BM of patients from various epithelial tumors, including breast, prostate, lung, and colon cancer [11, 17, 115]. Although DTCs may also be present in other organs at the same time, it can be envisaged that BM might serve as a reservoir of DTC from where they may recirculate into other distant organs, such as liver or lungs, where better growth conditions may exist. The observed correlation between DTCs in BM and local relapse in breast cancer suggests that these cells might even circulate back to the primary tumor site [116]. Again, the development of an experimental model in which these provocative and clinically relevant hypotheses can be tested will be of utmost importance. If BM is a particular reservoir for DTCs, drugs targeting the BM–tumor interaction (e.g., biphosphonates or antibodies to the RANK ligand) may be sufficient to prevent metastatic or even local relapse.

MOLECULAR DETERMINANTS OF METASTATIC SPREAD

Hematopoietic cells can be a source of false-positive cells, but it appears that most cytokeratin-positive cells



Tentative characteristics of a putative 'metastatic stem cell':

- Rare	- Long term persistence
- Quiescent (Ki-67$^-$)	- Causes metastatic relapse in patients
- Self renewal	- Response to EGF/FGF2
- Forms metastases in mice	- Epithelial-mesenchymal transition
- Chemotherapy resistance	- Phenotype: ALDH1$^+$, EpCAM$^+$, CD44$^+$/CD24$^{-/low}$, MUC1$^-$/CK19$^+$ (*breast*) CD44$^+$, CD133$^+$ (*prostate*)

Figure 44.1. Stem cell model of metastatic progression. The primary tumor releases circulating tumor cells (CTCs) into the blood circulation, and these cells home to secondary sites (e.g., bone marrow). At the secondary sites, the disseminated tumor cells (DTCs) with stem cell properties survive and give rise to progenitor cells, which will eventually stop proliferating and rest in a differentiated state. All these cell populations contribute to the formation of the overt metastasis. The secondary organ may provide special niches for the tumor cells, and their capacity to self-renew, proliferate, or differentiate might be influenced by the environmental factors provided by these niches. In bone marrow, it can be speculated that the hematopoietic stem cell niches may be also used by stem-cell–like DTCs. Evidence that DTCs have stem cell properties is summarized in the box.

in BM and blood samples are of epithelial origin, as indicated by the analysis of large cohorts of non-cancer control patients [117]. The most important question whether these cytokeratin-positive cells are indeed tumor cells was answered using whole-genome amplification and comparative genomic hybridization of single DTCs and CTCs [118–121]. All cytokeratin-positive cells seemed to show genetic changes, clearly indicating that the cells are tumor cells. However, DTCs in patients with breast cancer and other solid tumors (e.g., esophageal cancer [92]) did not usually contain the same genetic changes as the primary tumor [121]. From these surprising findings published by the Klein group, it was concluded that DTCs may disseminate early from their primary tumor and undergo an independent genetic progression [11]. However, whole-genome amplification of a single immunostained DTC or CTC is a very challenging technique, which by definition does not allow any repeat experiments. Thus, it would be desirable that the exciting present data, which have been published by one group over the past ten years, could be reproduced by other laboratories using ring experiments with standardized protocols. Using a different technical approach (i.e., loss-of-heterozygosity [LOH] analyses of specific genomic regions) the Brandt group recently showed that in prostate cancers, genetic aberrations of CTCs in early-stage patients are identical to those in distinct, even small, areas of the primary tumor [122], which suggests that a metastatic subclone already exists in the primary tumor [123]. These subclones might also exist in other solid tumors, such as breast cancer, but can be easily missed by the routine analyses of a few sections of the primary tumor.

Besides the direct analysis of DTCs and/or CTCs, the genetic profiling of primary tumors in relation to the presence or absence of DTCs or CTCs might provide unique information on putative molecular determinants of micrometastasis in cancer patients. This is one of the main goals of the European DISMAL consortium (www.dismal-project.eu). Early stage cancer patients without lymph node metastasis (stage N0) and with no signs of overt metastasis (stage M0) are selected, and both groups (DTC and/or CTC positive or negative) are matched for all other relevant parameters, such as age, tumor stage, or differentiation grade. The best results are obtained from analysis of fresh frozen tumor tissue. To avoid contamination with normal tissue present in all tumors, areas containing

tumor cells are laser-microdissected and the DNA and RNA from these areas are isolated. RNA is hybridized to arrays containing probes representing the entire pattern of expressed human genes. The extracted DNA is analyzed by comparative genomic hybridization (CGH) using arrays that cover the whole genome. The complex pattern obtained by these array experiments require a sophisticated bioinformatics to reveal the signatures significantly associated with the presence of DTCs and/or CTCs. A further validation of the resulting candidate genes is required and can be performed rapidly on tissue microarrays containing hundreds of tumor samples from an independent cohort of cancer patients with known DTC and/or CTC status. This strategy has been successfully applied to tumors from patients with non–small-cell lung cancer and revealed that loss of 4q is significantly associated with the detection of DTCs, suggesting the presence of metastasis suppressor genes in this chromosomal region [124].

FUTURE DIRECTIONS

DTCs in BM have been detected in all solid tumor types, suggesting that the BM might be a preferred reservoir for bloodborne DTCs. Whether DTCs use this environment as a niche to persist in a dormant state over many years before they disseminate into other organs is subject of current investigations. Understanding this stage of dormancy and the conditions enabling DTCs to reactivate growth, as well as identifying the founder cells of overt metastases (metastatic stem cells), are some of the most important and challenging areas of basic research on early tumor cell dissemination.

Sequential peripheral blood drawings – in particular, for real-time monitoring of minimal residual disease in cancer patients undergoing systemic therapies – should be more acceptable than repeated BM aspirations. Although the prognostic significance of CTC could be reliably demonstrated for metastatic breast, colorectal, or prostate cancer patients, studies on the impact of CTCs in primary cancer patients are still ongoing. The identification of patients at increased risk for recurrence after chemotherapy is an application of high clinical relevance. Recently, encouraging results on monitoring of CTCs during primary systemic or adjuvant chemotherapy in breast cancer patients were obtained. In summary, DTCs/CTCs have the potential to become important biomarkers for real-time monitoring of the efficacy of systemic adjuvant therapy in individual patients. Furthermore, phenotypical and molecular characterization of these cells will contribute to more "tailored" and personalized antimetastatic therapies.

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45 Lymphatic Mapping and Sentinel Lymph Node Biopsy

Robert H. I. Andtbacka and Jeffrey E. Gershenwald

Primary tumor cells can metastasize to local, regional, and distant sites through four main mechanisms: direct invasion, lymphatic spread, hematogenous spread, and celomic spread. In most solid tumors, the initial form of tumor spread is lymphatic metastasis to regional lymph nodes.

In many types of cancer, the presence of regional lymph node metastasis is one of the most important predictors of recurrence and survival. Consequently, the presence of regional lymph node metastasis often leads to recommendations for additional treatment, such as more extensive lymph node surgery, radiation therapy, and/or systemic therapy (i.e., chemotherapy, biological therapy, or targeted therapy). Given the clinical relevance of regional lymph node metastasis in many types of cancer, evaluation of the regional lymph nodes is an essential component of the staging of those cancers.

In this chapter, we provide an overview of the importance of the lymphatic system in tumor metastasis, explain the concept of the sentinel lymph node (SLN) as it relates to cancer patients, describe and illustrate how SLNs are identified and assessed in clinical practice, and provide clinically salient examples of the impact of SLN metastasis on recurrence and prognosis.

THE LYMPHATIC SYSTEM

The lymphatic system in humans has three major interrelated functions: (1) transport of interstitial fluid to the lymphoid tissue; (2) absorption of fatty acids and their transport as chyle to the circulatory system; and (3) transport of antigen-presenting cells, such as dendritic cells, to lymph nodes for activation of the immune system.

The lymphatic system is organized into two major components: the conducting system, made up of lymphatic capillaries, lymph vessels, and the thoracic ducts; and the lymphoid tissue, including lymph nodes and lymphoid follicles. The conducting system collects interstitial fluid that has leaked out from blood capillaries into the interstitial space to provide nutrients for tissue. The collected interstitial fluid is transported as lymph via the afferent lymphatic vessels to the lymphoid tissue, which functions to defend the body against infection and the spread of tumors. Lymph nodes are generally bean-shaped organized collections of lymphoid tissue with white blood cells – most prominently, lymphocytes – packed in tight clusters called *lymphoid follicles*. Several afferent lymphatic vessels generally transport lymph to a single lymph node. The lymph percolates through the lymph node, in which it is scanned by the immune system for foreign antigens; it exits the lymph node via efferent lymphatic vessels and is eventually returned to the blood circulation via the thoracic ducts. Humans have 500 to 600 lymph nodes located at intervals along the lymphatic system. Lymph nodes are particularly abundant in the neck, axilla (armpit), ilioinguinal (groin) region, chest, and near the intestines. The areas in which lymph nodes are particularly abundant are called *lymph node basins* [1].

IMPORTANCE OF THE LYMPHATIC SYSTEM IN TUMOR METASTASIS

A hallmark of cancer is the ability of primary tumor cells to metastasize to vital organs and other distant sites. Metastasis of primary tumor cells to regional lymph nodes via the afferent lymphatic system is an important and often the initial route by which cancer cells gain access to the rest of the body. Although metastasis via the lymphatic system to regional lymph nodes was previously thought to be a passive process by which tumor cells detached from the primary tumor and entered preexisting, thin-walled lymphatic capillaries with incomplete basement membranes [2–4], more recent clinicopathologic studies have shown

that primary tumors and stromal cells can secrete lymphangiogenic growth factors that promote the growth of new lymphatic vessels [5]. These lymphangiogenic growth factors include vascular endothelial growth factor (VEGF)-C and VEGF-D, as well as VEGF-A; the last, in addition to being a well-established angiogenic factor, has been shown to be lymphangiogenic. [5] The secreted factors activate VEGF receptor (VEGFR)-2 and VEGFR-3 on lymphatic endothelial cells that form the luminal surface of lymphatic vessels, resulting in a signaling cascade that contributes to lymphangiogenesis and facilitates lymphatic metastasis [6–13].

This secretion of lymphangiogenic factors by tumor and stromal cells may be considered an expanded variant of the "seed-and-soil" hypothesis [14], the hypothesis that primary tumor cells secrete factors to modify the "soil" in the lymph node to make it more hospitable for metastasizing tumor cells to settle and proliferate in the lymph node. In the lymph nodes, the tumor cells may induce lymphangiogenesis and potentially other changes to vessel morphology, resulting in abnormal connections between the lymphatic and vascular networks. This may further promote the spread of tumor cells from the lymph nodes to more distant sites – such as brain, lung, liver, or bone – via the vascular system.

Although lymphangiogenesis is exceedingly difficult to measure directly, various surrogates of lymphangiogenesis, including intratumoral and peritumoral lymphatic vessel density and lymphatic vessel number, have been shown to correlate with the presence of lymph node metastasis in cutaneous melanoma [15], inflammatory breast cancer [16], muscle-invasive transitional cell carcinoma of the bladder [17], non–small-cell lung cancer [18], and head and neck cancer [19].

THE SLN CONCEPT

Several studies support the concept that lymphatic fluid from a primary tumor drains via the afferent lymphatics to one or more specific lymph nodes, termed the *sentinel lymph nodes*. The drainage pathway to the SLNs can be assessed by lymphatic mapping, which involves injecting a mapping agent into the tissue around the primary tumor and following the agent in the afferent lymphatics to the SLN. If a radioactive agent is used, the procedure is referred to as *lymphoscintigraphy*.

The concept of lymphatic drainage from a body location to a specific lymph node and lymph node basin was first established by the German physician Rudolf Virchow in the mid-nineteenth century. In 1923, Braithwaite [20] referred to the first iliocecal lymph node receiving drainage from the appendix as the "glands sentinel." In 1939, Gray [21] introduced the concept that tumor cells spread in an orderly fashion through the lymphatic system, migrating first to a single nodal focus, the SLN, before migrating to the remainder of the lymph nodes in a lymph node basin. In 1960, Gould et al. [22] described a "sentinel node" to which direct lymphatic drainage occurred in patients with carcinoma of the parotid gland. If intraoperative evaluation of this node revealed micrometastatic disease, a radical neck dissection was performed to ensure that no other nodes harbored occult metastatic disease. Almost two decades later, Cabañas [23] reported on 100 patients with penile cancer who underwent lymphatic mapping and lymphoscintigraphy, in which he described a "sentinel lymph node" draining the penis. The SLN or SLNs were surgically removed, a procedure known as SLN biopsy, and then examined pathologically. This SLN or group of SLNs not only was the first site of metastasis from penile cancer, but, in many patients, also represented the only site of involvement. On the basis of these findings, Cabañas suggested that no further surgery was indicated if the SLN did not contain any metastases – surgical removal of the remaining lymph nodes in the lymph node basin should be performed only if the SLN contained metastasis.

The enormous clinical implications of lymphatic mapping and SLN biopsy were not fully appreciated until a landmark paper published by Morton et al. [24] in 1992. On the basis of their work in a feline model [25], Morton and colleagues described the use of lymphatic mapping and SLN biopsy in patients with melanoma. [24] Their work, later confirmed in studies from other groups [26–29], established that in melanoma, (1) different areas of the skin have different drainage patterns to the regional lymph node basins, (2) for a given area of the skin, a specific lymph node or group of lymph nodes – that is, a specific SLN or group of SLNs – is the first to receive the lymphatic drainage to a lymph node basin (Figure 45.1), and (3) if the SLN or SLNs harbor no metastasis, the rest of the lymph nodes in the specific lymphatic basin will also be free of metastasis [26–29]. The SLN biopsy technique was subsequently also shown to be useful and predictive of lymph node metastasis in other cancers, such as cancers of the breast [30–34], colon and rectum [35–37], stomach [38, 39], esophagus [40, 41], lung [42–44], and genitourinary tract [45–50]. However, to date, lymphatic mapping, lymphoscintigraphy, and SLN biopsy have been used most widely for melanoma; selected high-risk non-melanoma skin cancers; and breast cancer.

RATIONALE FOR SLN EVALUATION

There are several reasons for evaluating SLNs in patients with melanoma, breast cancer, and other solid tumors: (1) to obtain information about the cancer

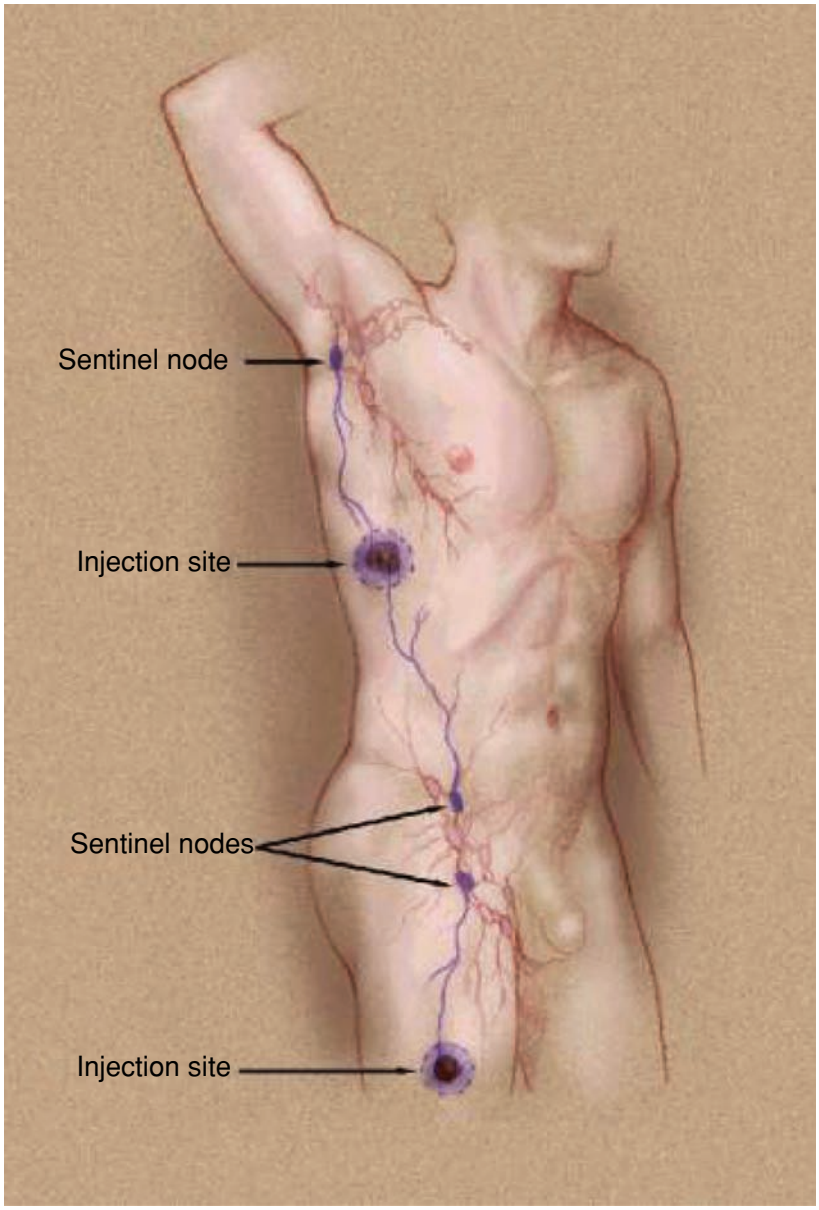

Figure 45.1. Schematic illustrating concept of lymphatic mapping and sentinel lymph node (SLN) biopsy in melanoma. Afferent lymphatic drainage from a flank melanoma leads to SLNs in both the inguinal and axillary regions, and drainage from a thigh melanoma leads to an SLN in the groin. (Courtesy of Jeffrey E. Gershenwald, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

stage and the patient's prognosis – the presence of regional lymph node metastasis is one of the strongest independent predictors of tumor recurrence and survival; (2) to guide treatment decision-making – SLN status guides decisions about additional treatments, such as surgical removal of the remaining lymph nodes in the basin (termed lymph node dissection or lymphadenectomy) to decrease the risk of in-basin tumor recurrence, adjuvant systemic therapy, and radiation therapy; (3) to offer patients a potential therapeutic benefit – early removal of SLNs containing microscopic disease may in itself provide a therapeutic benefit for some patients; (4) to minimize morbidity for patients with clinically negative lymph nodes – removing only SLNs is associated with much lower morbidity than removing all of the lymph nodes in a basin; and (5) to permit more in-depth analysis of a few lymph nodes (rather than "routine" histologic assessment of all lymph nodes in a lymph node basin), allowing for a more accurate evaluation of the presence of regional lymph node metastasis.

Reasons 1 through 3 apply equally to traditional regional lymph node dissection and SLN biopsy; reasons 4 and 5 are specific to SLN biopsy. These reasons are discussed in the following sections.

Determine Cancer Stage and Patient Prognosis and Thereby Guide Treatment Selection

In many types of cancer, the presence of regional lymph node metastases is associated with an increased risk of tumor recurrence and a worse prognosis. Knowing the metastatic status of regional lymph nodes aids risk stratification, determining prognosis, and deciding appropriate treatments for patients. Regional lymph node dissection (removal of all lymph nodes in a lymph node basin) and SLN biopsy both facilitate identification of regional lymph node metastases (if present), but SLN biopsy offers several advantages over regional lymph node dissection, as outlined below.

Offer a Potential Therapeutic Benefit

Removal of SLNs may offer a therapeutic benefit to some patients, depending on how tumor spread from primary tumors occurs – a concept not yet fully understood. In melanoma, for example, two competing hypotheses have been proposed regarding the impact of regional lymph node metastases on the subsequent development of distant metastatic disease.

According to the *incubator hypothesis*, tumor cells from the primary melanoma initially metastasize via the lymphatic system in an orderly fashion to SLNs. In the SLN, the metastatic clone can grow and avoid destruction by the immune system because of immunosuppressive factors released by the primary tumor. The metastatic cells may grow (incubate) in the SLN and become a source of distant metastasis. Hence, according to this hypothesis, removal of the tumor-involved SLN before there is further spread may prevent distant metastasis and may provide a survival advantage.

According to the *marker hypothesis*, metastasis from the primary tumor occurs simultaneously via both lymphatic and hematogenous routes, and the presence of tumor cells in the SLN is merely a marker that the primary tumor has gained the ability to metastasize. According to this hypothesis, removal of the tumor-involved SLN is unlikely to have an impact on the development of distant metastasis and therefore unlikely to provide a survival advantage [51].

The extent to which either of these hypotheses represents the predominant mechanism of distant metastasis across the spectrum of solid tumors remains controversial and is likely to be dependent on the malignant potential of different tumor types. In melanoma, surgical removal of the primary tumor and immediate removal of lymph nodes with micrometastatic disease (clinically nonpalpable lymph nodes) has been shown to improve recurrence-free, distant metastasis-free, and overall survival compared to removing the lymph nodes only when they become macrometastatic (clinically palpable) [52]. This recent observation indirectly strengthens the incubator hypothesis in melanoma. Whether the incubator hypothesis may also apply to other solid tumors is not well established.

Minimize Morbidity for Patients with Clinically Negative Lymph Nodes

Traditional regional lymph node dissection is associated with potentially significant adverse effects (e.g., paresthesia, surgical infection, seroma, lymphedema, and increased long-term risk of infection). Moreover, patients without lymph node metastasis are not likely to benefit from lymph node dissection but are still subjected to the associated risks. Patients without lymph node metastases represent a significant proportion of all patients with clinically negative lymph nodes. For example, in melanoma, four randomized clinical trials have evaluated the impact of performing routine elective lymph node dissections (ELNDs) in patients with clinically negative lymph nodes. All of these trials have failed to show an overall survival benefit from routine ELND [53–58]. One of the basic challenges with these trials is the fact that only a small fraction of melanoma patients have occult lymph node metastasis at the time of diagnosis and as such may benefit from the ELND, whereas the majority of patients have uninvolved lymph nodes and cannot benefit from the procedure, making it statistically very difficult to power a trial to show an overall benefit from ELND.

In melanoma, the risk of lymph node metastasis is dependent on primary tumor features such as Breslow tumor thickness and tumor ulceration – patients with thicker and ulcerated primary melanomas have a higher risk of lymph node metastasis [59]. In patients with thin (Breslow thickness 1.0 mm or less), nonulcerated primary melanoma, the reported incidence of microscopic lymph node metastasis at diagnosis is less than 10 percent in patients with no clinical evidence of lymph node metastasis, whereas in patients with thick (Breslow thickness greater than 4.0 mm), ulcerated melanomas, the reported incidence of microscopic lymph node metastasis at diagnosis is over 50 percent [59, 60]. Overall, only 15 percent to 20 percent of melanoma patients with clinically negative lymph nodes thought to be at risk will actually harbor occult lymph node metastasis. Using lymphatic mapping and SLN biopsy to determine which patients with clinically negative lymph nodes harbor occult metastasis prior to performing a lymph node dissection would spare many patients from the potential morbidities of a lymph node dissection.

Permit More In-Depth Evaluation of Regional Lymph Node Status

Historically, before the advent of the SLN biopsy technique, patients with melanoma and breast cancer routinely underwent a regional lymph node dissection to assess whether there was metastatic spread to a lymph node basin. This surgery often involved removal of ten to sixty-five lymph nodes that needed to be assessed for metastatic disease. This was a very labor-intensive process for the pathologist, and most lymph nodes were assessed only in a limited fashion, with conventional hematoxylin and eosin (H&E) staining of generally one section per lymph node. When only one section is analyzed, small micrometastases in lymph nodes can be missed in up to 25 percent of cases [61, 62]. However, with the advent of the SLN biopsy technique, generally only two or three SLNs are identified. This limited specimen affords the pathologist the opportunity to perform enhanced histologic evaluation of the lymph nodes.

In most cancer centers, contemporary SLN histologic approaches include analysis of "more" of the lymph node (i.e., analysis of serial sections, obtained in a process similar to slicing a loaf of bread in multiple slices) and the use not only of H&E staining but also of immunohistochemical approaches with antibodies to tumor-specific proteins. In melanoma, for example, antibodies against S100-B, HMB-45, and Melan-A are often used (Figure 45.2). In breast cancer, use of anti-cytokeratin antibodies against CAM 5.2 and pancytokeratins AE1-AE3 has been shown to increase the rate of detection of microscopic metastasis [63, 64]. In Merkel cell carcinoma, an aggressive neuroendocrine carcinoma of the skin, CK20 and pancytokeratin AE1-AE3 staining also aid in detecting SLN micrometastasis.

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of the SLN has also been studied as an approach to further enhance detection of submicroscopic SLN metastasis [64–70]. Although the hypothesis has been proposed that this approach may identify clinically meaningful disease by measuring putative tumor-associated gene expression, the clinical impact of an RT-PCR–positive SLN on patient prognosis is not well established, and the use of RT-PCR for molecular staging of SLNs is currently not recommended for solid tumors outside a clinical trial.

A particular challenge of using RT-PCR for the detection of SLN metastasis is the selection of appropriate molecular targets for a particular tumor type. For example, in the largest study to date to evaluate the usefulness of RT-PCR in melanoma [68], SLNs from 1446 patients in whom SLNs were negative for metastasis on H&E and immunohistochemical testing were evaluated using semiquantitative RT-PCR for the following genes:

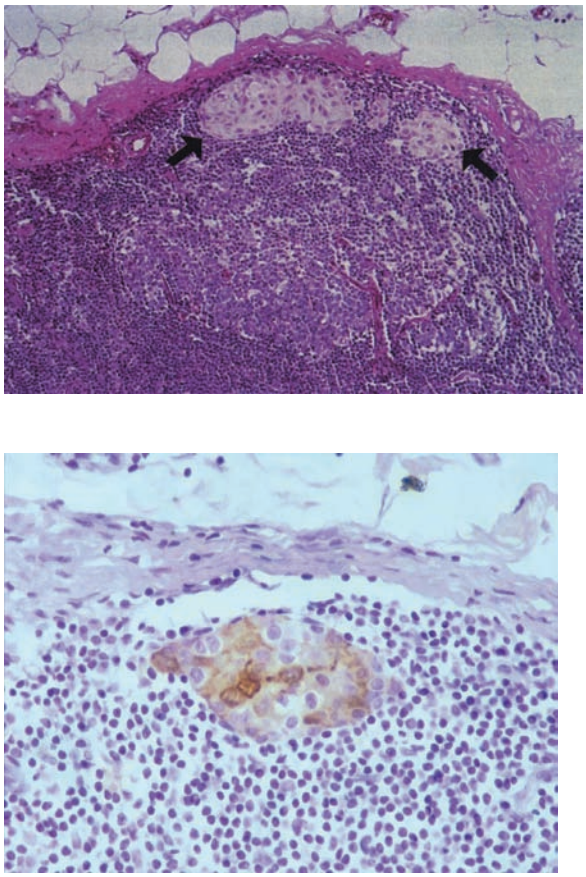

Figure 45.2. Sentinel lymph node metastases. (A) Metastases in SLN stained with H&E. Arrows denote SLN metastasis in subcapsular space. (B) Metastases in SLN stained with antibody against HMB-45 (aminoethylcarbazol, ×40). Light hematoxylin used as a counterstain. Note that not all melanoma cells are labeled with the antibody. [(A) From Gershenwald JE, Colome MI, Lee JE, Mansfield PF, Tseng C, Lee JJ, Balch CM, Ross MI. Patterns of recurrence following a negative sentinel lymph node biopsy in 243 patients with stage I or II melanoma. *J Clin Oncol* 16(6):2253–60, 1998. (B) Courtesy of Victor G. Prieto, MD, PhD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.]

tyrosinase, the rate-limiting enzyme in melanin biosynthesis, and the melanoma-associated genes *MART-1*, *MAGE-3*, and *gp100*. Although a subset of the SLNs in these patients demonstrated "molecular" evidence of melanoma (defined as positivity for tyrosinase and at least one of the other three markers), long-term follow-up demonstrated no difference in disease-free, distant disease-free, or overall survival between RT-PCR–positive and RT-PCR–negative patients. On the basis of these data, the authors concluded that RT-PCR provided "no additional prognostic information beyond standard histopathologic analysis of SLNs" [68]. In contrast, other smaller studies have shown a negative impact of RT-PCR positivity on recurrence and survival [65, 71–74]. This discrepancy has several potential explanations, including variations in marker selection,

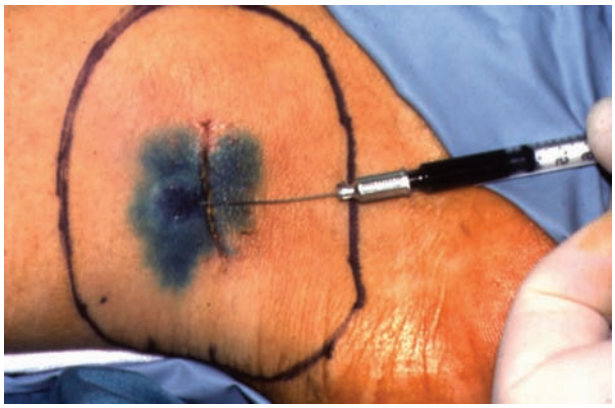

Figure 45.3. Intradermal injection of isosulfan blue dye around melanoma biopsy site. (Courtesy of Merrick I. Ross, MD Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

specimen processing, and RT-PCR methodology (e.g., contemporary approaches include use of quantitative real-time RT-PCR) and short follow-up that may not accurately allow for difference in survival to be detected between PCR-positive and -negative patients. This discrepancy in results between different studies highlights the effect changing technology may have on molecular detection of SLN metastases and the need for additional research before molecular analysis can be adopted as standard of care in SLN evaluation.

IDENTIFYING SLNs IN CLINICAL PRACTICE

SLNs are identified through lymphatic mapping and lymphoscintigraphy. Lymphatic mapping entails injecting a mapping agent, usually a blue dye, around a primary tumor and then tracking the flow of that agent through the tumor-draining lymphatics to SLNs. In addition, some patients undergo a preoperative lymphoscintigraphy, which involves injection of a radiocolloid into tissue, followed by dynamic monitoring of the movement of the radiocolloid over time from the tissue into the lymphatic vessels and ultimately to the lymph node(s) draining that tissue. This allows for the identification of the draining nodal basins and the general location and number of SLNs.

Early Experience: Use of Vital Blue Dye Only

In their initial report of lymphatic mapping and SLN biopsy in patients with cutaneous melanoma who had clinically uninvolved lymph nodes, Morton and colleagues used a vital blue dye to map the lymphatic system [24]. The dye was injected intradermally into the skin around the primary melanoma site (Figure 45.3). An incision was then made over the expected draining lymph node basin, and all blue lymphatic channels were then traced to the draining SLN (Figure 45.4). The SLN was removed and submitted for histopathologic analysis. With this technique, Morton and colleagues successfully identified the SLN in 194 (82%) of 237 lymphatic basins. Subsequently, several other investigators also showed that using a vital blue dye, the SLN could be identified in 82 percent to 94 percent of patients with melanoma [24, 75–78].

In 1994, Giuliano and colleagues extended the lymphatic mapping and SLN biopsy technique to patients with breast cancer [31]. They injected vital blue dye into the breast tissue surrounding the breast tumor, and after approximately five minutes, they made an incision in the ipsilateral axilla. There was a clear learning curve with the technique: early in their experience, the SLN was successfully identified in 66 percent of patients, but at the conclusion of their initial study, the SLN identification rate was 78 percent.

Several different blue dyes have been used, including 1% isosulfan blue and 1% methylene blue; their ability to reveal the SLN in melanoma appears very similar [79]. In breast cancer, most clinicians prefer to use 1% isosulfan blue, as 1% methylene blue has been associated with skin necrosis of the breast [12, 80].

Later Experience: Advantage of Using a Radiolabeled Colloid

One of the challenges with lymphatic mapping is determining to which lymph node basin the lymphatic vessels from a tumor drain. Whereas in many parts of the body the course of afferent lymphatic drainage to regional lymph node basins is generally predictable (e.g., the upper extremities drain to the axilla and the lower extremities drain to the groin), in other locations, such as the head and neck, trunk, and abdominal cavity, not only are lymphatic drainage patterns quite variable but drainage to multiple nodal basins is common [81]. Even in areas of the body in which lymphatic drainage is seemingly well defined, SLNs are sometimes

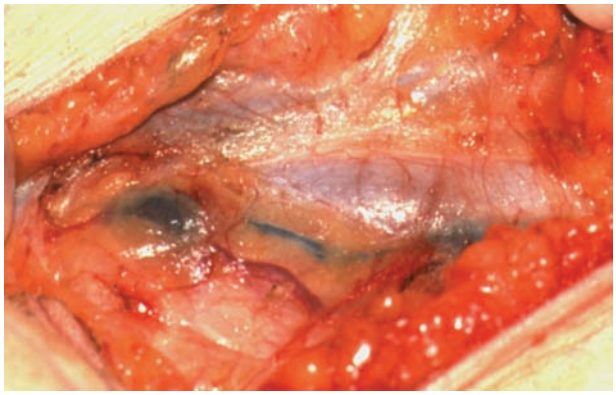

Figure 45.4. Early-experience example of relatively large incision required to identify afferent lymphatic leading to blue-stained sentinel lymph nodes in regional nodal basin before use of gamma probe (see also Figure 45.8). (Courtesy of Merrick I. Ross, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

identified in ectopic or unusual locations outside the traditional named nodal basins [81, 82]. The details of this issue are beyond the scope of this chapter; however, because of the frequency of SLNs in unusual locations, preoperative lymphoscintigraphy is widely used to facilitate identification of nodal basins at risk, and ectopic locations (Figure 45.5A–C).

Compared with vital blue dyes, radiocolloids offer several advantages: (1) the radiocolloid can be traced in real time with a handheld gamma probe, which facilitates the intraoperative detection of drainage to more than one basin as well as unusual drainage patterns (Figure 45.5B); (2) the gamma probe can be used to transcutaneously localize the precise location of the SLN(s), allowing for a smaller, directed incision to be made to retrieve the SLN; and (3) the gamma probe can be used to guide the surgeon intraoperatively to the SLN and confirm that the correct lymph nodes are removed.

Krag and colleagues performed preoperative lymphoscintigraphy using a ^{99m}Tc-labeled radiocolloid in patients with melanoma and a handheld gamma probe to identify the radioactive SLNs. Using this technique, they were able to identify the SLN in 98 percent of melanoma patients [83, 84]. They expanded the use to patients with breast cancer and were able to detect the SLN in 82 percent of patients. [33] This was a substantial improvement compared to the use of vital blue dye alone.

Successful use of radiocolloids to facilitate accurate detection of SLNs requires that the radiocolloid [1] efficiently enter the lymphatic system, (2) move through the afferent lymphatic vessels to the draining lymph node, (3) be retained in the SLNs, (4) distinguish SLNs from secondary-echelon or second-tier nodes (i.e., nodes that may stain "blue" or take up radiocolloid but are not really SLNs, because they receive drainage from the SLNs themselves, not the afferent lymphatics; Figure 45.6), and (5) accurately demonstrate all SLNs.

Of these requirements, perhaps the most important is efficient entry of the radiocolloid into the lymphatic system itself. Such entry is highly dependent on the size of the radiocolloid used. Particles smaller than 5 nm can enter into the vascular capillaries rather than the afferent lymphatics, whereas particles larger than 75 nm do not easily pass into the lymphatic system and tend to remain at the injection site. The ideal particle size is 5 to 75 nm. In Australia, for example, ^{99m}Tc-antimony sulfur colloid with a uniform particle size of 10 to 15 nm is preferentially used. In Europe, many centers use ^{99m}Tc-nanocolloid albumin with a slightly larger particle size variation – between 3 and 80 nm; approximately three-fourths of these particles are smaller than 30 nm. The specific radiocolloid used is dependent on the approval mechanisms in different countries [82]. In the United States, small-diameter radiocolloids are not approved for human use by the Food and Drug Administration, which has led to the use of ^{99m}Tc-sulfur colloid, which has a particle size range of 50 to 2000 nm and an average size of 300 nm. This size is not ideal for efficient lymphoscintigraphy; to improve on the uptake of the radiocolloid into the lymphatic capillaries, particles larger than 200 nm can be removed by filtering the solution through a 0.2-μm filter. Massaging the tissue at the radiocolloid injection site may also promote entry into the lymphatic system [82, 85].

Enhancing Clinical Utility: Evolution of the Combined-Modality Approach

In 1998, Gershenwald et al [78] reported on the early experience with SLN biopsy in 626 patients with primary cutaneous melanoma at the University of Texas MD Anderson Cancer Center. The SLN was identified in 87 percent of patients who underwent lymphatic mapping and SLN biopsy using 1% isosulfan blue dye alone, compared with 99 percent of patients who underwent lymphatic mapping and SLN biopsy with concomitant use of 1% isosulfan blue and ^{99m}Tc-labeled sulfur colloid. The superiority of a combined approach compared to using either method alone has also been shown by others in melanoma and breast cancer [34, 78, 86, 87]; currently, most centers favor using a combined-modality approach. In experienced hands, this allows for detection of the SLN in more than 98 percent of melanoma patients [78, 86] and more than 95 percent of breast cancer patients [34, 87].

DETAILS OF SLN BIOPSY FOR MELANOMA AND BREAST CANCER

Lymphatic mapping and SLN biopsy are currently performed as a staging tool, primarily in patients with melanoma, selected high-risk non-melanoma skin cancers, and breast cancer. The technique for skin-based primary tumors differs somewhat from that for breast tumors, and specific protocols also vary between centers. Regardless of the tumor type and center, successful SLN identification and analysis requires a multidisciplinary collaboration among experts in nuclear medicine, surgery, and pathology.

Melanoma and High-Risk Non-melanoma Skin Cancers

In cutaneous melanoma, patients with a Breslow tumor thickness of at least 1 mm and subsets of patients with tumor thickness less than 1 mm are routinely offered SLN biopsy. Patients with high-risk non-melanoma skin cancer are also commonly offered SLN biopsy, although clinical practice varies widely in this regard. The contemporary approach to lymphatic mapping and SLN biopsy for cutaneous melanoma and non-melanoma skin cancers generally consists of three

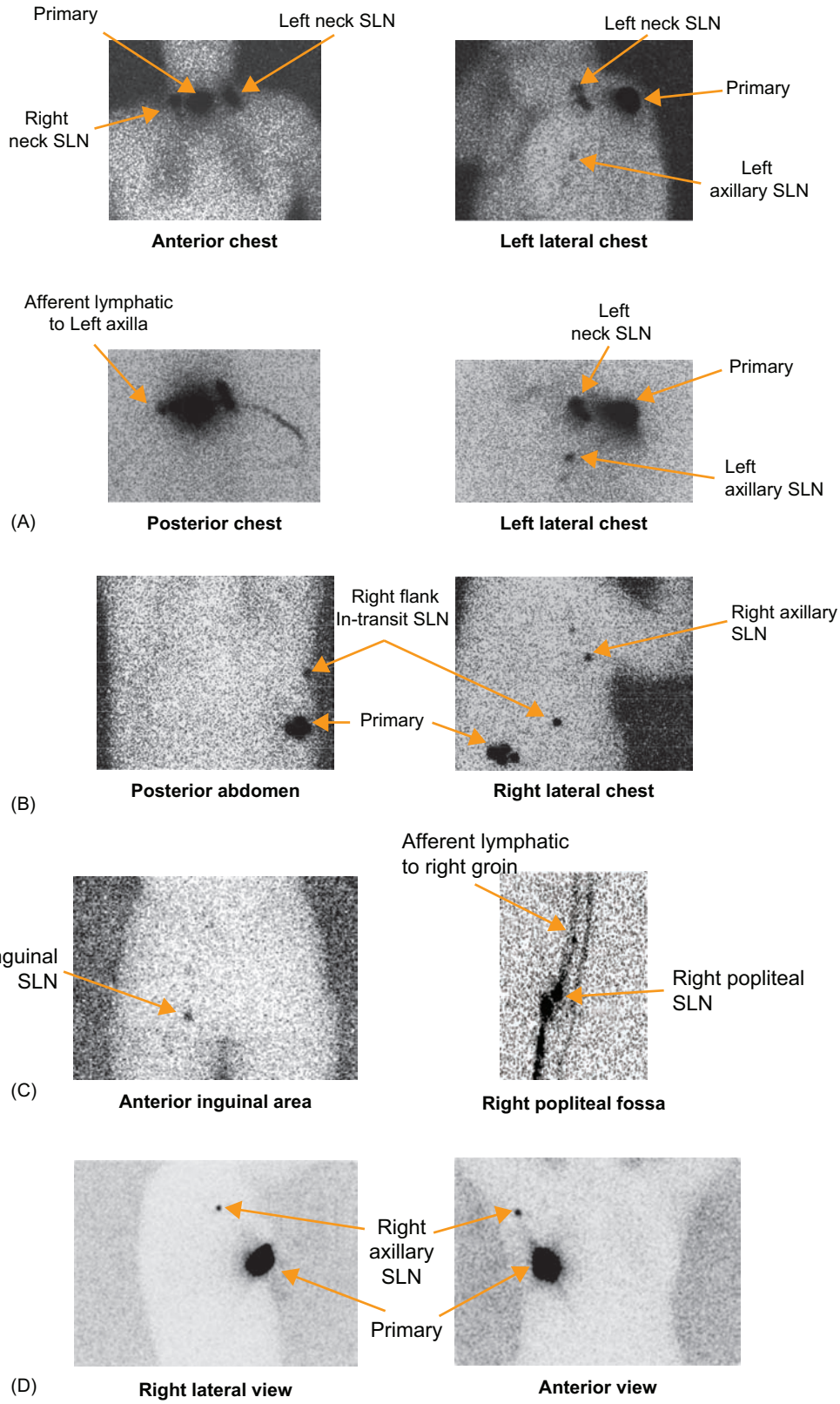

Figure 45.5. Preoperative lymphoscintigraphy. After injection of technetium 99m (^{99m}Tc)-labeled sulfur colloid at the primary cutaneous melanoma site, preoperative lymphoscintigraphy reveals drainage to (**A**) multiple nodal basins (bilateral neck and left axilla) from a tumor of the upper midline back; (**B**) "in-transit"/ectopic SLNs in the right flank region and right axilla from a primary tumor of the right lateral back; and (**C**) SLNs in a right lower extremity popliteal fossa lymph node basin as well as a right inguinal lymph node basin from a primary tumor of the heel. (**D**) Breast lymphoscintigraphy demonstrating drainage to ipsilateral axilla (anterior and lateral views) following injection of technetuium 99m (^{99m}Tc)-labeled sulfur colloid at primary breast cancer site. (Courtesy of Jeffrey E. Gershenwald, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

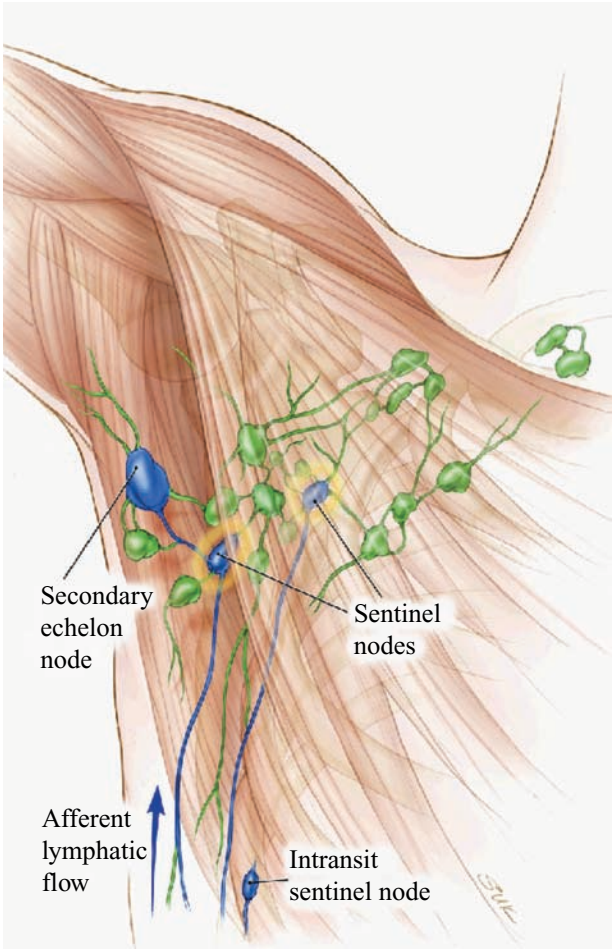

Figure 45.6. Schematic demonstrating afferent lymphatic vessels leading to true sentinel lymph nodes (SLNs) deep within the axilla. Note focal radiotracer uptake (yellow halo effect from ^{99m}Tc-labeled sulfur colloid injection) in SLNs but not secondary-echelon (i.e., second-tier) lymph nodes situated more superficially. (Courtesy of Jeffrey E. Gershenwald, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

principal components: (1) preoperative lymphoscintigraphy with a radiocolloid to identify the regional nodal basins at risk and the general location and number of SLNs within the basins; (2) intraoperative lymphatic mapping performed using a blue dye, identification of the SLN with the aid of a handheld gamma probe, and excisional biopsy of all SLNs in all regional basins at risk; and (3) careful pathologic evaluation of the SLNs.

In the United States, 0.4 to 1.0 mCi of ^{99m}Tc-labeled sulfur colloid is injected intradermally at four different points around the primary cutaneous melanoma either the day before or the day of surgery by members of the nuclear medicine or surgical team. The radiocolloid enters into the lymphatic system and travels to the SLNs draining the specific part of the skin. To track the flow of the radiocolloid, dynamic imaging is performed with a gamma detector after radiocolloid injection. The time it takes for the radiocolloid to reach SLNs varies among patients, but in most cases the SLN is reached within thirty minutes. However, in some patients with drainage to multiple lymph node basins, it may take more than an hour for the tracer to reach the SLNs in some nodal basins.

After the patient is asleep in the operating room, 1 to 5 ml of vital blue dye (usually 1% isosulfan blue or 1% methylene blue) is injected intradermally around the primary melanoma. Drainage of the vital blue dye to the SLN is quite rapid – uptake is usually visible in the SLNs within several minutes (Figure 45.7). Using a handheld gamma probe, the surgeon detects the radiocolloid in the SLN transcutaneously and then makes an incision in the skin overlying the SLN (Figure 45.8A–B). A SLN is defined as any lymph node that is radioactive and/or blue. The SLN is then surgically excised and sent to the pathologist for serial sectioning and histopathologic analysis. Importantly, intraoperative use of the gamma probe greatly facilitates identification of additional SLNs in the nodal basin, thus contributing to accurate identification of all SLNs in a given regional nodal basin (Figure 45.8C–D). In some centers, lymph nodes that are neither radioactive nor blue but that on palpation seem suspicious may be routinely removed.

Breast Cancer

In breast cancer, drainage from the breast tumor is primarily to the axilla, but depending on the specific location of the tumor within the breast, drainage may sometimes be observed to the internal mammary lymph nodes, supraclavicular nodes, intramammary interval nodes, and interpectoral nodes [82]. In contrast to the approach used by most centers for patients with melanoma, preoperative lymphoscintigraphy is not routinely performed for patients with breast cancer

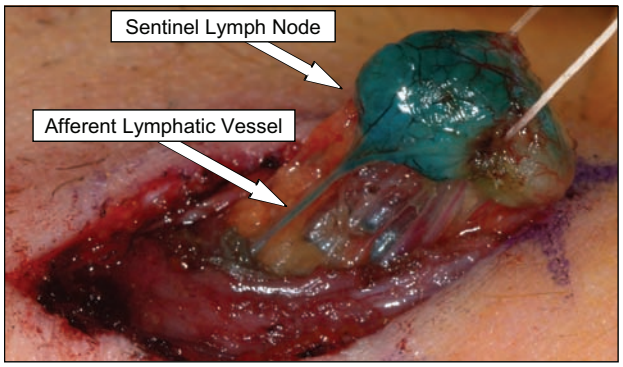

Figure 45.7. Intraoperative identification of afferent lymphatic vessel and SLN. Injection of a vital blue dye around the tumor leads to uptake of the dye by the lymphatic system and transport of the dye to the draining regional nodal basins, thereby allowing for identification of SLNs. Note isosulfan blue-stained afferent lymphatic vessel leading to blue-stained SLN. (Courtesy of Jeffrey E. Gershenwald, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

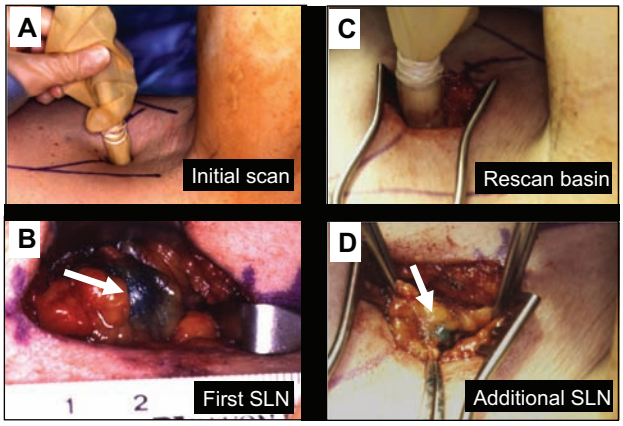

Figure 45.8. Use of gamma probe to locate and ensure removal of all SLNs from a lymph node basin in a patient with melanoma. Following preoperative intradermal injection of both technetium 99m-labeled sulfur colloid and 1% isosulfan blue dye, (A) a gamma probe is used to transcutaneously localize areas of increased focal radiotracer uptake (corresponding to SLNs) in the regional nodal basin. (B) Using a small, directed incision overlying this area, the surgeon identifies and harvests a blue-stained SLN. (C) After this first SLN is harvested, rescanning of the basin reveals an additional area of increased focal radiotracer uptake. (D) An additional blue-stained SLN is identified and harvested. (Courtesy of Jeffrey E. Gershenwald, MD. Copyright retained by the author and the University of Texas MD Anderson Cancer Center.)

in most centers. Thirty minutes to four hours before the planned surgery, 0.4 to 0.6 mCi of filtered ^{99m}Tc-labeled sulfur colloid in a volume of 5 to 6 ml is injected at four different points around the primary breast tumor or biopsy site. If the tumor is not easily palpable, ultrasonography can be used to localize it. In some centers, the sulfur colloid is injected not around the tumor but instead just deep to the areola or within the skin around the areola. In the operating room, when the patient is asleep, 3 to 5 ml of vital blue dye (usually 1% isosulfan blue) is injected at four different points around the tumor or biopsy site and the breast is massaged to promote entry of the radiocolloid and the vital blue dye into the peritumoral afferent lymphatics. From this point forward, the intraoperative approach to identifying SLNs is similar to that for melanoma. However, there is one important difference. In melanoma, immediate intraoperative analysis of SLNs with frozen section examination is not routinely performed because it is difficult to accurately detect melanoma SLN metastases by frozen section technique. In breast cancer, in contrast, metastases in SLNs can frequently be detected by frozen section examination; frozen section examination is therefore employed in most centers. If breast cancer SLN metastases are identified intraoperatively by frozen section pathologic analysis, the patient usually undergoes a level I and II axillary lymph node dissection during the same operation.

CLINICAL IMPLICATIONS OF SLN METASTASES

Numerous studies have provided significant insight into the clinical relevance of SLN metastases in patients with cancer. To introduce some of the relevant concepts, we will focus on the clinical implications of SLN metastases in melanoma and breast cancer.

Melanoma

In melanoma, SLN status is a critical determinant of disease stage and has been shown to be the most important predictor of survival in patients with clinically negative lymph nodes [78, 88–92]. The five-year survival in patients without SLN metastasis is 88 percent to 93 percent, whereas it decreases to 51 percent to 67 percent in patients with the same tumor thickness and evidence of SLN micrometastatic disease [92]. Moreover, patients with metastatic disease have a higher risk of cancer recurrence. Knowing whether a patient has microscopic spread of disease to the SLN also enables clinicians to recommend additional treatment, such as completion lymphadenectomy (i.e., removal of the remainder of the lymph nodes in the regional nodal basin in which at least one SLN was positive).

Completion lymphadenectomy following early identification of micrometastatic nodal disease using the SLN biopsy approach may have a significant impact on survival. In the prospective Multicenter Selective Lymphadenectomy Trial, Morton and colleagues [52] randomized patients with clinically node-negative primary melanoma to a wide local excision of the primary melanoma and nodal observation or wide local excision and SLN biopsy. If the SLN showed evidence of metastatic disease, patients then underwent completion lymphadenectomy. Data presented after the third of five planned interim analyses indicated that patients who underwent completion lymphadenectomy following a positive SLN biopsy had a significantly improved five-year survival compared with patients in the observation arm who developed clinically palpable metastatic melanoma in their regional nodal basin and underwent completion lymphadenectomy only after clinical metastasis was confirmed (72% vs. 52%, $p = 0.004$). This apparent survival advantage for the SLN-positive group provides evidence that early intervention in the nodal basin in patients who actually have regional nodal disease is clinically important. Interestingly, in patients with melanoma, the amount of metastatic disease (i.e., tumor burden) in the SLN has been shown to predict the risk of involvement of non-SLN lymph nodes in the lymph node basin, the risk of tumor recurrence, and survival [93–95].

SLN biopsy also identifies patients with micrometastatic lymph node disease who may benefit from adjuvant systemic treatments to reduce the risk

of melanoma recurrence after surgery. Interferon alfa-2b, an immunomodulatory agent, is currently the only approved adjuvant treatment for lymph node metastatic melanoma in the United States. The ECOG E1684 study randomized patients to observation versus high-dose interferon alfa-2b for 1 year. Compared with observation, interferon alfa-2b increased the five-year recurrence-free survival from 26 percent to 37 percent and the five-year overall survival from 37 percent to 46 percent [96]. Other studies have also found that interferon alfa-2b can increase recurrence-free survival and overall survival [97]. However, other studies have not confirmed the overall survival benefit in patients receiving adjuvant interferon alfa-2b [98, 99]. The effect of adjuvant interferon alfa-2b in patients with micrometastatic lymph node disease is currently under investigation in the ECOG E1697 clinical trail.

Breast Cancer

In breast cancer, SLN metastasis is also associated with a worse prognosis. According to the sixth edition of the American Joint Commission on Cancer staging system, lymph node metastasis in breast cancer is classified as follows: pN0(i+), metastasis 0.2 mm in diameter or less; pN1mi, metastasis >0.2 mm but less than 2.0 mm; and pN1, metastasis >2.0 mm [100]. In a large study of 2408 patients with invasive breast cancer, Cox and colleagues [101] found significantly worse disease-free and overall survival in patients with a pN1mi SLN metastasis than in patients with no metastasis. Increasing tumor burden in the SLN was also associated with an increased risk of non-SLN lymph node metastasis. Overall survival in patients with pN0(i+) disease was not significantly different from that of patients with no SLN metastasis. However, among patients with pN0(i+) disease, patients who did not undergo a completion lymph node dissection had significantly worse survival than patients who did. Based on these data, a completion lymphadenectomy is recommended in patients with SLN metastasis in breast cancer. Other ongoing studies also address the role of completion lymphadenectomy in patients with SLN metastasis.

The presence of SLN metastasis in breast cancer has less of an impact on adjuvant treatment (chemotherapy and hormonal therapy) than in melanoma, as many patients with breast cancer 1.0 cm or greater without lymph node metastasis receive adjuvant treatment regardless of SLN status.

FUTURE DIRECTIONS: POSSIBLE ALTERNATIVES TO SLN BIOPSY

Although the morbidity associated with SLN biopsy is low, a noninvasive method of diagnosing early regional lymph node metastasis would be welcome, as it would obviate the need for any surgical lymph node evaluation procedure. Noninvasive methods that have been studied include preoperative ultrasonography, 2-fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET), and other imaging approaches.

Preoperative ultrasonography has been explored in several studies. Importantly, ultrasonography is user-dependent, and the ability to detect lymph node metastasis is determined by both the equipment used and the expertise of the ultrasonographer. In selected specialized centers, tumor metastases measuring 2 to 4 mm in diameter have been detected in SLNs, and ultrasonography is clearly superior to palpation for the detection of small nodules. However, ultrasonography has not been shown to be as sensitive as SLN biopsy followed by histopathologic evaluation of the SLN(s), which still represents the "gold standard" for evaluation of the regional nodal basin in at-risk patients with clinically node-negative melanoma and breast cancer [102]. A newer technique using ultrasound microbubble contrast agents has, at least in animal studies, been shown to increase the ability to detect smaller SLN metastases [103].

FDG-PET detects fluorine 18-labeled FDG. FDG is an analog of glucose that is taken up by high-glucose-using cells such as cancer cells and brain and kidney cells, as well as inflammatory cells. FDG-PET imaging has become a very useful tool for the diagnosis of distant metastatic disease for many cancers. However, numerous studies have demonstrated that it has little role in detecting SLN metastasis, principally because of low sensitivity in detecting such disease (i.e., less than 20% in most series) [104].

Computed tomography and magnetic resonance imaging have similarly not been shown to be useful in detecting SLN metastasis [104, 105].

Newer techniques using fluorescent compounds such as fluorescent cobalamin (vitamin B_{12}) and indocyanine green fluorescence have been shown to detect SLNs in animal models and in human breast and gastric cancers [106–109]. SLNs can also be detected in esophageal, gastric, lung, and breast cancer using near-infrared fluorescence technology and quantum dots [110–115]. The fluorescence and infrared technologies are currently still experimental but offer potentially new exciting methods of detecting SLNs and possibly SLN metastasis. Whether they will facilitate detection of micrometastatic disease in SLNs without their surgical removal is still unknown.

SUMMARY AND CONCLUSIONS

Lymph node metastasis is one of the most common forms of tumor spread and is one of the strongest predictors of prognosis in patients with clinically

node-negative cancers. The technique of lymphatic mapping and SLN biopsy has revolutionized the treatment of such patients, particularly in melanoma and breast cancer. Key benefits of lymphatic mapping and SLN biopsy include (1) accurate regional nodal staging (particularly because of the ability to perform enhanced histologic analysis on a limited specimen, generally comprised of only two or three SLNs per basin), (2) reduced morbidity compared to the morbidity of performing complete lymphadenectomy in all clinically node-negative patients, (3) potential therapeutic benefits, and (4) possible survival benefits associated with early identification and removal of microscopic regional nodal disease. Consequently, lymphatic mapping and SLN biopsy have become the standard of care in many patients with clinically node-negative melanoma and breast cancer. Although noninvasive approaches to identifying microscopic regional lymph node disease have been explored, none currently has the sensitivity to supplant lymphatic mapping and SLN biopsy. Further improvements in technology will be required before an entirely noninvasive approach to evaluating the status of the regional lymph nodes enters the clinical arena.

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46 Molecular Imaging and Metastasis

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With the advancement in modern genomic and proteomic technologies in the past decade, knowledge of the molecular and cellular mechanisms of cancer initiation and progression is expanding at an unprecedented rate. A prudent approach for clinicians and scientists would be to extract salient information and apply it to address significant challenges in the current practices of cancer management. An important issue is how best to query the molecular and physiological information relevant to cancer in patients. Molecular imaging is a particular useful technology in the pursuit of this quest, as it allows the visualization of critical molecular signaling pathways in action in living subjects, in a noninvasive and longitudinal manner. Metastasis, manifested often in the late stages of cancer (although most work today supports metastasis as an earlier event than previously recognized), is the main cause of mortality in patients with solid tumors. To be able to prevent or control metastasis is considered one of most significant challenges in clinical oncology.

Whole-body in vivo molecular imaging is ideally suited to assess the very complex process of metastasis, in which the location(s) and magnitude of disseminated lesions are changing in time. For cancer metastasis, the common imaging modalities employed for repetitive, noninvasive imaging include positron emission tomography (PET), computed tomography (CT), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), and optical imaging by bioluminescence (e.g., firefly luciferase [FL or Luc]) or fluorescence (e.g., green fluorescent protein [GFP]).

Each imaging modality has its strengths and limitations in regard to molecular imaging applications. Because of tissue absorption and scattering of low-energy photons, the optical methods using bioluminescence and fluorescence are better suited for in vivo imaging of small animals.

PET and SPECT employ radioactive tracers that emit positrons and gamma rays and are used widely in molecular imaging involving novel radioactive molecular tracers. However, they differ in the detection instrumentation. The PET scanner uses a ring of detectors to capture two directly opposite high-energy gamma rays emitted during the positron annihilation process, and computer processing of the two coincident signals are used to reconstruct a three-dimensional image. The principal detection unit in SPECT produces a two-dimensional planar image from the gamma-emitting isotopes that can be rotated around the subject to construct tomographic images. Because PET captures more events than SPECT, the resolution of the images and the level of detection of PET are more sensitive. For instance, in clinical oncological studies, FDG-PET uptake of the radiolabeled tracer probe ^{18}F-FDG (^{18}F–2-deoxy-2-fluoro-D-glucose) in the order of 1 fmol/L [1] is sufficient for tumor detection. CT and MRI especially afford excellent anatomical information on the subject, but their ability to reveal molecular signaling information is more limited.

Because no single modality can capture all the desirable imaging capabilities, the emergence of combinations of two technologies, such as PET-CT and PET-MRI, is gaining momentum to improve the precision of visualization of signals. Given the unique strengths, limitations, and imaging physics behind each imaging modality, a comprehensive discussion of all of these imaging platforms and their applications is beyond the scope of this chapter. We refer the interested reader to several current reviews that cover the principles of molecular imaging and their application in oncology [2, 3].

In the past two decades, significant progress has been achieved in the applications of molecular imaging techniques in clinical oncology, with the use of several modalities, such as CT, MRI, PET, and SPECT, being the mainstays of modern cancer treatment. However, timely detection of metastasis via imaging still remains challenging because of factors such as limited

TABLE 46.1. Common imaging reporter genes used in cancer research

Imaging modality	Reporter gene	Probe/substrate	Applications
Optical	Fluorescent proteins: GFP, RFP, etc. Bioluminescence proteins: luciferases (firefly, renilla, gaussia)	None D-luciferin, coelenterazine	Cellular and microscopic imaging; IR better for spectral in vivo imaging [52, 54]. Cell marking, small animal *in vivo* imaging [129].
MRI	Mutant transferrin receptor, ferritan	MION-transferrin	In vivo gene transfer, preclinical setting [130].
Radionuclide	Somatostatin receptor (SSTR) Na/I symporter Dopamine D2 receptor HSV1-tk	^{99}Tc or ^{111}In SSTR analogs $^{124, \text{ or } 131}$I-NaI, ^{99}Tc pertechnetate ^{18}F-FESP ^{18}F-GCV, ^{18}F-FHBG; $^{124 \text{ or } 131}$I-FIAU	In vivo tumor gene transfer, preclinical models [131, 132]. Imaging and radiotherapy capability, preclinical models [131]. Preclinical in vivo imaging [133]. Tested in preclinical and clinical settings [134, 135].

The imaging modality, probe, and/or substrate and application suited for a particular reporter gene are listed. In general, optical imaging approaches are more suitable for cellular and small animal imaging, whereas radionuclide imaging can be used in both preclinical and clinical settings.

instrument resolution and sensitivity and the lack of suitable imaging markers for resolving metastatic lesions. This chapter focuses on ongoing research efforts in developing improved molecular imaging applications in cancer and metastasis. We focus primarily on optical and PET imaging strategies. The chapter is further divided into four categories of tracer technology, covering (1) reporter gene imaging, (2) nanoparticle-based tracers, (3) antibody-based approaches, and (4) small-molecule imaging tracers. These categories are arranged in the order in which they evolved, transitioning from research-oriented applications toward current clinically utilized technologies.

IMAGING APPROACHES USING REPORTER GENES IN PRECLINICAL MODELS

Reporter genes have been widely used in molecular imaging applications to study metastasis. A list of commonly used imaging reporter genes, outlining each gene's enabling substrate or tracer, and some of the applications are summarized in Table 46.1. These genes include the bioluminescence reporter FL, renilla luciferase (RL), GFP, red fluorescent protein (RFP), the recently developed near-infrared fluorescent protein (IFP), the PET reporter gene HSV1-thymidine kinase (HSV1-tk), the sodium/iodine symporter (NIS), and the somatostatin receptor SSTr2 for SPECT imaging (see Table 46.1 and the references therein). Investigations of MRI reporter genes have also recently begun. A majority of these reporter genes are used to genetically mark tumor cells, often via viral gene transfer vectors. However, because of safety and immunogenicity issues related to the viral vectors, the practice of reporter gene imaging is limited largely to preclinical studies in animal models.

The development of molecular imaging technologies for oncological research has accelerated at a rapid pace in the past decade. In this section we highlight three strategies of reporter gene applications that are relevant to metastasis research: (1) the use of tumor cells stably marked with reporter genes to track their in vivo expansion and dissemination, (2) exploiting luciferase protein domain complementation to engineer imaging reporter system to spy on critical cellular signaling, and (3) the use of viral-vector–based transcriptional targeted expression of imaging reporter genes to detect metastasis in preclinical models. Readers interested in the science of reporter genes based on molecular imaging are recommended to consult the recently published book on this subject [4].

Tumor Cells Marked with Imaging Reporter Genes

One of most widely used approaches to study the metastasis process in living animals is to engineer tumor cells with a stable label by an optical reporter gene such as FL or GFP, which enables the noninvasive, repetitive detection of primary tumors and disseminated lesions in an individual animal over time. Some of the strengths and limitations of this application of bioluminescence imaging (BLI) are shown in Figure 46.1. The low background activity and the ease of use of luciferase-based BLI are key advantages of this modality. However, because of light absorption and scattering by tissues, BLI is semiquantitative and does not allow three-dimensional localization of the signal. To overcome this inherent limitation of optical imaging, we and others use ex vivo imaging of isolated organs to provide better localization and quantification of the signals emanating from the disseminated tumor cells (Figure 46.1B). Moreover, in our experience, the

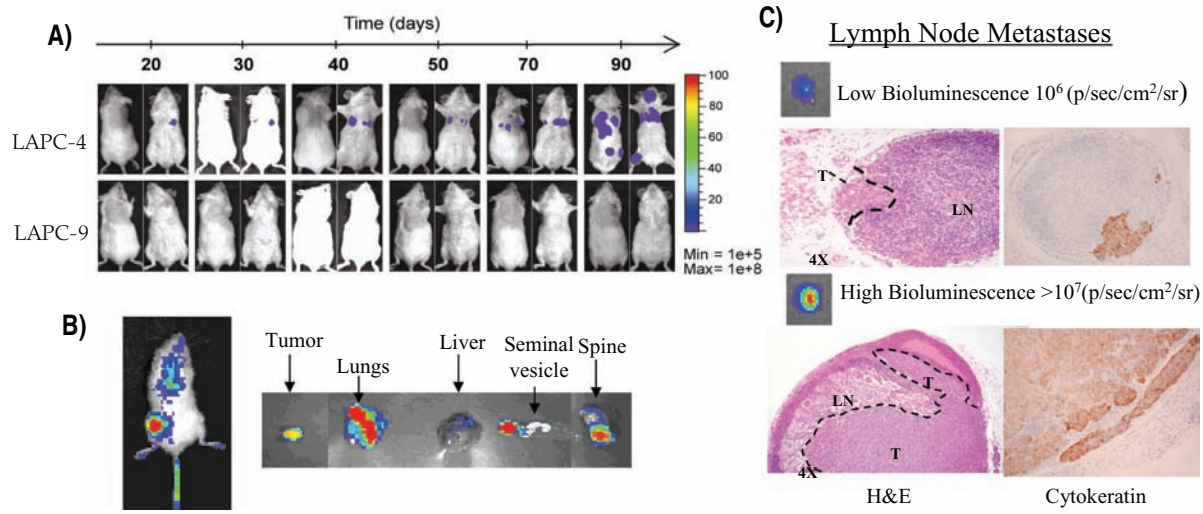

Figure 46.1. Strengths and limitations of bioluminescence imaging (BLI) to monitor metastasis. (A) The use of BLI to assess metastatic potentials of two prostate xenograft models (LAPC-4 and -9) longitudinally over time. The ease of use and low background of luciferase reporter genes are clear advantages. (B) BLI provides a two-dimensional semiquantitative signal, which is difficult to localize in a metastatic setting. Imaging of harvested organs ex vivo can aid in analyzing the magnitude and localization of tumor spread. (C) The magnitude of bioluminescence signals in isolated organs correlates well with volume of metastasis, as assayed in the lymph node metastasis. These results are adapted from ref. [136].

bioluminescence signal intensity of the isolated organs correlates well with the volume of metastases, as assayed by conventional histological means (Figure 46.1C). The main advantage of the BLI approach is its ability to provide a rapid computation assay of metastatic volume, which saves a significant amount of time and effort compared with complete histological analyses of the tissue.

As noted previously, each reporter gene imaging modality has its own strengths and weaknesses. PET reporter-gene–based imaging is superior to optical imaging in achieving quantitative three-dimensional localized signal detection. As shown in Figure 46.2A, PET signals emanating from the prostate tumor can be distinguishable from the closely situated bladder signal. The inability for a single imaging modality to achieve optimal detection of metastasis has encouraged researchers to adopt the approach of coupling different imaging reporter genes together to achieve complementary imaging results.

Three successful strategies have been applied to link two or more reporter genes together in a single genetic expression unit. The first, and most common, approach is the bicistronic method, which involves

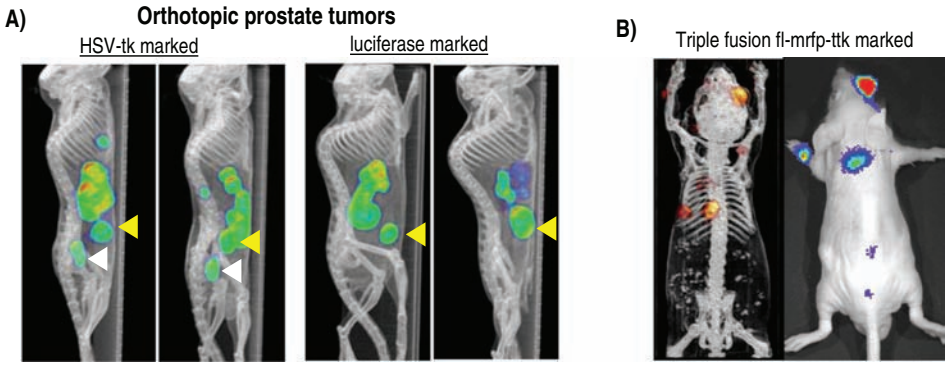

Figure 46.2. Strengths and limitations of the HSV-tk PET reporter gene. (A) HSV-tk marked prostate tumors can be imaged by its specific tracer F18-FHBG, denoted by the white arrowhead. This prostate tumor PET signal is lacking in luciferase (control) marked tumors. The quantitative, three-dimensional localized PET signals enable the clear separation from the bladder signal (yellow arrowhead), owing to excreted tracer accumulated in the bladder. Nonspecific uptake of this tracer in the GI track is observed. (B) Marking tumors with a multimodal triple fusion gene fl-mrfp-ttk, encoding firefly luciferase linked to monomeric RFP and truncated HSV-tk component, enables the visualization of metastasis with both PET and BLI modality in this B16 melanoma-bearing animal.

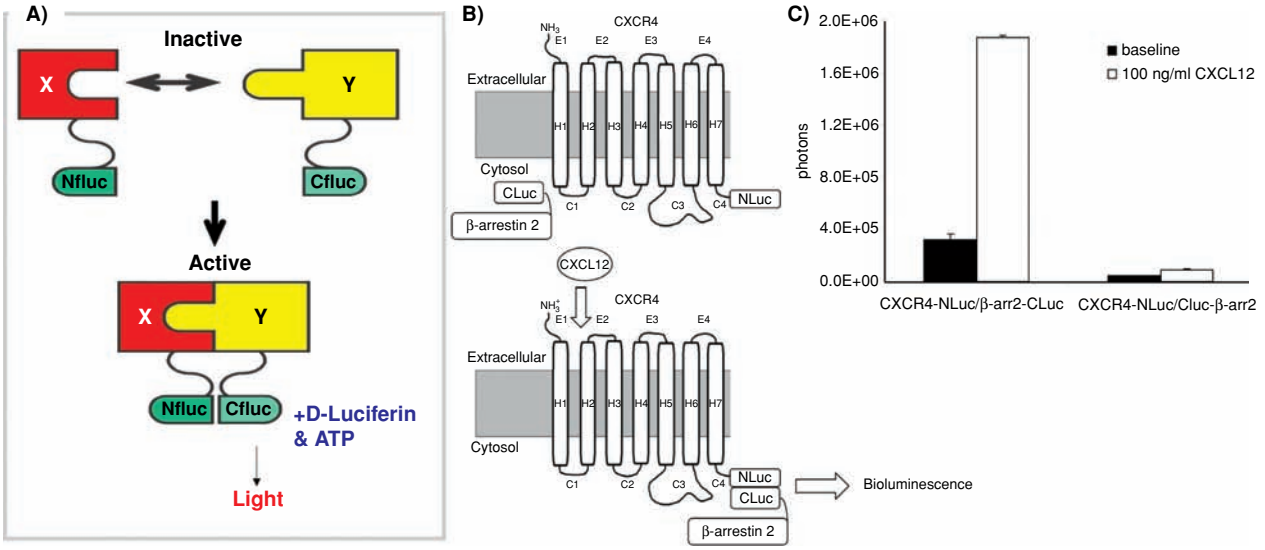

Figure 46.3. Luciferase complementation to report pathway activation in vivo. (A) The concept of split reporter strategy. N- and C-terminal luciferase protein segments can be linked to a partner of a interacting pair of proteins (X, Y). The approximation of the N- and C-luciferase segment brought on by the binding of protein X and Y reconstitutes the bioluminescence activity of intact luciferase. (B) The application of this split luciferase reporter strategy to CXCR4 and its interactive partner b-arrestin. Upon activation by the its chemokine ligand CXCL12, the signaling cascade initiates the pairing of the reporter complementation system, resulting in bioluminescence production. (C) Demonstration of the chemokine responsive activation of bioluminescence production in cells bearing the proper configured CXCR4-Nluc, and b-arr2-Cluc, presumably, joining the Cluc fragment to the N-terminal of b-arrestin intefers with the protein-protein interaction. (Adapted with permission from refs. [9] and [19].)

the incorporation of an internal ribosome entry site (IRES) sequence between two genes. Under the control of a single promoter, both genes are transcribed into a single mRNA but translated into two proteins directed by the IRES sequence [5]. A second bidirectional approach uses a centrally located enhancer element to drive expression of two genes in opposite orientation. For instance, centrally located tetracycline-responsive regulatory elements can direct the coexpression of HSV1-tk and the dopamine receptor D2R gene [6]. The third approach relies on the creation of an effective fusion protein that consists of preserved functional domains of the parental imaging reporter proteins. The first successful example is a fusion gene that combines the PET/fluorescence capability of HSV1-tk and GFP [7]. Further extension of this concept resulted in the creation of a series of triple fusion reporter genes that enable simultaneous use of three imaging reporter functions of bioluminescence, fluorescence, and PET, such as the fl-mrfp-ttk construct [8]. An experimental metastasis model of melanoma was established by systemic injection of B16 murine melanoma cells marked with this triple fusion gene (Figure 46.2B). Metastatic lesions can be visualized simultaneously by microPET and BLI. These triple fusion proteins were designed to maximize the ability to interrogate biological events spanning a multitude of length scales, from single cell measurements by in vitro fluorescence microscopy to multicellular detection in living animals by in vivo bioluminescence and PET imaging. As such, the application of the triple fusion reporter approach holds enormous promise for the study of metastasis. However, an unresolved concern is the stability of these large multidomain artificial proteins, which could hinder the efficient long-term application of this very powerful concept.

Luciferase Protein Complementation Reporter System

Successful metastatic spread requires tumor cells to sense and act in response to their environmental stimuli. The tumor cells will integrate external stimuli and propagate internal signals to execute specific cellular functions. Molecular imaging approaches that enable the visualization of the complex intracellular signal transduction program in the cancer cell will be instrumental to unravel the critical regulatory mechanisms of the metastatic cascade. Paulmurugan et al. first reported the use of a "split protein" strategy with FL to image critical protein–protein interactions along signal pathways [9]. This approach was inspired by earlier studies that described the protein-fragment complementation assay, in which splitting an enzyme into two fragments abolishes its function, whereas approximation of the two fragments together restores the enzymatic activity [10, 11]. The premise of the split luciferase protein strategy to image protein–protein interaction is illustrated in Figure 46.3A, and several groups have further substantiated the feasibility of

this strategy for both FL and RL [12, 13]. Here, we describe two representative examples of use of the split luciferase strategy to report the functional status of critical signaling pathways in cancer metastasis.

Chemokines belong to a family of small, secreted proteins that are considered to be the environmental cues that are critical in regulating immune cell trafficking and hematopoietic cell mobilization from bone marrow [14]. Chemokine receptor CXCR4 belongs to the family of seven transmembrane G-protein–coupled receptors, and it mediates cytoskeletal rearrangement and cell migration upon activation by its cognate ligand CXCL12 or SDF-1 [15]. Normal physiological functions of CXCR4-signaling are often coopted by cancer cells. The evidence supporting CXCL12–CXCR4 signaling promoting metastasis is particularly evident in breast cancer [16] but is also implicated in other malignancies. CXCR4 expression was detected in 67 percent of breast cancer lesions in bone in one study [17] and is associated with shorter disease-free survival and increased risk of recurrence [17, 18].

Luker et al. [19] applied the split luciferase complementation approach to develop a BLI system that reports the activation of CXCR4 signaling axis. Binding of CXCL12 to CXCR4 results in phosphorylation of the intracellular C-terminus of the receptor by protein kinase C and G-protein–coupled receptor kinases (GRKs), which then in turn recruits cytosolic scaffolding protein β-arrestin 2 to the receptor [20]. As shown in the schematic of Figure 46.3B, the juxtaposition of the two fusion reporter constructs, CXCR4-N-luc and β-arrestin 2-Cluc, upon CXCL12 activation, leads to the reconstitution of bioluminescent activity. A sevenfold induction of bioluminescent signal was observed in cells expressing the two correct constructs (Figure 46.3C). Incorporating such a CXCR4 reporter system into tumor cells will provide a real-time monitoring of functional activation of this chemokine signaling axis during the metastasis process. This is just one of many innovative luciferase complementation systems that have been developed to identify critical growth regulatory pathways. Interested readers may find more comprehensive coverage of this topic in recent reviews [4, 21].

Transcriptional-Targeted Imaging Using Tissue- and Tumor-Specific Promoters

Transcriptional-targeted or gene-expression–based imaging has emerged as a facile and flexible approach for studying signaling pathways during tumor progression in live animals [4]. In transcriptional-targeted imaging, a pathway- or cell-specific promoter is placed upstream of an imaging reporter gene to drive its expression; this reporter cassette is introduced into tumor cells in a live animal either by genetically engineered transgenic methods or by using viral gene delivery vectors. Transcriptional-targeted imaging has been successfully demonstrated for specific pathways such as TGF-β, p53, HIF1α, or NF-κB [4] and several tissue- and tumor-specific promoters [4]. However, the transcriptional potency of specific promoters is often much weaker relative to constitutive viral promoters, leading to inefficient expression and limited imaging capability. Hence, our research group has developed an amplification method termed the *two-step transcriptional activation* (TSTA) system, which activates a strong transcriptional activator that induces an imaging reporter gene [22]. The functionality of the TSTA system is illustrated with a prostate-targeted adenovirus. In Figure 46.4A, the genomic organization of this prostate-specific recombinant adenovirus, AdTSTA-tk, is shown. In this virus, a modified prostate-specific antigen (PSA) promoter was used to control the expression of the synthetic Gal4VP16 transcription activator, which is composed of the strong VP16 transactivation domain fused to the Gal4 DNA-binding domain (Gal4DBD). Gal4-responsive elements were placed upstream of the promoter that drives the expression of an imaging reporter gene. In doing so, Gal4VP16 protein can strongly transactivate the expression of the imaging reporter genes, such as HSV1-tk or the FL gene [23]. The potency of gene expression of AdTSTA-tk is very comparable to the constitutive cytomegalovirus (CMV) promoter-driven vector (AdCMV-tk) in prostate cancer cell lines (Figure 46.4B). A clear distinction is that the prostate specificity of AdTSTA-tk enabled the expression of the HSV-tk PET reporter gene in the prostate tumor while restricting gene expression in a nonprostate tissue, such as the liver, despite the inadvertent gene delivery to this site (Figure 46.4C).

In principle, the TSTA method can be applied to amplify any pathway-, cell-, or tumor-specific promoter. Extensive use of this method by our group and others verified its ability to greatly enhance transcription of a wide range of cancer- and tissue-specific promoters, such as survivin, mucin1, vascular endothelial growth factor (VEGF), and cardiac-specific promoters [4, 24, 25]. Using the amplified TSTA vector-mediated imaging report method, we were able to image endogenous prostate gland and prostate tumors after tumor-directed vector delivery by microPET-CT or by BLI [12, 23, 26, 27]. Moreover, because of the cell-restricted expression capability of the AdTSTA vector, systemic-directed vector delivery can also successfully detect metastatic lesions in the lungs, lymph nodes, and peritoneum in murine models of prostate and breast cancer (Figure 46.5). Several recent reports have demonstrated the feasibility of using an adenoviral vector to deliver reporter genes such as HSV-tk and NIS to achieve specific imaging signals in hepatic and prostate tumors in patients [28, 29]. Therefore, we foresee the translation

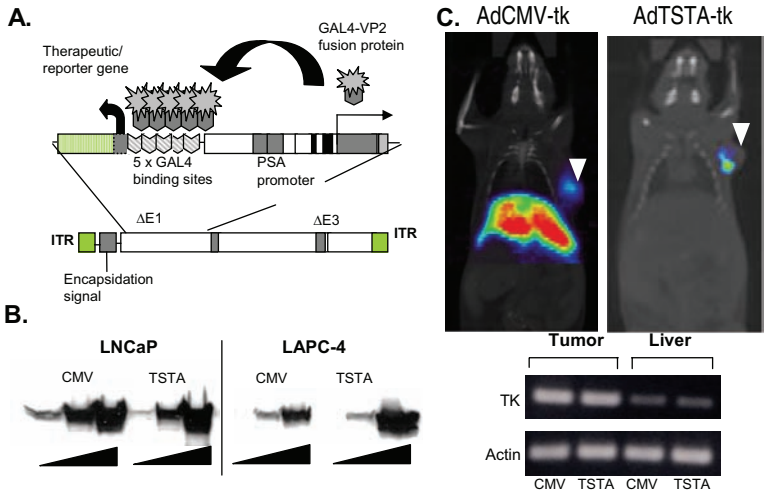

Figure 46.4. Amplified prostate-specific HSV-tk imaging vector. (A) The Ad containing the TSTA system. The enhanced prostate-specific PSA promoter drives the expression of the potent synthetic activator, composed of 2 herpes VP16 activation domains (aa 413–454) fused to the GAL4 DNA binding domain. The GAL4-VP2 activators bind to the GAL4 binding sites, activating the expression of the HSV-tk gene. These two components were inserted into the E1 genome region of an Ad5 vector. (B) The magnitude of TSTA-driven HSV1-tk expression is comparable to strong constitutive CMV promoter. LNCaP and LAPC-4 prostate cancer cells were infected with increasing dose strong constitutive AdCMV-tk or prostate-specific AdTSTA-tk at MOI 0.2, 1, and 5 (triangle). The level of HSV1-tk protein expression as assessed by Western blot was quite comparable between the two vectors. (C) Prostate-restricted AdTSTA-tk safeguards cancer gene therapy. Top diagram shows the scheme of PET-coupled gene therapy. Intratumoral injection of both AdCMV-tk and AdTSTA-tk (1 × 109 infectious units) resulted in visible tumor signal. Due to systemic vector leakage from the tumor, both vectors were delivered to the liver but expression of HSV-tk and, hence, strong PET signal was only observed in the liver of the AdCMV-tk injected animal. Both vectors achieved tumoricidal effects after treatment with suicide pro-drug ganciclovir (GCV) as noted by the positive apoptotic stain (TUNEL) of nearly the entire tumor, with a small viable area in the tumor periphery (blue in H&E stain). Liver cell apoptosis (TUNEL) was observed only in the liver of the CMV-treated but not in the TSTA group.

of a wide range of transcriptional-targeted reporter gene imaging techniques for use in clinical oncology in the near future.

NANOPARTICLE-BASED MOLECULAR IMAGING AGENTS

Characterization of nodal metastases in regional and distant lymph nodes is a critical step in cancer staging and patient management. The development of minimally invasive and accurate detection techniques for nodal metastases calls for a collaborative effort among interdisciplinary teams of experts. This type of cross-disciplinary collaboration is exemplified in the development of nanoparticle (NP) lymphotropic imaging contrast agents for detection of nodal metastases [30–34]. The sizes of these NPs range from tens to hundreds of nanometers and make them well suited for use as lymphotropic contrast agents for imaging of the lymphatic system [35].

Clinical Sentinel Lymph Node Assessment Techniques

In breast cancer and melanoma, it has been well established that lymph node status is the single most powerful prognostic indicator [36–39], with the presence of axillary lymph node metastasis associated with reduced overall survival. Histopathological determination of the lymph node is routinely performed for patients with breast cancer and melanoma, and this information is used for both cancer staging and patient management [36, 39]. Until recently, axillary lymph node dissection (ALND) was the standard of care in many breast cancer centers for patients diagnosed with invasive breast cancer [36, 39]. ALND, however, is associated with a significant rate of morbidity and a high cost of staging. In recent years, to circumvent the issues stemming from the invasiveness of ALND, the sentinel lymph node (SLN) concept was introduced, referring to the lymph nodes that drain directly from the primary tumor [35, 40, 41]. The sentinel lymph node dissection (SLND) procedure is now being offered in many breast cancer centers as an alternative to the ALND, based on the rationale that a histological examination is best performed on the lymph nodes that are most likely to receive lymphatic drainage from the primary tumor. If the status of these sentinel nodes is negative, the probability of metastasis in more distant nodes is low [36]. In addition to breast cancer and melanoma, the SLND technique has been applied to several other types of solid tumors with lymphatic metastasis, such as head and neck [38], cervical [42], colorectal [43], bladder [44], and prostate cancers [45].

Identification of the SLNs is the crucial first step during SLND, which relies on the trafficking and accumulation of lymphotropic NPs to the SLNs. The size and surface properties of these NPs are key determinants of their lymphotropic properties [46]; the NPs used in many clinics typically range in size from 50 to 200 nm [39]. Retention of the NPs in the lymph nodes is mainly the result of phagocytosis by the macrophages [47]. It has been advocated that the optimal NP size for lymphoscintography is ~100 nm [39].

Clinically, the localization of the SLNs is carried out by the injection of a blue dye, such as methylene blue (either alone or in conjunction with radiolabeled nanocolloid), followed by lymphoscintigraphy. The lymphoscintigraphic agent of choice differs by geographic location: in the United States, it

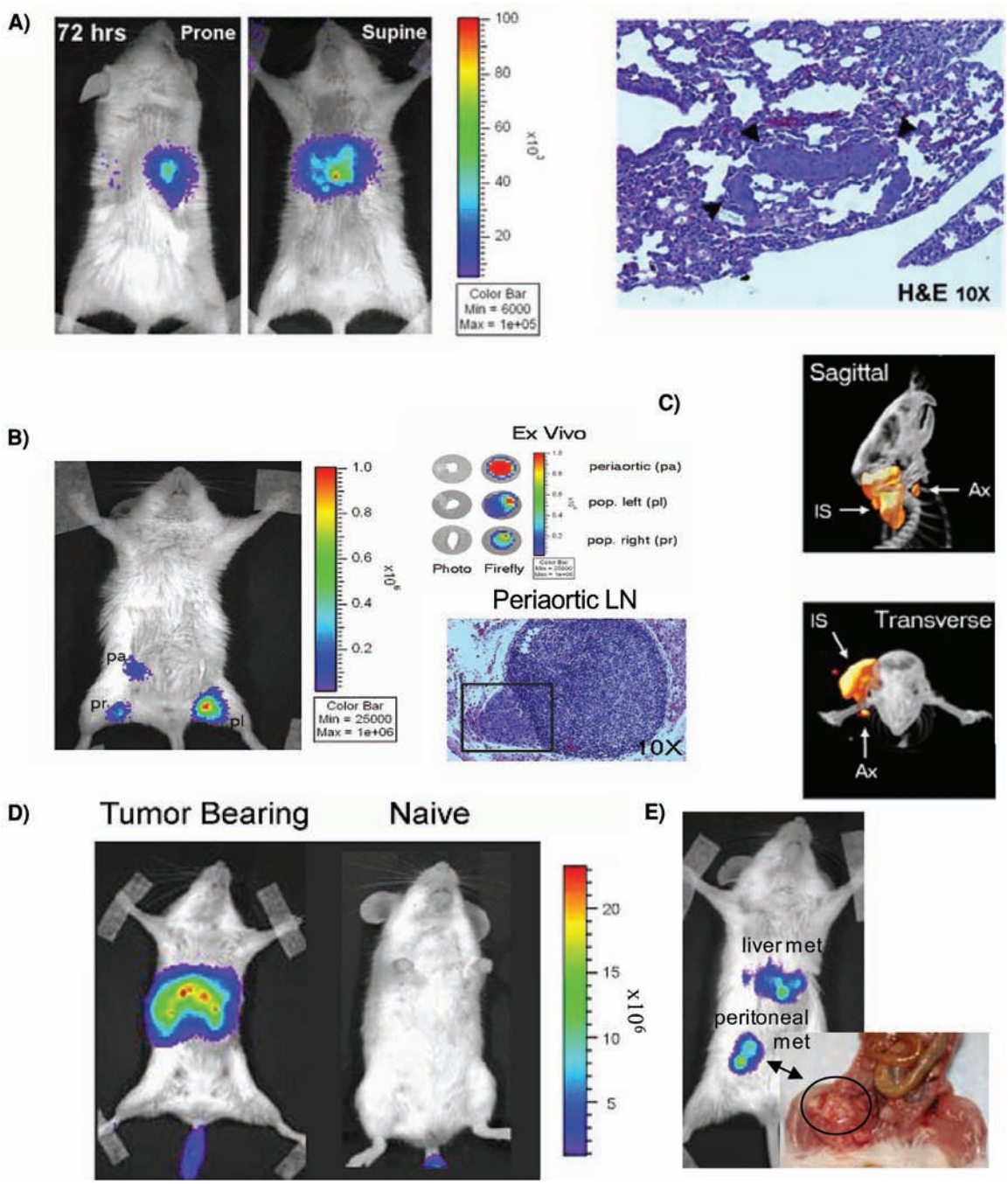

Figure 46.5. Application of TSTA to image metastases. (A) The prostate-specific, PSA promoter-based AdTSTA-firefly luciferase (fl) vector can be used to detect lung metastases of prostate cancer. Twenty-one days after orthotopic implantation of prostate tumors (LAPC9), 10^8 infectious units of AdTSTA-fl were injected intravenously, and mice were imaged for FL expression three days later. (B) AdTSTA-fl can be used to detect small lymph node metastases of prostate cancer. AdTSTA-fl was injected in both hind paws of an animal harboring an established orthotopic human prostate tumor that typically is able to metastasize to lymph nodes (LAPC9/VEGF-C; left). Ex vivo imaging of the harvested pelvic nodes indicated that the largest volume of metastasis was located in the periaortic lymph node, as noted by its signal intensity. Histology (H&E) revealed a small subcapsular lesion (marked by a square) within this periaortic node. The number of cells detected within the node metastases was estimated to be $\sim 4 \times 10^3$ cells, as determined by morphometry. (C) Detection of occult lymph node metastasis by microPET/CT imaging. A mouse bearing a tumor with nodal metastasis received AdTSTA-tk via intratumoral injections. The hypothesis tested was that Ad could drain out of the tumor through peritumoral lymphatic vessels and into the regional sentinel lymph nodes. Signals were detected in both the tumor and the draining sentinel lymph node using ^{18}FHBG PET imaging. Representative sagittal (top) and transverse (bottom) microPET/CT images show that PET signal is emitted from the tumor injection site (IS) as well as the draining axillary sentinel lymph nodes (Ax). (D) A cancer-specific mucin1 promoter-driven AdMuc-TSTA-fl luciferase-expressing vector was injected intravenously, at a dose of 10^8 infectious units, into an animal bearing experimental liver metastasis of KPL-1 breast tumor. A robust signal in the liver was visualized up to three weeks post-vector injection (image shown). No signal was detected in a similarly treated tumor-naïve animal. (E) Similar systemic administration of AdMuc-TSTA-fl was able to visualize both KPL-1 liver and peritoneal metastases in another animal. These results suggest that AdTSTA is a powerful and specific tool to reach distant prostate and breast cancer metastases for their detection and potential eradication.

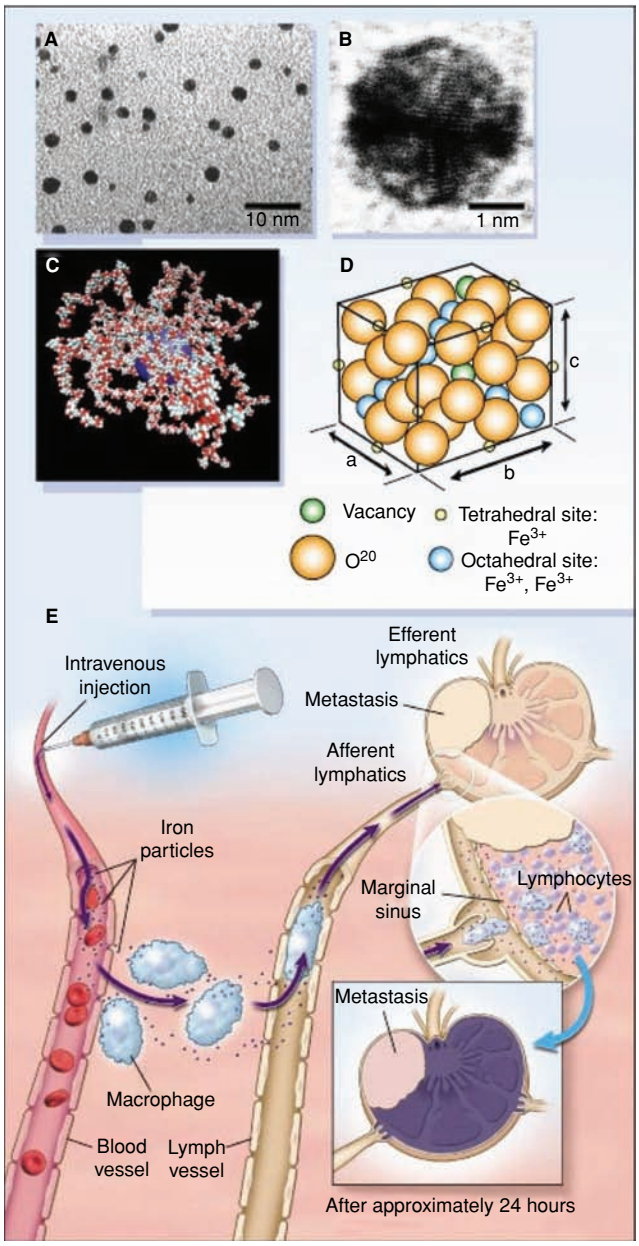

Figure 46.6. Electron micrograph of hexagonal lymphotropic superparamagnetic nanoparticles (panels A and B), molecular model of surface-bound 10-kDa dextrans and packing of iron oxide crystals (panels C and D), and mechanism of action of lymphotropic superparamagnetic nanoparticles (panel E). The model lymphotropic superparamagnetic nanoparticles shown here measure 2 to 3 nm on average (panels A and B). The mean overall particle size of the 10-kDa dextrans is 28 nm (panels C and D). In panel E, the systemically injected long-circulating particles gain access to the interstitium and are drained through lymphatic vessels. Disturbances in lymph flow or in nodal architecture caused by metastases lead to abnormal patterns of accumulation of lymphotropic superparamagnetic nanoparticles, which are detectable by MRI. (Reprinted by permission from *New England Journal of Medicine* [30], copyright 2003.)

is technetium-99–labeled sulfur colloid; whereas in Europe it is technetium-99–labeled human serum albumin nanocolloid. The current method of intraoperative lymphoscintigraphy involves the use of a gamma camera to monitor the trafficking of the radiolabeled NP from the peritumoral injection site into the SLN, which is then harvested for histopathological examination [48].

Nanoparticle-Based SLN Lymphoscintography

In addition to the blue dye and radiolabeled nanocolloids for SLN lymphoscintigraphy, several other classes of NPs are currently being investigated for use with other imaging modalities, such as MRI and NIR imaging, for SLN staging and mapping [49].

Compared with CT and PET, MRI offers excellent soft-tissue contrast. Assessment of the lymph node status by conventional MRI is achieved via observation of nodal morphological and signal intensity changes [49, 50]. These parameters alone, however, are difficult and insufficient to reproducibly identify metastatic lymph nodes [49, 50]. Ultrasmall superparamagnetic iron oxide particles (USPIOs) were developed to enhance the specificity of MRI imaging for nodal metastasis [30, 31, 49, 50] via interaction of these NPs with the reticuloendothelial system. When delivered systemically, these nanometer-sized USPIOs extravasate from the blood vasculature into the lymphatic system and are taken up by circulating macrophages. Accumulation of these USPIO-containing macrophages in the normal lymph nodes leads to decreased nodal signal intensity. In contrast, the infiltration of the node by tumor cells alters the nodal architecture and results in reduced NP uptake and retention. The reduction in NP uptake causes an increase in signal intensity and/or signal heterogeneity across the node (Figure 46.6). To date, this technique has been applied to image nodal metastases in head and neck and prostate cancers and appears to be effective in detecting metastatic lymph nodes in the pelvis and abdominal regions [49]. Although the technique appears promising in differentiating between malignant and benign nodal tissues with high specificity, the lack of universally accepted guidelines for this technology has limited its use; thus, it is used mostly in preclinical settings [50].

Near-infrared (NIR) fluorescence imaging is a relatively new imaging modality that has been developed in recent years [32–34, 51–53]. Compared with other molecular imaging modalities, such as PET or MRI, in vivo fluorescence imaging is far less expensive in terms of instrumentation and operational cost and is devoid of the potential health hazards caused by ionizing radiation and magnetic fields. Most current applications of the in vivo fluorescence imaging technique employ fluorescent probes emitting in the NIR region, in which

attenuation of light from tissue absorption and scattering is at a minimum compared with other wavelength regimes [32]. Studies using NIR NPs for SLN mapping have been reported [32, 33, 51, 54] in the hope that these fluorescent tracers can serve as alternatives to the blue-dye and radiolabeled nanocolloids currently being used in clinics. Many of these studies used NIR quantum dots as the lymphotropic imaging tracer. The quantum dots were functionalized with organic molecules to increase their solution stability and biocompatibility; while reducing their toxicity [55]. Although significant improvements to the safety and biocompatibility of quantum dots have been achieved in recent years, their applications in human in vivo imaging await further safety validation.

In a study by Kim et al., the retention of paw-injected quantum dots in draining axillary lymph nodes was detected in vivo [32]. In a second study, by Kobayashi et al., quantum dots of five different emission wavelengths were injected simultaneously into various injection sites in the upper body, and their respective drainage and retention into the draining lymph nodes in the neck and upper body were captured [53]. These studies demonstrated principally that NIR NPs can be used in conjunction with in vivo fluorescence imaging techniques for the mapping of shallow SLNs, such as those in breast cancer and melanoma. As mentioned earlier, one of the greatest technical challenges facing the translation of fluorescence in vivo imaging technique is the attenuation of the emitted light by tissue. Although improvements on the NIR imaging techniques are currently being developed to address this issue, at present the NIR imaging technique is limited mostly to preclinical studies.

In recent years, the fusion of nanotechnology and molecular imaging techniques has resulted in the development of several classes of NP-based lymphotropic contrast agents for SLN staging and mapping. We are hopeful that in the near future the ongoing research efforts in this multidisciplinary field will lead to the creation of the next generation of lymphatic contrast agents, which not only will have superior lymphotropic properties but will also be able to functionally and noninvasively image tumor metastases in the lymph node when coupled with different clinical imaging modalities.

RADIO IMMUNOIMAGING OF METASTASIS

Recent advances in genetic and protein engineering technologies have propelled unprecedented growth in the development and applications of monoclonal antibody (mAb)-based agents in medicine. The development and commercialization of antibody (Ab)-based therapies represent a significant market segment of the pharmaceutical industry, with the projected sales of recombinant protein-based therapeutics reaching $15 billion US by 2010 [56]. As of 2007, eighteen Ab-based therapeutics have received U.S. FDA approval [57], and among those, eight were approved to be used in the diagnosis and treatment of different malignancies. Among this small group of Ab-based cancer drugs are rituximab (Rituxan), trastuzumab (Herceptin), and bevacizumab (Avastin), which are widely used in cancer treatments today. Four of the eight approved Ab agents were intended for cancer diagnostic imaging purposes: arcitumomab (CEA-Scan) for imaging of colorectal cancers, nofetumomab merpentan (Verluma) for small-cell lung cancer, satumomab pendetide (OncoScint) for colorectal and ovarian cancers, and capromab pendetide (ProstaScint) for prostate cancer [57]. All four were conjugated to radionuclides for in vivo radio immunoimaging (RII) with SPECT.

Theoretically, Ab imaging agents with high specificity to their targets are uniquely suited for cancer diagnostic imaging, because they are able to differentiate cell phenotypes in vivo through binding to cell surface biomarkers. When used with quantitative imaging modalities such as PET-CT or SPECT-CT, Ab-based agents can identify the location and extent of tumor infiltration in patients. This information can be especially useful when planning the course of treatment tailored to the individual needs of each patient. Compared with a cell metabolic rate-dependent tracer such as ^{18}F-FDG, Ab-based RII tracers have the unique advantage of being able to image tumors with intrinsically low metabolic rates, such as low-grade lymphoma or prostate cancer [57–59]. However, despite their enormous potential, this class of molecular imaging agents has yet to reach clinical viability or solid commercial success: only capromab pendetide (ProstaScint) remains in use today, whereas the other three Ab-based imaging agents have become obsolete [57, 60]. In recent years, the field of Ab RII agent development is undergoing resurgence owing to improvements on imaging instrumentation, and more significantly, the creation of Ab fragments with high binding affinity and superior pharmacokinetic properties compared with intact Abs.

Criteria of Ideal RII Agents

A successful RII Ab agent should fulfill several criteria. First, the targets of the Ab agent should be readily accessible by the Ab agent, and should be overexpressed only in tumor tissues with minimum expression in normal tissues. Examples of targets that are currently under evaluation in clinical trials include CD20, CD22, prostate-specific membrane antigen (PSMA), mucin 1, carcinoembryonic antigen (CEA), HER2/neu receptor, epidermal growth factor receptor (EGFR), and VEGF, to list just a few [57, 58].

Second, to achieve high quality imaging, the Ab must demonstrate optimal pharmacokinetics and be cleared efficiently from the plasma while maintaining a reasonable tumor uptake. The tracer should exhibit minimal uptake by the reticuloendothelial system, particularly in the kidney and liver. Elimination of the tracer through the kidneys rather than the hepatobiliary system is preferable so that the signals originated from abdominal lesions can be distinguished from the liver signals. For antibody fragments, the size for renal clearance is ~60 kDa, with particles lower than 60 kDa cleared mainly through the kidneys.

Third, the circulation half-life ($t_{1/2}$) of the Ab tracer must match that of the radionuclide used to label the Ab agent. Given the dominance of SPECT and PET usage in the oncological molecular imaging, the ideal $t_{1/2}$ of an Ab agent should be in the range of hours to days [57, 58, 60]. Reference [57] contains a list of radionuclides that are potential candidates for Ab imaging, and their half-lives.

Commercial Ab-Based RII Agents

The most advanced efforts in the development of Ab-based RII agents are for gastrointestinal cancers [57], with two of the four FDA-approved Ab imaging tracers, arcitumomab (CEA-Scan) and satumomab pendetide (OncoScint), targeted to colorectal cancer. However, both products have become obsolete [57, 60]. In the following section, the applications of CEA-Scan and ProstaScint are analyzed to illustrate the challenges faced by Ab-based cancer imaging agents.

CEA is a key antigen that is believed to be correlated with the progression of colon cancer. It is a large glycoprotein that is produced in the normal fetus, but only a trace amount is found in normal adult cells. Both clinical and preclinical studies have established CEA as a biomarker for gastrointestinal tumors [61–64], as it is produced in more than 90 percent of primary colon cancers [64]. Elevated preoperative CEA levels are correlated with poor overall survival even after the surgical resection of the primary colon cancer [64, 65]. However, a number of nonmalignant conditions have also been found to be correlated to elevated CEA levels [64].

CEA-Scan is a murine mAb Fab′ fragment conjugated to ^{99}mTc to be used for the detection of recurrent or metastatic colorectal cancer [57]. It received FDA approval in 1996, but in 2006 its commercial production was terminated. Potential complications in using CEA as an imaging target arise from the fact that glycosylphosphatidylinositol-linked CEA from the cell surface is cleaved from the cell surface by phospholipases and is released into circulation, which interferes with tumor cell binding with the CEA-Scan and reduces the tumor-to-blood contrast ratio of the tracer. Further, the secreted CEA is metabolized through the liver [64], which leads to high tracer accumulation in the liver. In a side-by-side comparison study by Libutti et al. of the use of ^{18}F-FDG PET and CEA-Scan to detect recurrent colon cancer in patients after surgical resection, the ^{18}F-FDG PET outperformed CEA-Scan [66]. The imaging results were validated by "blind" second-look laparotomy for patients who had undergone surgical resection of primary colon carcinoma and were suspected of tumor recurrence. The aim of the study was to detect tumor recurrence and to determine whether the recurrent tumor, when found, was resectable via ^{18}F-FDG and CEA-Scan. Of the twenty-eight patients who were subject to the combination treatment of imaging and surgical techniques, twenty-six were found to have tumor recurrence, and among those, ten had unresectable tumors. The FDG-PET correctly predicted 90 percent of the unresectable cases, whereas CEA-Scan failed to predict any case. For the rest of the sixteen patients who had resectable disease, the FDG-PET correctly predicted the status in 81 percent of patients, whereas the CEA-Scan had a success rate of only 13 percent. A similar conclusion was drawn in another comparison study by Wilkomm et al. [67], which involved the use of FDG-PET and a radiolabeled anti-CEA antibody fragment for the detection of colon cancer local recurrence and metastases [67].

Despite of ^{18}F-FDG's wide application in oncologic imaging today, its application in diagnosis and disease management of prostate cancer remains limited, owing to several factors. First, as noted previously, the uptake of FDG by tumors is low. Second, significant bladder uptake of the tracer interferes with the tumor signal. Finally, it is perceived that PET imaging offers limited advantages in diagnostic imaging of prostate cancer compared with other imaging modalities, such as MRI [57, 68]. Therefore, the development of Ab-based RII agents for prostate cancer could fill a unique niche. ProstaScint was developed from the antibody 7E11, which was originally used for the discovery of PSMA [57]. PSMA is the most widely studied marker for prostate cancer. It is a 100-kDa transmembrane protein with a short cytoplasmatic domain and a relatively large extracellular domain [69]. In contrast to CEA, PSMA is not secreted into the circulation. As an imaging marker for prostate cancer, PSMA expression is restricted mainly to prostate tissues, whereas significant lower expression levels are found in other tissue types [69]. In prostate tissues, increasing levels of PSMA are positively correlated with tumor aggressiveness [69]. The presence of higher amounts of PSMA in metastatic lesions of the bone, lymph nodes, soft tissue, and lungs compared with nontumor tissues has been well established [70–72].

Originally, ProstaScint was approved for diagnosis and staging of patients who were at high risk of lymph node metastasis [57]. Unfortunately, despite the

advantages of PSMA as a favorable target for prostate cancer imaging, ProstaScint targets only the intracellular domain of PSMA and is incapable of binding to the extracellular domain of the protein. Therefore only nonviable prostate tumor cells, whose cytoplasm is accessible to the circulating Ab, could be targeted [57, 69]. This limits the ability of ProstaScint to image bone metastases, which are not associated with large populations of apoptotic or necrotic cells. Other Ab agents that target the PSMA extracellular domain, such as J591, are being developed [69].

Use of Long–Half-Life Radionuclides

The clinical impact of the first generation of Ab agents was underscored by issues such as low target-to-background ratios and immunogenicity, which prevented their repeated administration in vivo [57, 58, 60, 68]. Despite their theoretical potential, Ab-based RII agents have yet to achieve their full potentials. In recent years, advances in molecular imaging instrumentation, improved radionuclide conjugation techniques, the commercial availability of long-lived positron emitters, such as ^{89}Zr ($t_{1/2} = 78.4$ hr) and ^{124}I ($t_{1/2} = 100.2$ hr), and protein engineering techniques have led to renewed interests in antibody oncologic imaging.

In a study by Perk et. al. [73], the ^{89}Zr-labeled mAb DN30 was injected into mice bearing human tumor xenografts positive for c-Met expressions to evaluate the capability of the Ab for imaging c-Met–positive tumors in vivo. The c-Met receptor is encoded by the MET oncogene and is the ligand to the tyrosine kinase receptor hepatocyte growth factor. The activation of c-Met receptor has been show to play a role in tumorigenesis and the acquisition of invasive phenotypes [74]. It is hoped that binding of DN30 mAb to c-Met can interfere with the c-Met signaling pathway, which subsequently controls the activation of the MET oncogene, and curb tumorigenesis and metastasis. The Ab agent was able to detect tumor as small as 11 mg. The xenografted tumors were visible beginning one day postinjection, and tumor signals were sustainable for up to four days postinjection. As reported by the authors, a humanized DN30 Ab is being developed for clinical study.

In a clinical study by Börjesson et Al., ^{89}Zr-labeled chimeric mouse-human mAb U36, which recognizes the CD44v6 antigen that is commonly expressed in head and neck tumors, was used to image primary and lymph node metastases in a group of 20 patients with squamous cell carcinoma of the head-and-neck [75]. The immuno-PET findings were compared to those obtained via CT/MRI and palpation. A subgroup of patients also underwent ^{18}F-FDG PET imaging, in addition to the above diagnostic modalities. No significant immune response was detected in the majority of the patients, except for two who developed antibody responses against the chimerical mAb. The imaging data showed comparable sensitivities using immuno-PET versus CT/MRI to detect lymph node metastases (72% vs 60%). Further, data from the subgroup of patients who also received additional ^{18}F-PET imaging showed comparable results when using immuno-PET (85%) and ^{18}F-FDG (62%). However, immuno-PET, CT, and MRI all reported false-positive findings, whereas the ^{18}F-PET did not. The authors acknowledged that at present neither immuno-PET nor ^{18}F-PET was capable of detecting micrometastases in the lymph nodes.

Engineered Antibody Fragments

In recent years, the focus on the development of Ab imaging agents has gradually shifted from using intact Ab to using recombinant Ab fragments. Intense efforts are currently being devoted to the development of protein fragments to improve the pharmacokinetics of Ab-based RII agents. Early Ab fragment agents were created via enzymatic digestion of intact Abs to produce either Fab or F(ab′)2 fragments, such as the articumomab/CEA-Scan. The emergence in the past decade of engineered protein fragments, such as single-chain Fv fragments, diabodies, and minibodies, whose pharmacokinetics and organ biodistribution can be tailored for particular molecular imaging applications, may finally unleash the potential of this class of molecular imaging agents.

It has been demonstrated that the circulation time of protein is strongly dependent on parameters such as their molecular weight (M_w) and their binding affinities to targets [76, 77]. The M_w of an intact Ab is ~150 kDa. The high M_w of an intact Ab, together with antibody interaction with the neonatal Fc receptor, causes it to remain in blood circulation for weeks postinjection, which gives the Ab sufficient time to accumulate at the tumor at the expense of decreased tumor-to-background signal ratio. Their large sizes and extended blood residence time lead to slow blood clearance, low tissue penetration, and immunogenicity against the Ab tracer [78]. To circumvent these challenges, there is a growing trend to gravitate towards the use of antibody fragments such as those shown in Figure 46.7, which have more optimal pharmacokinetic properties for tumor imaging.

Antibody fragments of a range of molecular weights can be produced via recombinant DNA technology. The target-specific Ab variable genes are first isolated and subsequently cloned, then expressed into desired Ab formats. These recombinant protein fragments range in sizes from 25 kDa (scFV) to 110 kDa (scFv-Fc) and have demonstrated their capabilities as cancer imaging agents in numerous preclinical and human studies because of their superior biodistribution as compared with intact antibodies [58, 60, 78]. In contrast to intact

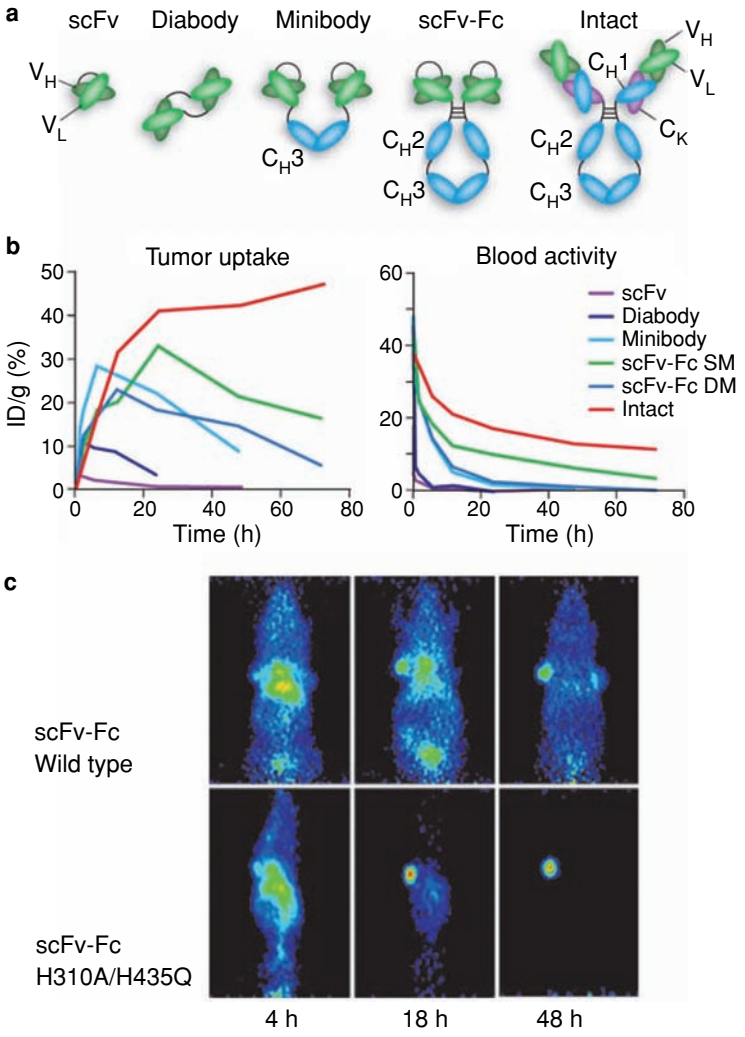

Figure 46.7. Engineered antibody-based PET. (a) Schematic showing domain composition of engineered fragments including scFv (25 kDa), diabody (55 kDa), minibody (80 kDa), scFv-Fc (105 kDa), and intact antibodies (150 kDa). (b) Tumor uptake and blood clearance curves for radioiodine-labeled anti-CEA scFv mAb fragments in athymic mice bearing subcutaneous LS174T human colon carcinoma xenografts. Uptake is expressed as percentage injected dose per gram (ID/g). The image marked scFv-Fc SM is a H310A single mutant of the mAb; scFv-Fc DM is a H310A/H435Q double mutant. (c) Serial microPET imaging of 124I-labeled anti-CEA scFv-Fc fragments (parental human gamma1 and H310A/H435Q double mutant) in LS174T xenograft-bearing mice. The difference in clearance rates is readily apparent in the serial images. (Reprinted by permission from Macmillan Publishers Ltd., *Nature Biotechnology* [78], copyright 2005.)

Abs, which remain in circulation for weeks and cannot be imaged until days after injection, the smaller Ab fragments can reach optimal tumor-to-blood distribution ratio within twenty-four hours, which makes them more suitable candidates for tumor imaging than intact Abs when used in conjunction with shorter half-life radionuclides.

Smaller protein fragments such as the scFvs, which are composed of heavy and light chains of the variable regions linked via a peptide linker, have limited use as imaging agents because of their very fast clearance times. However, the rapid clearance of scFvs from the blood and their reduced valence of binding compared with the parent Ab lower their tumor uptake. *Diabodies* are noncovalent dimers of scFv fragments linked by short peptides with molecular weight of ~55 kDa. Compared with scFvs, diabodies have superior tumor uptakes because of their bivalence binding to targets and longer circulation times; therefore, they are ideally used in conjugation with radionuclides having intermediate half-lives, such as ^{64}Cu ($t_{1/2} = 12.7$ hr) or ^{124}I ($t_{1/2} = 4.18$ days). Developments of larger fragments, such as minibodies composed of scFv-CH3 (80 kDa) or scFv-CH2-CH3 fusion proteins (110 kDa), are also being actively pursued. Currently, investigations of Ab-based RII agents have largely remained in the preclinical phase [58, 60, 78].

The pharmacokinetic and biodistribution properties of Ab-fragment RII agents can be further optimized via modification of the protein fragment structure to suit different in vivo applications. This approach is illustrated by the success of several preclinical studies of diabodies and minibodies derived from anti-CEA mAb T84.66 [79–85]. Extensive protein engineering has been carried out to fine-tune the structural–functional relationships of these protein fragments via the screening of the type of fragment (diabody vs. minibody), the peptide linker, and the radionuclides. In the following section we outline some of these studies to illustrate the versatility of Ab-fragment imaging agents.

The first minibody form of T84.66 was generated by fusion of the scFv domain to the IgG CH3 domain [80]. It was hoped that by retaining the bivalence of binding of the scFv domains, and the addition of the stabilizing CH3 domain, the overall tumor retention and pharmacokinetics of the minibody form would be superior compared with the original scFv form. Two constructs of the minibody were created and tested, which differed only in the peptide linker region between the scFv domains and the CH3 domain. The linkers varied in their lengths, which conferred different degrees of structural flexibility to the minibody. The radioiodinated minibodies were administered in vivo to target LS174T xenografts in athymic mice. Six hours after injection, tumor uptake as high as 32.9 percent ID/g was achieved, which compared favorably with the 30 to 40 percent ID/g obtained for intact Abs [80]. The properties of the minibody agents represented a significant improvement over those of scFv

agents, which remained in the 1 to 5 percent ID/g range.

In the follow-up work [84], a slightly modified version of the T84.66 minibody was radiolabeled with ^{64}Cu for PET imaging. Uptake in the LS174T xenograft in athymic mice five hours after injection was 17.91 %ID/g. This study demonstrated conceptually the feasibility of using this minibody with human PET scanners. Interestingly, the authors raised the issue of passive uptake of the minibody by the tumor via the passing targeting mechanism, as radiolabeled albumin has shown uptake as high as 8.2 %ID/g. Also of note is the high hepatic uptake of ^{64}Cu, which will hinder potential clinical applications.

In the work by Sundaresan et. al [86], PET imaging on ^{124}I-labeled anti-CEA diabody and minibody was conducted on mice bearing CEA-positive and CEA-negative xenografts. Mean tumor uptake of 4.5% ID/g was reported for the diabody at 18 hrs postinjection; and 20.6% ID/g for that of the minibody. The tumor-to-background ratios were comparable for both agents. However, the nontarget accumulation of the diabody was significantly lower compared with that of the minibody. The authors concluded that in this animal model the diabody was advantageous to the minibody, given its superior tumor-to-background contrast ratios. Interestingly, a comparison between ^{18}F-FDG and radiolabeled minibody imaging was also performed on a subset of the mice in this study. In contrast to the high specific uptake of the minibody in the CEA-positive xenografts, the ^{18}F-FDG uptake was 3 to 8% ID/g, which was comparable to that of the diabody. The authors suggested that RII can be applied as a complementary imaging tool to image cancers that are not suitable for ^{18}F-FDG imaging. Olafsen et al. have developed a cysteine-modified anti-CEA diabody, which contains a reducible disulfide bond. By conjugating radionuclides, or therapeutic payloads to the cysteine via site-specific conjugation reaction, instead of random conjugation to the surface amino acids, the binding regions on the protein surface are preserved. A similarly constructed anti-HER2 diabody has also been generated. Recent breakthroughs in radiochemistry have enabled ^{18}F labeling of the anti-CEA diabody, which may have better translational potential, compared with other radionuclides [83], and has a half-life suitable for diabody imaging.

A pilot clinical study using ^{123}I-labeled anti-CEA minibody was conducted with a group of ten patients with biopsy-proven colorectal cancer [79]. Minibody imaging using SPECT scans and CT scans were compared against surgical surveys. The minibody scan agent identified eight of ten tumors that were $\geq$ 1.0 cm in size, whereas CT scan captured five of ten tumors. Additionally, the minibody was well tolerated, and no significant immune response against the minibody was reported from any of the patients. This study marked the first attempt at validating the translational potentials of Ab fragments. Currently, one anti-CEA agent diabody is undergoing FDA clinical trial for immunoscintigraphy of colorectal cancer [87].

Great advances in Ab-based imaging technology have been made in the past decade, which are facilitated by developments of immunoPET instrumentation and recombinant protein technology. The intense level of preclinical investigations in Ab fragment–based RII agents signals the potential significance contribution of this technology in oncology.

SMALL-MOLECULE PET IMAGING OF CANCER METASTASIS

Advances in noninvasive imaging modalities within the past decade have opened endless opportunities for molecular diagnostic in cancer metastasis. In recent years, traditional morphology-based imaging modalities, such as CT [88], ultrasound, and MRI, have been complemented by functional and molecular imaging techniques such as SPECT and PET. The most promising is the fusion of PET with CT, which allows visualization of physiological processes with anatomical localization (Figure 46.8). PET detects high-energy gamma photons emitted from positron-emitting isotopes such as ^{15}O, ^{13}N, ^{11}C, and ^{18}F. These isotopes have relative short half-lives, ranging from 2 minutes to 110 minutes (e.g., ^{15}O and ^{18}F, respectively). Other less commonly used positron emitters include ^{14}O, ^{64}Cu, ^{62}Cu, ^{124}I, ^{76}Br, ^{82}Rb, and ^{68}Ga [2]. For imaging, these positron-emitting isotopes are chemically conjugated to molecules (tracers) that are metabolized or overexpressed in pathological tissues [89]. The tracer concentration is typically on the order of picomolars; thus, the radiolabeled tracers are able to interrogate in vivo physiology without significantly altering the functionality of the system. A tracer can be a metabolite, a drug, a peptide, a lipid, or an antibody. The rapid turnover of many tracers in metastatic cells enables in vivo detection of metastasis by PET. Other molecular events, such as angiogenesis, apoptosis, receptor expression, or hypoxia, are also being explored for oncological imaging by PET. A summary of tracers and the pathological process they are associated with is given in Table 46.2.

The conjugations of ^{18}F to 2-deoxy-2-fluoro-D-glucose (^{18}F-FDG); ^{11}C- and ^{18}C- to choline; and ^{18}F- to fluoride have shown great promise in detection and staging of cancer metastasis. We focus on these small-molecule radiotracers with emphasis on breast and prostate cancer in the following sections.

^{18}F-FDG

Many types of malignant cells have higher-than-normal glucose metabolism [90]. The glucose transporter

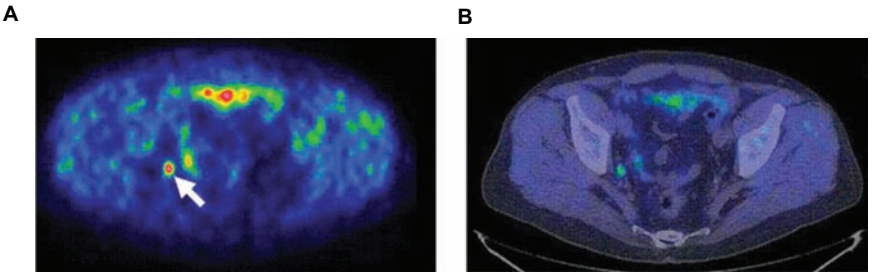

Figure 46.8. PET image fused to computerized tomography (PET-CT). ^{11}C-choline PET of a patient with prostate cancer lymph node metastasis. (A) Positive signals are localized in the pelvis. (B) Positive signal is located within a lymph node, increasing the specificity of the signal. (Reprinted with permission from Elsevier [138].)

GLUT-1 has been shown to be overexpressed when compared with normal cells in many types of cancer cells, including breast cancer [88], cholangiocellular carcinoma [91], non–small-cell lung carcinoma [92], and prostate cancer. Amplified glucose usage in malignant cells is the basis for cancer detection by PET imaging through uptake of the glucose analog, ^{18}F-FDG. ^{18}F-FDG is transported into cancer cells by the GLUT-1 glucose transporter and is then phosphorylated to FDG-6-phosphate by hexokinase II. FDG-6-phosphate is not further metabolized and is thus retained within the malignant cells. Recently, work by Cantley and coworkers have suggested that the pyruvate kinase enzyme M2-PK is responsible for the Warburg effect, which is the underlying mechanism of increased ^{18}F-FDG uptake in tumors, as well as rapid tumor cell proliferation. Further, the researchers stated that a switch in a splice isoform of the pyruvate kinase is responsible for the shift in cellular metabolism and tumorigenesis [93, 94].

Breast cancer is the leading type of cancer, and the second leading cause of cancer-related death, in women in Western society [95]. Studies showed that ^{18}F-FDG has an affinity for breast cancer with detection rates varying from 64 percent to 100 percent [96–98]. ^{18}F-FDG has also been used in the staging of axillary lymph node metastasis, with reported sensitivities from 79 percent to 100 percent [99–102]. Furthermore, ^{18}F-FDG has a relatively high sensitivity and specificity with regard to the status of axillary lymph nodes, distant metastasis, and recurrence as compared with conventional morphological imaging modalities [103–105]. In a recent report, Borkar and Pandit-Taskar reported the use of ^{18}F-FDG in the detection of a distant dermal metastasis from breast cancer [106] (Figure 46.9). However, one main drawback of using PET for nodal imaging is the high rate of false-negative results in the presence of a small volume of disease – specifically, axillary micrometastases – owing to the limited spatial resolution of current PET technology scanners (about 5 mm). This limitation of PET to detect micrometastases is compensated by the introduction of very accurate pathologic techniques, such as multislice sectioning and immunocytochemistry staining coupled with sentinel lymph node biopsy (SLNB). These techniques have significantly increased the rate of detection of micrometastases in removed axillary nodes. As a result, SLNB after lymphoscintigraphy is still the current standard diagnostic approach for staging of the axillary lymph nodes. More details on SLNB are given in the section on nanoparticle imaging agents

TABLE 46.2. Tracers that can image tumor metastasis by positron emission tomography

Process imaged	Tracers	Abbreviation	Transporter/enzyme/receptor	Isotope	Type of molecule	Examples of cancers
Metabolism	Fluorodesoxyglucose	FDG	GLUT1/Hexokinase II	^{18}F	Glucide	Many cancers
	Choline	-	CHT/Cholinkinase	^{11}C, ^{18}F	Vitamin	Prostate cancer
	Acetate	-	Acetyl-CoA synthetase	^{11}C, ^{18}F	Metabolite	Prostate cancer
	Methionine	-	LAT1/2	11C	Amino acid	Cerebral tumors
	Anti-amino-fluorocyclobutane-carboxylic acid	FACBC	LAT	^{18}F	Amino acid	Prostate cancer
	Fluoroethyl-L-tyrosine	-	-	^{18}F	Amino acid	Cerebral tumors
	Fluoro-estradiol	FES	Estrogen receptor	^{18}F	Lipid	Breast carcinoma
	Dihydrotestosterone	DHT	Androgen receptor	^{18}F	Lipid	Prostate cancer
Apoptosis	Annexin V	-	Phosphatidylserine	^{99m}Tc	Protein	Renal cancer
Angiogenesis	Vascular endothelial growth factor antibody	VEGF	VEGF receptor	^{124}I	Cytokine	Ovarian
Hypoxia	Fluoromisonidazole	FMISO	Membrane diffusion/nitroreductases	^{18}F	-	Head and neck, Lung
	Diacetyl-bis-methylthiosemicarbazone	ATSM	Membrane diffusion/nitroreductases	^{18}F	-	-
	Fluoroazomycin arabinoside	FAZA	Membrane diffusion/nitroreductases	^{18}F	-	-
Proliferation	Fluoro-deoxythymidine	FLT	ENT, CNT/Thimidine kinase	^{18}F	Nucleoside analog	Any tumor
Antigen expression	DOTA-octreotide		Somatostatin receptor 2	^{68}G	Endogenous ligand	Neuroendocrine tumors
	Cyclic Arg-Gly-Asp-(RGD) pentapeptides		αvβ3 integrin	^{18}F, ^{99m}Tc	Endogenous ligand	Melanoma
	Fluoro-hydroxymethylbutyl-guanine	FHBG	Thymidine kinase	^{18}F	Nucleoside analog	Gene expression monitoring
	cG250	-	CA-IX antigen	^{124}I, ^{131}I	Antibody	Kidney cancer
Chemoagents uptake	Gemcitabine	-	Nucleoside transporter/DCK	^{18}F	Nucleoside analog	Leukemia
Blood flow	Water	H_2O	None	^{15}O	-	-
	Carbon dioxide	CO_2	None	^{15}O	-	-

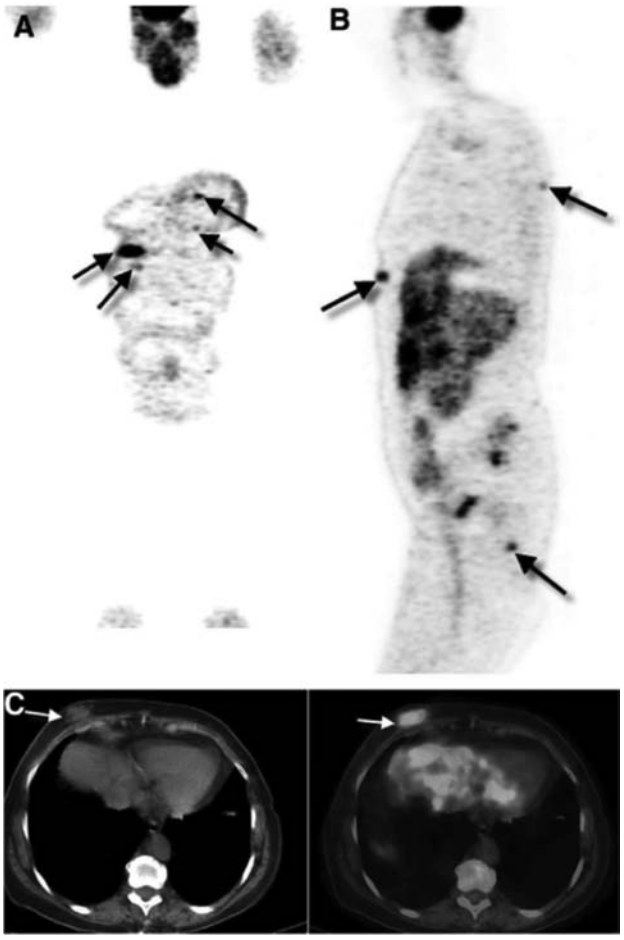

Figure 46.9. ^{18}F FDG PET/CT scan for metastatic breast cancer restaging. (A) Coronal ^{18}F FDG PET image in the 73-year-old woman demonstrates FDG uptake in the subcutaneous region of the left breast, left chest wall, and the right side of the abdominal wall. (B) Sagittal PET image demonstrates FDG uptake in the subcutaneous region of the abdominal wall, right upper back, and the right hip. (C) Axial CT and ^{18}F FDG PET/CT-fused images demonstrates FDG uptake in a subcutaneous nodule in the right lower chest wall. (Reprinted with permission from *Clinical Nuclear Medicine* [106].)

later in the chapter. Finally, the ability to predict the tumor response to chemotherapy is unique to PET/CT compared with conventional imaging techniques. PET/CT has been applied successfully to predict tumor response in breast cancer, soft tissue sarcoma [107], Hodgkin lymphoma, and gastrointestinal stromal tumors [108–110].

^{18}F-Choline and ^{11}C-Choline

Choline is one of the components of phosphatidylcholine, an essential element of phospholipids on the cell membrane [111]. Malignant tumors may show high proliferation and increased metabolism of cell lipid components, which will lead to an increase in the uptake of choline [112]. ^{11}C-choline and ^{18}F-labeled choline derivatives, including ^{18}F-fluoroethylcholine and ^{18}F-fluoromethylcholine (^{18}F-choline), have shown great promise in the evaluation of patients with prostate cancer [113–115]. In a recent study, Beheshti et al. demonstrated the potential role of ^{18}F-choline in the assessment of bone metastasis in patients with prostate cancer. The sensitivity, specificity, and accuracy of ^{18}F-choline PET/CT in detecting bone metastases from PCA were 79 percent, 97 percent, and 84 percent, respectively [116]. In some cases, ^{18}F-choline uptake was observed in the spine lesion while morphological changes in CT were not detected (Figure 46.10). In other cases, when significant morphological progress was seen on CT with Hounsfield units greater than 825, no ^{18}F-choline uptake was observed (Figure 46.10). These observations can be explained partly by the differences in the types of lesions present: lytic lesions may exhibit increased glycolysis or cell membrane proliferation that leads to ^{18}F-choline uptake. When a patient undergoes hormone therapy or other types of treatment, densely sclerotic lesions may develop as a result of treatment or apoptosis; therefore, the lesions do not readily take up ^{18}F-choline. The results of these studies show great promise in identifying lytic over blastic bone lesions but also suggest that care should be taken when interpreting the results.

^{11}C-choline has proven to be an appropriate probe for noninvasive imaging of prostate cancer in patients [117–119] and is shown to be rapidly and preferentially taken up in nodal and distant metastases [120–123]. In a preliminary human study, ^{11}C-choline PET/CT was able to detect recurrent lymph node metastases after radical prostatectomy [124] (Figure 46.11). Although the cohort is limited in size, there was strong evidence that the patients benefited from ^{11}C-choline PET/CT with a rather small salvage lymph node dissection. One advantage of ^{11}C-choline over ^{18}F-choline for PET imaging is the absence of high urinary excretion of the ^{11}C-choline radionuclide, which provides clear images of the small pelvis lymphatic drainage of that region. The disadvantage of using ^{11}C-choline for PET imaging is its short half-life (~20 minutes for ^{11}C, compared with ~110 min for ^{18}F), which requires a cyclotron to be located nearby.

^{18}F-Fluoride

Bone scans with ^{18}F-fluoride ions were first introduced by Blau et al. [125]. ^{18}F-fluoride undergoes ion exchange in an aqueous environment with hydroxyl groups on hydroxyapatite to form fluoroapatite, which subsequently is incorporated into the bone matrix [126]. In a preclinical animal model of bone metastasis using the LAPC-9 human prostate cancer model, the ^{18}F-fluoride uptake was confined primarily within the cortical bone of the tibia and extended distally

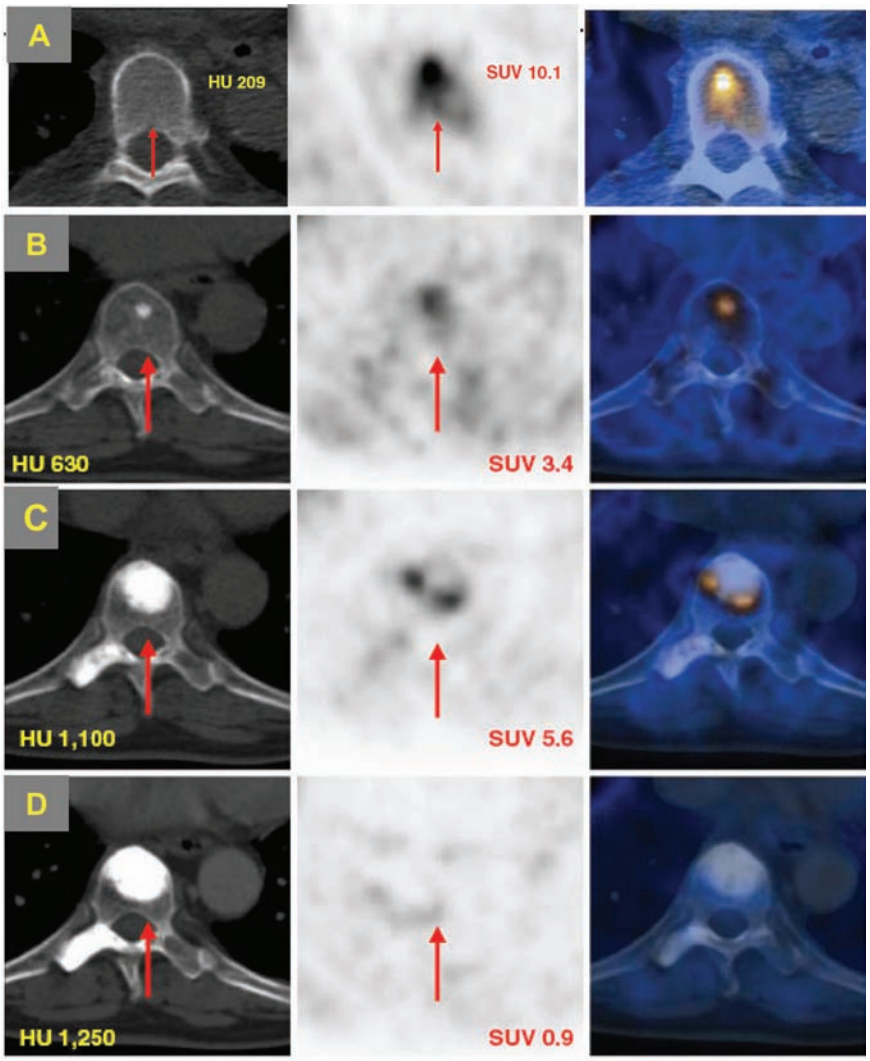

Figure 46.10. PET detection of the dynamic pattern of bone metastases of prostate cancer (A) ^{18}F-choline uptake of bone marrow metastasis in the thoracic spine T8 revealed by PET and PET/CT despite the normal CT in this patient. (B) Small sclerotic lesion with corresponding ^{18}F-choline uptake in the thoracic spine (T9); patient had no therapy. (C) Significant morphological progress in the sclerotic lesion (HU 1100), ^{18}F-choline uptake seen only at the rim of a sclerotic lesion in a patient under hormone therapy. (C) CT of a highly suspicious lesion with increasing in sclerotic density (HU 1250) but negative ^{18}F-choline in another patient under hormone therapy. (From Beheshti et al. [116], reprinted with permission from Springer.)

from the injection site in larger lesions (Figure 46.12) [127]. Along with PET/CT images, plain radiographs and histologic specimens were evaluated for the presence of an osteoblastic lesion at the four-, six-, and eight-week time points (Figure 46.12) [127]. On plain radiographs, osteoblastic lesions could not be identified until the six-week time point, whereas ^{18}F-fluoride tracer uptake as early as four weeks after tumor implantation [127] was detectable on PET/CT images. In a clinical study by Evan-Sapir et al. [128], the detection sensitivity of bone metastases in forty-four patients with high-risk prostate cancer using several modalities, including ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) planar bone scintigraphy, SPECT, ^{18}F-fluoride PET, and ^{18}F-fluoride PET/CT were compared. The results showed that ^{18}F-fluoride PET/CT is a highly sensitive and specific modality for detection of bone metastases in patients with high-risk prostate cancer. It is more specific than ^{18}F-fluoride PET alone and more sensitive and specific than planar and SPECT bone scintigraphy. Because of the relative ease of producing ^{18}F-fluoride without further chemical synthesis and the promising results of its use, the Centers for Medicare and Medicaid Services (CMS) is currently considering coverage of ^{18}F-fluoride PET for identifying bone metastasis.

PET imaging using small-molecule tracers such as FDG has demonstrated value in the diagnosis of and

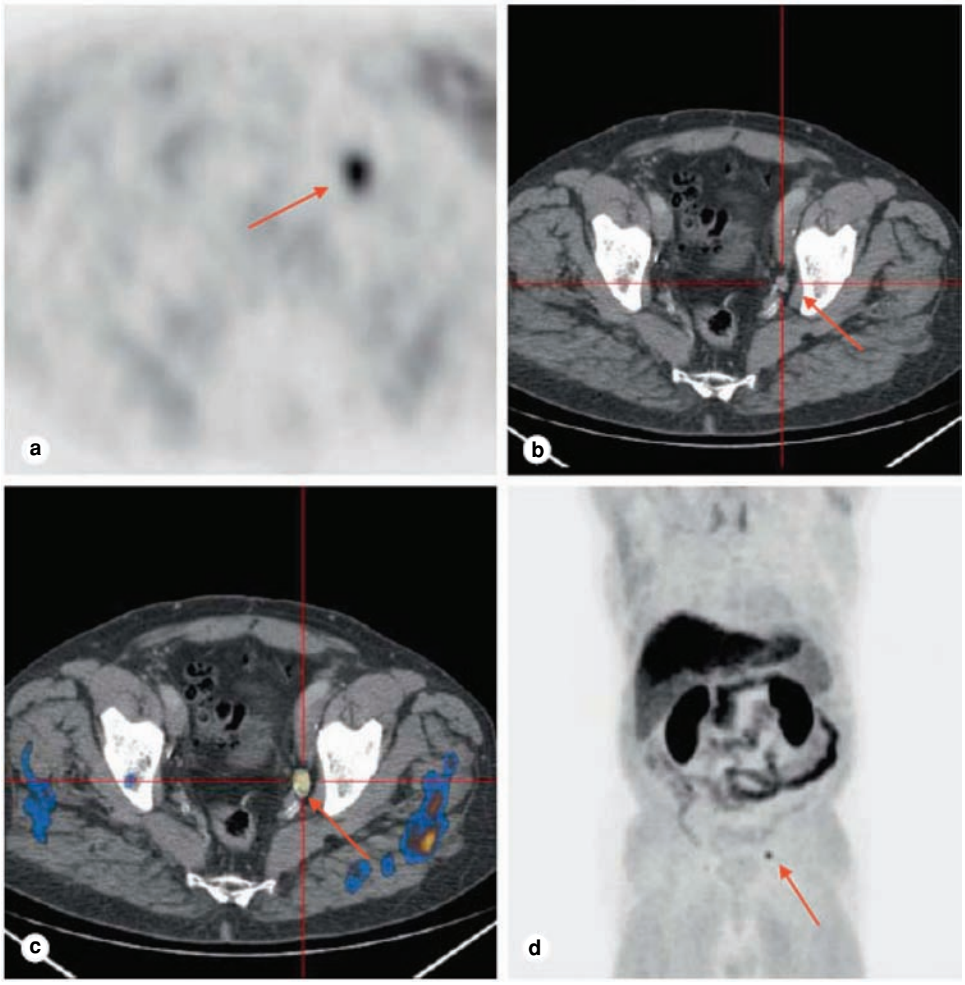

Figure 46.11. ^{11}C-choline PET/CT (transaxial slices) of a patient after radical prostatectomy, (a) ^{11}C-choline PET image (transaxial slice) in a patient stage at pT3b pN0 cM0 GII Gl. 7 R1. (b) Corresponding contrast-enhanced 4-row helical CT scan (transaxial slice). (c) Fused PET/CT image. (d) PET image (coronary slice, MIP maximal intension procedure) with one suspicious lymph node in the small pelvis (arrow). The PSA level at the time of imaging was 1.3 ng/mL. ^{11}C-choline PET/CT shows intensive focal uptake in the lymph node ventral A. iliaca externa. Pathohistology after salvage lymphadenectomy confirmed the presence 3 of 12 lymph node metastases. The subsequent treatment was watchful waiting. After a follow-up of six months, the PSA was stable at 0.1 ng/mL. (From Rinnab et al. [124], reprinted with permission from S. Karger AG, Basel.)

treatment planning for malignancies. Although small-molecule radiotracers have shown great promise in the diagnosis of metastatic lesions in prostate and breast cancer, there is still much debate regarding their specificity and consistency. Continual trials to assess the value of currently available small-molecule PET tracers are warranted to solidify their roles in managing metastases. Meanwhile, scientists should continue the search for new or more selective tracers for cancer detection.

CONCLUSION

Molecular imaging holds great promise to expose the hidden and often deadly process of cancer progression and metastasis. Many different forms of innovative imaging probes are under development in preclinical stages, from the viral vectors and nanoparticles of about 100-nanometer size, to biologic agents such as antibodies, to small-molecular radioactive tracers. In general terms, the larger viral vectors possess more functional capabilities and thus can be designed to produce highly selective imaging signals. However, their immunogenicity and poor biodistribution in circulation hinder their delivery to tumor sites. The clinical translation of viral vector-based molecular imaging faces a significant roadblock. Nanoparticle-based imaging is anticipated to gain in popularity at a rapid pace in the near future, driven in part by the great interests in

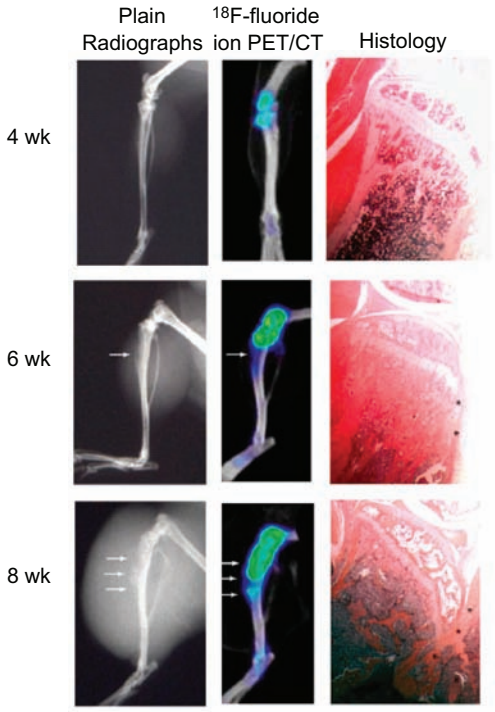

Figure 46.12. ^{18}F-fluoride PET-CT to detect bony metastasis. Plain radiographs, corresponding PET/CT images, and histologic specimens of representative LAPC-9 tumors using ^{18}F-fluoride ion at 4-, 6-, and 8-week time points. Although bone formation is visible on plain radiographs at the 6- and 8-week time points (arrows), a progressive increase of ^{18}F-fluoride ion uptake, indicating osteoblastic activity, is well visualized on successive PET images beginning at 4 weeks after tumor cell injection (arrows). Increasing signal intensity on PET scans corresponded to new bone formation seen on high-power histological analysis (asterisks). (From Hsu et al. [127], reprinted with permission from Society of Nuclear Medicine.)

the nanotechnology field to create a smart delivery vehicle. Active investigations will be needed to substantiate the in vivo performance capabilities of nanoparticles.

As a class of imaging agent, engineered antibody probes hold realistic promise for clinical translation, especially as there are continual advances in radiolabeling chemistry and in protein production to high clinical grade standard. Following the advances in antibody-mediated cancer therapeutics (e.g., trastuzumab and bevacizumab), the emergence of this class of imaging agent is on the horizon.

The clinical translation of small-molecular imaging tracers is most straightforward among the four groups of agents discussed. The bottleneck in this area lies in developing novel and more selective molecular probes that can reveal the functional status of critical regulatory pathways in cancer. Because of the heterogeneity of human cancers and host cells, the full clinical utility of small-molecular probes, like molecular targeted pharmaceutical agents, is difficult to predict in preclinical models and requires careful assessment in human patients. The radiolabeled probes are used at tracer doses, which are usually two to three orders of magnitude below the level to produce pharmacological effects. Hence, the current regulations to test imaging probes in pilot clinical studies are less stringent and time-saving compared with those for traditional pharmaceutical agents. This situation predicts that more novel molecular imaging tracers will be forthcoming.

The future of molecular imaging is very bright, as ongoing multidisciplinary research efforts involving clinicians and scientists from diverse fields of oncology, nuclear medicine, chemistry, and molecular biology promise to bring rapid advances to the field. The ability to widely, consistently, and sensitively detect cancer metastasis by a noninvasive imaging modality lies ahead in the near future.

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47 Preserving Bone Health in Malignancy and Complications of Bone Metastases

Robert E. Coleman

BONE COMPLICATIONS IN EARLY DISEASE

Recent advances in adjuvant therapy for early-stage cancer present new challenges in the management of bone health in cancer patients. For example, in breast cancer, aromatase inhibitors (AIs) have demonstrated superior efficacy over tamoxifen. However, the use of an AI is associated with increased bone loss and incidence of fracture.[1–4] Similarly in prostate cancer, androgen deprivation therapy (ADT), through surgical castration or with the use of gonadotropin-releasing hormone (GnRH) analogs, has been shown to correlate with a fall in bone mineral density (BMD) and an increased fracture rate.[5,6] Thus, as these therapies become more commonly used for patients with early-stage disease, preservation of bone health with bisphosphonates and other bone-targeted agents may play a key role in overall disease management for patients receiving adjuvant therapy.

BONE LOSS AND FRACTURE RISK

The rate of bone loss naturally increases with age, with one in three women over the age of fifty years sustaining an osteoporotic fracture of the wrist, hip, or vertebrae.[7] However, although the absolute risk of an osteoporotic fracture increases with decreasing BMD, an analysis of nearly 150,000 healthy postmenopausal women found that 82 percent of fractures occurred in nonosteoporotic women (T-score $\geq$2.5).[8] As a result, the World Heath Organization (WHO) working group has identified risk factors other than low BMD for osteoporotic fracture; these include increasing age, female sex, smoking, personal history of fracture over the age of fifty years, a parental history of hip fracture, a low body mass index (<20 mg/m^2), consumption of more than three units of alcohol per day, corticosteroid use, and other diseases such as rheumatoid arthritis.[9] These can be used in fracture prediction with or without information on BMD.

Although peak bone mass is, on average, higher in men, and the accelerated rate of bone loss seen in the sixth decade in women as a result of menopause does not occur, age-related bone loss still takes place, and osteoporosis is an increasingly important health care problem in men, with the risk factors identified by the WHO applying also to men. Additionally, the prognosis in men after a fracture, especially of the hip, is worse than that in women, with 30 percent mortality and a significant proportion of the remaining 70 percent requiring long-term institutional care after sustaining a hip fracture.

A valuable screening tool for osteoporotic fracture risk assessment (for men and women) is FRAX, developed by the WHO Collaborating Center for Metabolic Bone Diseases.[7] This tool gives a ten-year probability of fracture risk using the WHO clinical risk factors, identified from previous meta-analyses, either alone or combined with the BMD T score, if a dual energy X-ray absorptiometry (DXA) scan is available, to give a ten-year fracture risk. FRAX also takes into account the country of origin of the patient and the patient's cultural identity (in the United States only) but does not advise on treatments, which should be based on clinical judgment. It is a very useful adjunct in the clinic, especially if DXA is either not available. Assessment can be done online at www.shef.ac.uk/FRAX.

Early Breast Cancer

The management of early breast cancer has become increasingly successful, leading to ten-year survival rates of around 80 percent to 85 percent. Approximately 75 percent of breast cancers express estrogen binding receptors; these are usually managed in the adjuvant

setting with targeted hormonal agents plus, if indicated, cytotoxic chemotherapy. These treatments are not without side effects; of particular relevance is their impact on skeletal health.

Until recently, tamoxifen was the cornerstone of hormonal therapy in postmenopausal women. However, AIs have now largely superseded tamoxifen in this treatment setting. Large randomized adjuvant trials have shown superiority of the AIs over tamoxifen in terms of disease-free survival, cancer recurrence, and a number of important toxicities, such as thromboembolic and endometrial complications.[1–4] As a result, most postmenopausal hormone–receptor-positive early breast cancer patients now receive an AI as part of their management, either up front for five years, or following two to five years of treatment with tamoxifen. An AI is also the treatment of choice for women receiving primary endocrine therapy either instead of or prior to surgery.

Postmenopausal women normally retain a low level of circulating estrogen, resulting from the conversion of androgens to estrogen in peripheral tissues by the enzyme aromatase. These postmenopausal levels are important for the maintenance of bone health and are inversely correlated with the risk of fragility fractures.[10] Aromatase inhibition by both nonsteroidal reversible and steroidal irreversible inhibitors reduces residual circulating estrogen to nearly undetectable levels. Because estrogen is important for bone health, the very low levels of estrogen induced by the AIs have, as expected, a negative impact on skeletal health.

All AIs are associated with accelerated bone loss. As part of the Arimidex, Tamoxifen Alone, or in Combination (ATAC) trial, a bone substudy evaluated changes in lumbar spine and total hip BMD.[11] In anastrozole-treated patients, there was a reduction in BMD at five years of 6.1 percent at the lumbar spine, and 7.2 percent at the hip, as compared with an increase in BMD of 2.8 percent and 0.7 percent with tamoxifen at the same sites. The most recent 100-month analysis of ATAC[12] showed an increased annual rate of fractures in the anastrozole group compared with the tamoxifen group of 2.93 percent versus 1.9 percent, respectively ($p < 0.0001$), while on treatment. Following completion of treatment, fracture rates became similar in the two groups. Similar changes in BMD and increases in on-treatment fracture rates have been reported in other studies, both with anastrazole[13] and the other widely used AIs, letrozole[14] and exemestane.[15]

The skeletal effects of the different AIs appear to be similar, with the clinical impact of using an AI depending more on the duration of exposure and incorporation (or not) of tamoxifen into the long-term adjuvant therapy program, rather than the choice of agent.[15] This observation is supported by the Letrozole, Exemestane, and Anastrozole Pharmacodynamics (LEAP) study in postmenopausal women, in which the on-treatment profile of biochemical markers of bone resorption and formation was similar with all three agents.[16]

Management of Aromatase Inhibitor-Induced Bone Loss

All women prescribed an AI should undergo assessment of bone density. DXA scanning of the hip and lumbar spine is the preferred imaging technique, as it is generally available, sensitive, and accurate. Other techniques, such as quantitative computed tomography, quantitative ultrasound, high-resolution magnetic resonance imaging, and ultrasound transmission velocity densitometry, are not currently used routinely but may have scope for the future.

The 2003 American Society of Clinical Oncology (ASCO) guidance on the role of bisphosphonates and bone health issues in women with breast cancer include a management algorithm for treatment-induced bone loss that uses only BMD, as measured by DXA scan, to determine treatment recommendations.[17] However, all women about to start treatment with an AI should also be clinically assessed for the risk factors for fracture outlined earlier. These issues are addressed by recently published European[18] and British guidelines[19] and are further discussed below, along with recommendations for treatment with bisphosphonates.

Both sets of guidelines recommend that older women (Europe <65 years, UK <75 years of age) who require an AI should also receive a bisphosphonate for bone protection if they have one or more risk factors (other than being female) for osteoporotic fracture, regardless of BMD score**.** These women are also at greater risk for having secondary osteoporosis, and should be investigated for this. If the patient is deficient in calcium, supplements of calcium and vitamin D should be prescribed. Suboptimal vitamin D levels are very common in women with breast cancer, particularly in the elderly, as housebound women are even more prone to vitamin D deficiency.[20]

Management of all other postmenopausal women depends on BMD, which should be measured by DXA within three months of starting AI therapy (Figure 47.1). Three groups are defined: high risk (T score < –2), medium risk (T score between –1 and –2) and low risk (T score > –1). Low-risk patients can be reassured and do not require any specific additional monitoring, although the more cautious European guidelines recommend repeat DXA after two years.[19] At the other end of the spectrum, the high-risk group should receive bone protection with a bisphosphonate, along with calcium and vitamin D supplements, and be given lifestyle advice regarding diet, smoking, and exercise. Patients of medium risk require regular monitoring of BMD every one to two years in addition to lifestyle advice,

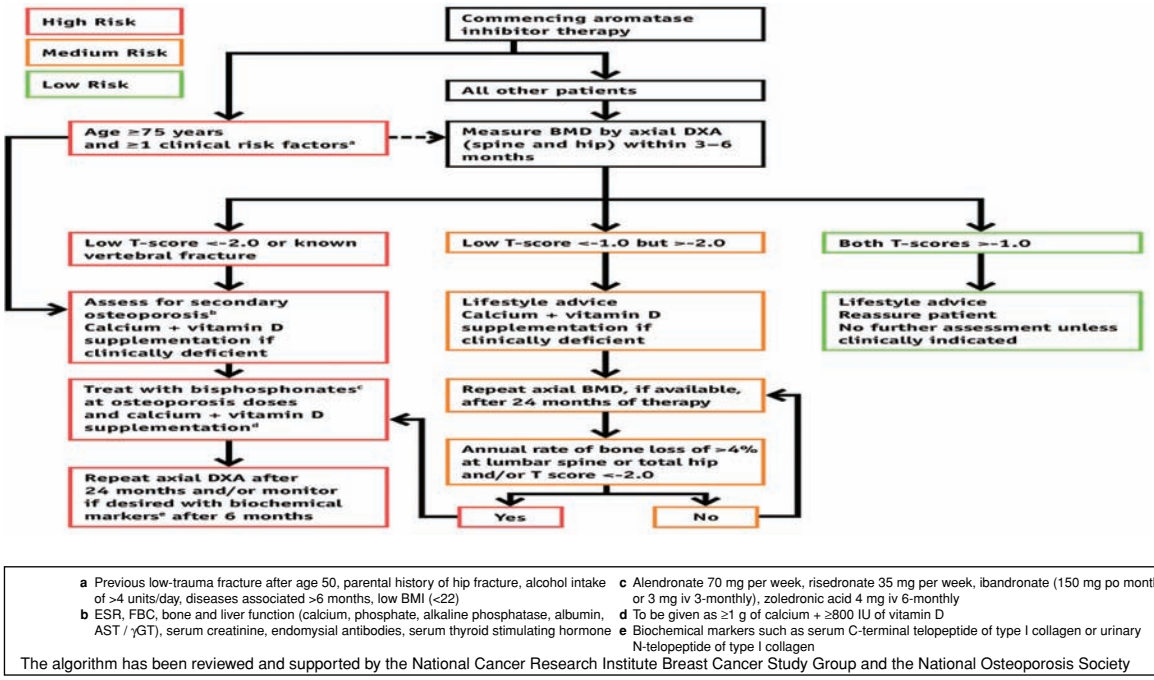

Figure 47.1. UK guidance algorithm on management of bone loss in postmenopausal women receiving an aromatase inhibitor for early breast cancer.

plus calcium and vitamin D supplementation if necessary. If BMD drops below T = –2 or the annual rate of bone loss exceeds 4 percent to 5 percent, then bone protection with a bisphosphonate is recommended.

Bisphosphonates for Treatment of Aromatase Inhibitor-Induced Bone Loss

Three large zoledronic acid and letrozole synergy trials (Z-FAST, ZO-FAST and E-ZO-FAST; $n > 2000$) to assess the efficacy of the bisphosphonate zoledronic acid in the prevention of AI (letrozole)-induced bone loss, have shown that this agent effectively inhibits letrozole-induced bone loss.[21–23] Postmenopausal women with either normal BMD or osteopenia (T score > –2) requiring adjuvant letrozole were randomized to immediate or delayed zoledronic acid (4 mg intravenously every six months). The delayed group received zoledronic acid when the BMD score at the lumber spine or hip decreased to less than –2, or they incurred a nontraumatic (osteoporotic) fracture. In all three trials, mean BMD increased in those receiving immediate zoledronic acid and fell in those in the delayed group. At twelve months, the differences in lumbar spine BMD between the groups were 4.4 percent in Z-FAST, 5.7 percent in ZO-FAST, and 5.4 percent in E-ZO-FAST. Similar benefits in hip BMD were also seen. Subsequent analyses have confirmed that these BMD advantages to immediate zoledronic acid are maintained at two to three years. To date, no significant differences in fracture incidence between the two treatment strategies have emerged. However, follow-up is short and the absolute number of fractures small; continued observation of the fracture rate is required to define who really needs prophylactic treatment.

The oral bisphosphonate, risedronate, already globally approved for the treatment of postmenopausal osteoporosis, has also been investigated in postmenopausal women with early breast cancer receiving anastrozole, as part of the SABRE (**S**tudy of **A**nastrozole with the **B**isphosphonate **R**isedronat**E**) study.[24] Two hundred thirty-four women were first stratified by baseline hip and lumbar spine T score. Patients who were osteopenic ($n = 154$), and so at moderate risk of fracture, were enrolled in a double-blind fashion and randomized to anastrozole and risedronate (35 mg weekly) or anastrozole and placebo. Risedronate increased BMD at twelve months in the osteopenic group by 1.7 percent, whereas those receiving placebo had a decrease in BMD of 0.4 percent.

Similarly, the ARIBON (prevention of anastrozole-induced bone loss with monthly oral ibandronate during adjuvant aromatase inhibitor therapy for breast cancer) study[25] evaluated the efficacy of monthly oral

ibandronate on BMD in postmenopausal women taking anastrozole for early breast cancer. Patients were stratified according to baseline BMD at the lumbar spine and total hip. All patients received anastrozole and calcium and vitamin D supplements daily. Further management depended on baseline BMD; fifty women identified as osteopenic (T score > –2.5 and < –1.0, either at hip or spine) were randomized to receive oral ibandronate (150 mg every 28 days, $n = 25$) or identical-in-appearance placebo tablets, every 28 days ($n = 25$). For osteopenic patients treated with anastrozole and ibandronate, the increases in BMD at the lumbar spine and total hip were 2.8 percent and 0.5 percent, respectively, at two years. For the placebo group, the changes in BMD at two years were –3.4 percent and –4.0 percent at the spine and hip, respectively. All differences between the treated and placebo groups were statistically significant ($p < 0.01$).

Clodronate has not been investigated in the treatment of AI-induced bone loss but has been shown to increase BMD in postmenopausal women receiving antiestrogens (tamoxifen or toremifene), compared with women receiving antiestrogens alone.[26] Alendronate has been approved for treatment of postmenopausal osteoporosis and has also been investigated in postmenopausal women being treated for early breast cancer. Because these studies were small or did not show statistically significant results, definite conclusions on the use of this agent in the cancer setting cannot be made.[27]

Denosumab

This agent is a fully humanized antibody to receptor activator of nuclear factor kappa B ligand (RANKL). In women receiving treatment with an AI, denosumab was highly effective in preventing bone loss, with a 7.6 percent difference in spinal BMD at two years between the active-treatment and placebo-treated patients.[28]

Early Prostate Cancer

The use of ADT as primary therapy or as adjuvant treatment after radiotherapy for localized prostate cancer has increased dramatically in the past twenty years, especially among elderly men. However, ADT is associated with a rapid fall in BMD. Additionally, because low BMD is prevalent among men diagnosed with prostate cancer, the clinical impact of further bone loss is significant.[29] Indeed, a decade ago, a startling increase in fracture rates was identified among prostate cancer patients who had received surgical ADT (orchiectomy).[5] Two large retrospective analyses of national and medical claims databases ($N = 50{,}613$ and $N = 12{,}120$, respectively) illustrated that fracture risks also increase significantly during ADT with GnRH agonists,[30,31] with the risk increasing with both age of the patient and cumulative number of doses received ($P < 0.001$).[30]

In addition to loss of mobility and functional independence, fractures have been associated with decreased survival in men with prostate cancer. One retrospective analysis ($N = 195$) reported an almost forty-month survival decrease in men with prostate cancer who experienced a fracture versus men who did not.[32]

Bone health management has become an important consideration throughout the continuum of care in prostate cancer, and management of bone loss is now an integral component of the ADT guidelines produced by specialist organizations, including the European Association of Urology (EAU)[33] and the National Comprehensive Cancer Network (NCCN) in the United States.[34]

Treatment of Androgen–Deprivation-Related Bone Loss

Lifestyle changes and supplementation with calcium and vitamin D are not sufficient to prevent BMD loss during ADT; therefore, bone-targeted treatments have been evaluated recently and have demonstrated promising activity. Pamidronate (60 mg every three months) significantly prevented ADT-associated BMD loss versus placebo during a small one-year trial ($N = 47$),[35] and oral alendronate (70 mg once weekly) has demonstrated similar efficacy in two separate trials in men receiving ADT for nonmetastatic prostate cancer ($N = 47$ and $N = 112$).[36,37] In four randomized controlled trials in men with nonmetastatic prostate cancer receiving ADT ($N = 106$, 120, 200, and 215, respectively), zoledronic acid (4 mg iv every three months for one year) consistently prevented BMD loss at the lumbar spine and total hip and was significantly superior to placebo ($P \leq 0.001$ for all).[38–41] Additionally, in a small study in men undergoing ADT ($N = 40$), a single dose of zoledronic acid (4 mg) appeared sufficient to prevent BMD loss versus placebo at twelve months ($P \leq 0.004$ at all sites); however, because of the accelerated bone turnover associated with ADT for prostate cancer, biochemical markers of bone metabolism began to increase toward baseline at three months after zoledronic acid treatment, suggesting that once-yearly dosing may be insufficient to fully compensate for the effects of ADT on bone.[42]

Denosumab has also been evaluated in men receiving treatment, on the rate of fractures, as well as changes in BMD and bone biomarkers. This agent is highly effective when given as a 60-mg subcutaneous injection every six months, and has been approved

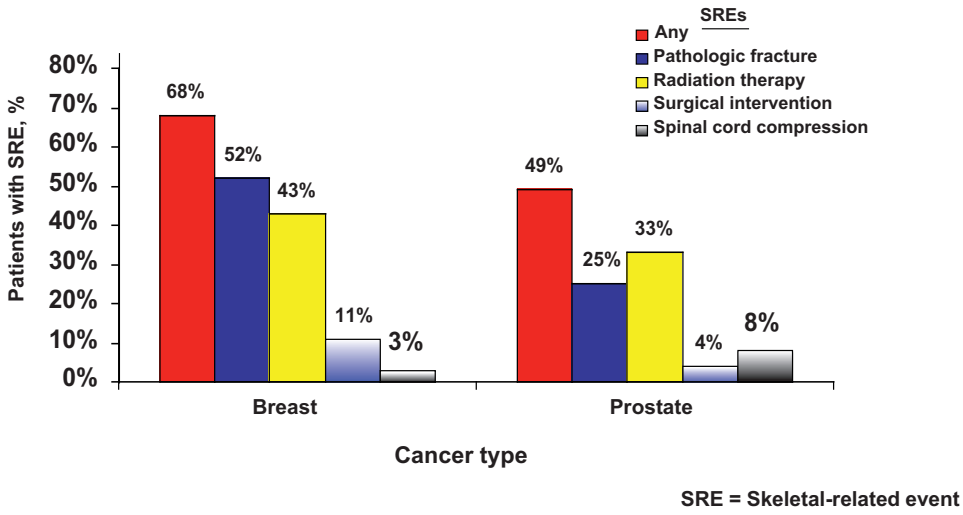

Figure 47.2. Skeletal event rates in patients with bone metastases from breast or hormone-refractory prostate cancer in the absence of bisphosphonates (maximum duration of observation 24 months).

by the regulatory agencies for this specific indication, unlike the bisphosphonates that are used "off-label."

METASTATIC BONE DISEASE

The cellular interactions between tumor and bone in patients with bone metastases profoundly alter bone homeostasis; this may subsequently lead to decreased skeletal integrity. These changes place patients at high risk for potentially debilitating and life-limiting skeletal complications, such as pathological fractures, spinal cord compression, and hypercalcemia of malignancy, as well as often resulting in a need for surgery or palliative radiotherapy to the bone (Figure 47.2).[43] For example, in patients with bone metastases receiving typical anticancer treatments but not a bisphosphonate, pathological fractures were experienced by 52 percent of patients with breast cancer ($n = 387$),[44] 22 percent of patients with hormone-refractory prostate cancer (HRPC; $n = 250$)[45], and 46 percent of patients with non–small-cell lung cancer (NSCLC) or other solid tumors ($n = 250$).[46] After a patient experiences a first skeletal event, the risk of subsequent events increases further. On average, in the absence of bone-directed therapy, the number of events per year is 3.7 in breast cancer, 1.47 in hormone HRPC,[45] and 2.7 in NSCLC.[46]

Causes of Bone Metastases

Bone is a fertile soil for metastatic tumor growth. Bone metastases are most common in the axial skeleton and limb girdles, thought to be as a result of the drainage of blood via the vertebral–venous plexus. Additionally, and of considerable importance, are the biological and molecular characteristics of tumor cells that promote their colonization in the bone microenvironment. Furthermore, the interactions between tumor and bone cells in the bone microenvironment are critical to the development of metastasis and thus the focus of intense research.[47]

The presence of tumor cells within the bone (marrow) microenvironment destroys the normally balanced coupling of osteoclastic bone resorption and osteoblastic bone formation. Release of tumor-cell–derived factors, such as PTHrP, and a variety of growth factors and cytokines stimulate osteoclastic activity, leading to accelerated bone resorption and the formation of lytic and destructive bone lesions. This occurs mainly through the osteoblastic activation of RANKL and subsequent binding to its receptor, RANK, on osteoclasts (Figure 47.3).

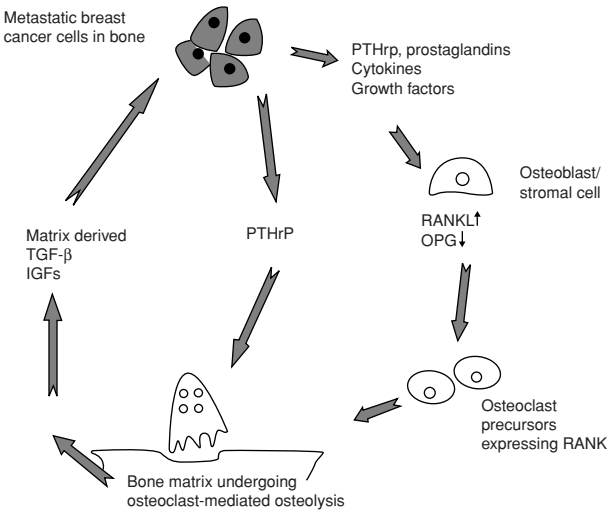

Figure 47.3. Metastatic bone disease – schema of intracellular interactions.

Because of the increased rate of bone turnover, there is excessive release of bone-derived growth factors from the bone matrix; this, in turn, may stimulate tumor growth. This creates the formation of a self-sustaining vicious cycle of cancer-induced bone disease. Inhibition of bone resorption and blockade of these molecular pathways within the vicious cycle have become therapeutic targets and strategies in both the treatment of bone metastases and, more recently, in their prevention.

Skeletal Morbidity

Complications of malignant bone disease are associated with consequences that include reduced quality of life and increased health care costs.[48] The occurrence of a pathologic fracture or radiation to bone has been shown to significantly decrease patients' physical and emotional well-being,[49] and after a fracture in a weight-bearing bone, patients report an ongoing lack of confidence in performing daily activities.[50] Skeletal-related events also contribute to increased health care costs in patients with bone metastases from cancer. Treatments to prevent skeletal complications, such as bisphosphonates, have been shown by cost analyses to significantly reduce this economic burden of malignant bone disease.[51]

Specific Complications

Bone Pain

Many patients with metastatic skeletal disease experience severe bone pain, and this remains a clinically challenging problem to treat rapidly and effectively. The pathophysiology of cancer-induced bone pain (CIBP) is not well understood, although animal models have revealed potential mechanisms that may be used as strategies for targeted therapies.[52] CIBP may be caused by a combination of a neuropathic-type nerve injury, direct tumor compression or ischemia, and sensitization of peripheral nociceptors or primary afferent neurons as a result of the release of a variety of growth factors and cytokines, such as prostaglandins, endothelins, and TGF-β.

In general, the treatment of CIBP initially requires analgesics, starting with nonopioid analgesia, including paracetamol and nonsteroidal antiinflammatory drugs, followed by opioids, and the addition of adjuvant analgesics when appropriate, such as glutamate inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. Beyond analgesics, treatment for metastatic skeletal disease includes external beam radiotherapy, radioisotopes, local surgery, and systemic therapies, including endocrine treatment, chemotherapy, and bisphosphonates. The choice of therapy often depends on whether the disease is focal or widespread, and on the presence or absence of visceral metastases. The clinical course may be characterized by periods of disease response or stability, interspersed by progressive disease, at which point changes in therapy are warranted in an attempt to gain disease control. However, ultimately, resistance to treatment is inevitable.

External beam radiotherapy is an effective treatment for metastatic bone pain, with up to 80 percent of patients experiencing some pain relief and approximately one-third achieving complete pain relief.[53] The optimal radiotherapy regimen has been debated over several years. Numerous published studies and meta-analyses have reported equal efficacy in terms of pain relief between single and multiple fractions in the management of painful bone metastases.[8] However, there appear to be a somewhat higher retreatment requirement and pathological fracture rate with the use of single large fractions. Despite this, many patients prefer the convenience of a single-fraction radiotherapy regimen, and this should be the preferred schedule, especially in frail or elderly patients with coexisting comorbidities. In patients who have disease extending widely within one region of the body, wide-field radiotherapy may be suitable, although this is invariably associated with significant toxicities.

The use of radioisotopes such as strontium-89 and samarium-153 has been shown to reduce bone pain in the palliative setting in patients with widespread painful bone metastases.[54] Radioisotopes are administered systemically and preferentially bind to the bone matrix at sites of active bone turnover. Most of the published data relate to studies in hormone-resistant prostate cancer, but a number of trials in breast cancer patients have been reported that indicate both improvement in pain and a reduction of analgesic use with acceptable hematological toxicities. Repeated dosing is possible, and further research in breast cancer patients is certainly warranted. In addition, novel agents such as radium-223, an alpha particle emitter, show great promise, with the potential to not only relieve bone pain but also modify the course of the disease.[55]

Prevention and Treatment of Fractures

Metastatic destruction of bone causes a reduction in load-bearing capability, and the accumulation of microfractures leads to pathologic fractures, most commonly occurring in ribs and vertebrae. However, the most devastating disabilities of metastatic skeletal disease are fracture of a long bone or epidural extension of the tumor into the spine. These represent the most significant complications that require surgical intervention, with the main aims of surgery being to relieve pain, provide structural stability, restore mobility, and, in the case of vertebral metastases, reduce neurological

deficit or risk of nerve compression. Increased attention is focused on the prediction of long-bone metastatic sites at risk of fracture, and in these patients, referral to an orthopedic surgeon should be considered to evaluate the need for prophylactic surgery. Prophylactic internal fixation should be generally followed by radiotherapy to maximize disease control and prevent disease progression around the implant.

Fractures are most common through lytic metastases, especially in weight-bearing bones, with the proximal femora being the most commonly affected sites. Damage to both trabecular and cortical bone is structurally important, but it is the extent of cortical destruction that is most clearly associated with fracture. Risk factors for fracture that need to be taken into account include pain, the anatomic site and size of a lesion, and its radiological characteristics.[56] Although intensity of pain per se is not clearly associated with fracture risk, pain that is exacerbated by movement appears to be an important factor in predicting impending fracture. Presumably, such functional pain indicates loss of mechanical strength of a bone.

As far as radiologic appearances are concerned, there is a consensus that lytic lesions carry a much higher risk of fracture than either mixed or osteosclerotic lesions. Accordingly, a particularly high fracture rate is found in association with metastases from lung cancer. Given the poor prognosis of this tumor, however, such fractures rarely lead to prolonged disability. In contrast, in breast cancer, which follows a much more protracted course, pathologic fracture is a major cause of prolonged disability.

Radiologic assessment also yields information about the size of a lesion and the extent to which the cortical bone is destroyed. When less than two-thirds of the diameter of a long bone is affected, pathological fracture is relatively unusual, but above this limit the fracture rate increases markedly, with an incidence of approximately 80 percent for such lesions. A practical scoring system incorporating anatomic, radiographic, and symptom-related factors has been described to guide the selection of patients for prophylactic fixation.[57]

Spinal Instability

Spinal instability can cause excruciating mechanical bone pain that is not relieved by radiotherapy or systemic treatment. As with pathologic fractures of long bones, stabilization is required for pain relief, accepting that this may be associated with significant morbidity and mortality. There are several methods for spinal stabilization, but in general, the posterior approach is technically easier and allows stabilization of a larger area of the spine. With careful selection of patients, excellent results can be obtained.

Percutaneous vertebroplasty and kyphoplasty, a new approach to treating spinal pain and instability, involves injecting an acrylic polymer into a diseased vertebral body.[58] The technique was developed initially for the treatment of painful vertebral hemangiomas, and considerable experience with it has been obtained in the treatment of osteoporotic compression fractures. Its use has now been extended to the treatment of malignant spinal disease. The technique provides effective pain relief, which is achieved more rapidly than with radiotherapy, and the procedure confers the added benefit of providing structural support to the spinal column, thus reducing the risk of further vertebral collapse and instability. Although generally safe, vertebroplasty can be complicated by leakage of the polymer, which predisposes to spinal cord or nerve root compression. The risk of this is less with kyphoplasty.[59] These interventional radiological techniques have the potential for wider use, particularly among patients with limited vertebral disease and those for whom major surgical spinal stabilization procedures are unsuitable.

Spinal Cord and Cauda Equina Compression

Spinal cord or cauda equina compression is a medical emergency and requires immediate radiologic assessment and treatment, including high-dose steroids and urgent referral for radiotherapy or surgical decompression and spinal stabilization. Early diagnosis is paramount to enable early intervention and successful rehabilitation.

The choice between surgical decompression and radiotherapy depends on a variety of clinical features. Surgical decompression is indicated for patients with recent onset of symptoms and with progressive paraplegia and urinary retention of less than thirty hours' duration.[60] The site of compression should be localized to no more than two or three vertebral segments, and the patient should have a life expectancy of at least several weeks. For patients in whom the paraplegia has been established for several days or urinary retention has been present for more than thirty hours, surgical decompression rarely results in the recovery of bladder or motor function. Radiotherapy is indicated for those who are either unfit for surgery or do not meet the criteria for surgical decompression and have pain.

Hypercalcemia of Malignancy

Hypercalcemia is an oncological emergency often associated with metastatic bone disease, although demonstrable bone metastases are not always present. Hypercalcemia causes a number of signs and symptoms, which vary considerably from patient to patient. These are often nonspecific, affecting many systems in the

body; they include nausea, vomiting, dehydration, and confusion, and can be mistaken for symptoms of the underlying cancer or associated treatment. If untreated, a progressive rise in serum calcium leads to a deterioration in renal function and decline in the level of consciousness. Death ultimately ensues as a result of cardiac arrhythmias and renal failure.

It is now clear that various mechanisms are involved in the pathogenesis of malignant hypercalcemia. These include increased bone resorption (osteolysis) and systemic release of humoral hypercalcemic factors. In some tumors, such as squamous cell cancers, humoral mechanisms are dominant, increasing both renal tubular calcium reabsorption and phosphate excretion. In others – multiple myeloma and lymphoma, for example – osteolysis predominates, whereas in breast cancer, both osteolysis and humoral mechanisms appear to be important.

Doubt about the etiology of hypercalcemia in patients with cancer is unusual, but nonmalignant causes should be considered, particularly in the absence of metastases. In the community, hyperparathyroidism is the most common cause of hypercalcemia and may be encountered also in patients with cancer. Measurement of parathyroid hormone (PTH) using a modern, specific radioimmunoassay is worthwhile if there is any doubt about the diagnosis; levels of PTH tend to be low or undetectable in malignancy and inappropriately high in hyperparathyroidism.

Intravenous bisphosphonates, in conjunction with rehydration with three to four liters of normal saline over twenty-four hours, are now established as the treatment of choice for hypercalcaemia. Approximately 70 percent to 90 percent of patients will achieve normocalcemia, resulting in relief of symptoms and improved quality of life. Zoledronic acid is the most effective bisphosphonate for the acute treatment of this metabolic emergency.[61] The duration of normocalcemia after an infusion of a bisphosphonate is typically around four weeks, and repeated chronic treatment to prevent recurrence will usually be required.

Treatment of Bone Metastases to Prevent Skeletal Complications

Bone metastases from almost any primary cancer, other than germ cell malignancies and lymphoma, represent incurable disease. However, as the median survival of patients with metastatic disease in the skeleton may be measurable in years, both symptomatic treatment and prevention of longer-term risks of bone-related events form essential parts of clinical care. The primary goals of treatment are to palliate symptoms and reduce the risk of bone events, thereby maintaining quality of life. Patients require input from a multidisciplinary team including involvement of medical and radiation oncologists, radiologists, orthopedic and spinal surgeons, palliative care physicians, and specialist nurses.

Systemic Therapy

Systemic therapy for the treatment of bone metastases can potentially have direct or indirect antitumor effects. Endocrine therapy, cytotoxic chemotherapy, biologically targeted agents, and radionuclides aim to directly reduce skeletal tumor burden and the release of tumor–cell-derived growth factors and cytokines. Alternatively, bone targeted treatment, such as with bisphosphonates, may be aimed at inhibiting the effects of these tumor–cell-derived factors on host bone cells.

Endocrine treatment is the preferred initial treatment option in the treatment of patients with prostate cancer and hormone–receptor-positive breast cancer. Chemotherapy is indicated in patients with hormone-insensitive tumors, in those with rapidly progressing life-threatening disease (other than hormone-naïve prostate cancer), and in patients who progress after endocrine therapy. The use of trastuzumab should be considered in patients with HER2/neu-positive breast cancer. Treatment decisions should be based on the severity of comorbidities and the wishes of the patient.

The chief aim of palliative chemotherapy is relief of symptoms, with pain relief and resumption of functional activity the main priorities. In general, responses to treatment are only partial, with a median duration of response varying from a few weeks in the case of NSCLC to several years in men with hormone-sensitive prostate cancer or in some women with endocrine-responsive advanced breast cancer. Strict on-treatment review of these patients is required to ensure avoidance of overtreatment and to monitor the impact of therapy on quality of life, toxicity, and the need for dose modification and supportive care. Chemotherapy may be potentially hazardous in those with disease-induced poor bone marrow reserve, and the use of hematopoietic growth factors may be required.

Bisphosphonates

Bisphosphonates, as potent inhibitors of osteoclast-mediated bone resorption, have become firmly established in the treatment of patients with bone metastases, and represent the current standard of care.[62] The indications for their use in the metastatic setting are the treatment of hypercalcemia of malignancy, relief of metastatic bone pain, and prevention of the complications of malignant skeletal disease. Guidelines suggest that starting bisphosphonates should be considered in patients with breast cancer as soon as bone metastases are confirmed by radiographs, even in absence of symptoms,[17,63] in castrate-resistant prostate cancer

with bone metastases, and in other solid tumors with symptomatic bone disease.

Clinical trials that have investigated the benefits of bisphosphonates in the setting of bone metastases have used a variety of clinical endpoints. Endpoints such as assessments of quality of life and pain can be affected by subjective bias; therefore, trials have assessed the occurrence of objective bone complications, otherwise known as skeletal-related events (SREs), as a composite endpoint. These are defined as events including pathologic fracture, spinal cord compression, radiotherapy to bone, the need for surgical intervention to prevent or treat bone complications, and hypercalcemia of malignancy.[9] Effective treatments that prevent or delay these events are clearly of clinical importance, having a positive impact on quality of life and clinical outcome.

Numerous clinical trials of bisphosphonates have demonstrated the beneficial effects on skeletal morbidity in patients with skeletal metastases, including reducing the risk and rate of development of a skeletal event and increasing the time to first SRE and rate of subsequent events.[62] Both intravenous and oral bisphosphonates have shown significant clinical benefits. Oral clodronate and ibandronate are approved for the management of patients with breast cancer and bone metastases, and an oral regimen should be considered for patients who are not able to attend regular hospital care.[63] However, oral bisphosphonates are poorly absorbed from the gut and absorption is negatively affected by food intake. Furthermore, oral formulations need to be taken on an empty stomach in the upright position, and patients should continue to fast and remain upright for at least thirty minutes postdosing. There are also significant compliance issues with chronic oral bisphosphonate therapy. Recent recommendations of an international expert panel on the use of bisphosphonates in solid tumors suggest that the intravenous route is preferable, as compliance can be more effectively monitored.[63]

Bisphosphonates in Advanced Breast Cancer

A Cochrane meta-analysis of eight trials including 2276 women with breast cancer and clinically evident bone metastases demonstrated a 21 percent reduction in the risk of developing a skeletal event for patients on bisphosphonate therapy (at recommended dosing) compared with placebo (RR 0.79; 95% CI 0.7 –0.86, $p < 0.0001$),[64] with the greatest risk reduction in SREs observed with zoledronic acid. Further potential clinical benefits include improvement in quality of life and effective reduction in bone pain, although bisphosphonates should be used as an addition to analgesia, rather than as first-line therapy for the treatment of bone pain.

In the only direct comparison in an appropriately powered comparison of two bisphosphonates, zoledronic acid was shown to reduce skeletal complications more effectively than pamidronate in patients with bone metastases from breast cancer. The proportion of patients with at least one SRE was similar for both drugs, but multiple event analysis showed that zoledronic acid significantly reduced the risk of SREs by an additional 20 percent beyond that achieved with pamidronate ($p = 0.025$).[65]

Bisphosphonates for Advanced Prostate Cancer

Bone metastases from prostate cancer are typically osteoblastic in appearance on radiographs. However, increased bone resorption within the metastatic lesions also occurs in addition to the localized increases in bone formation. Therefore, bisphosphonate therapy may provide multiple benefits to men with bone metastases from prostate cancer who are receiving ADT, by delaying the onset of SREs from the bone lesions and protecting the entire skeleton from the systemic effects of ADT and the cancer on bone.

Studies with oral clodronate and intravenous pamidronate had failed to achieve statistically significant benefits in prostate cancer.[62] However, the more potent agent zoledronic acid does achieve important clinical benefits. In 643 patients with HRPC and documented bone metastases, zoledronic acid was significantly more effective than placebo across all primary and secondary endpoints.[45] The zoledronic acid treatment group experienced significantly fewer SRE(s), and the time to first skeletal complication was extended by more than four months ($p = 0.011$). Using the Andersen-Gill multiple-event analysis, it was calculated that 4 mg of zoledronic acid reduced the overall risk of skeletal complications by 36 percent.[66]

Bisphosphonates for Tumors Other than Breast and Prostate Cancers Involving Bone

In the absence of bone-targeted therapies, patients with bone metastases from NSCLC or solid tumors other than breast or prostate cancer experience SREs at a rate comparable with that of HRPC patients who have bone metastases. This is a growing concern in the lung cancer setting because recent advances in treatment have extended survival to almost one year, but the median time to first SRE after the diagnosis of bone metastases is only slightly greater than one month.[48] Therefore, patients are living long enough for the effects of tumor on bone to manifest in potentially debilitating SREs that can reduce quality of life for the duration of their survival. Median survival after an SRE has been reported to be four months[67] but may increase with new therapeutic modalities. Zoledronic acid is the only bisphosphonate that has been formally evaluated in these tumor types.[46] In a randomized trial of

patients with NSCLC or other solid tumors ($N = 773$), zoledronic acid, compared with placebo, significantly reduced the proportion of patients with an SRE (39% vs 48%, respectively; $P = 0.039$), and the overall risk of developing an SRE (by 31%, $P = 0.003$). Zoledronic acid also significantly extended the median time to first SRE by eighty-one days compared with placebo ($P = 0.009$), a meaningful increase in this patient population with a median survival of only six months.[46]

In addition, exploratory analyses have suggested that the addition of zoledronic acid to standard treatment in patients with biochemical evidence of accelerated bone resorption (high levels of the type I collagen breakdown product N-telopeptide) actually improves survival in NSCLC, presumably owing to the prevention of life-threatening skeletal complications and avoidance of a decline in the level of general fitness that precludes the use of chemotherapy and disease-specific targeted agents.[68]

Zoledronic acid has also demonstrated efficacy in renal cell carcinoma and, when compared with placebo, significantly reduced the proportion of patients with SREs (41% vs. 79%; $P = 0.011$), and also delayed bone disease progression (586 days vs. 89 days; $P = 0.014$).[69]

In addition to reducing the risk of SREs from metastatic bone disease, bisphosphonates also significantly reduce elevated pretreatment levels of the biochemical markers of bone metabolism, such as N-telopeptide of type I collagen (NTX) and bone-specific alkaline phosphatase (BALP), in the majority of patients.[70–72] Normalization of bone resorption is an important aim of treatment, with effects on the risks of both further skeletal complications and of dying of the underlying cancer. Elevated levels of bone turnover markers have been shown to be predictive of both death and skeletal complications in patients with bone metastases from prostate cancer, lung cancer, or other solid tumors.[73] Among patients with breast cancer treated with zoledronic acid whose NTX levels normalized on study at three months, survival was significantly longer compared with that of patients who had persistently elevated NTX ($P = 0.0004$),[71] and similar to that of patients with normal baseline NTX. Similar results suggesting a significant correlation between zoledronic acid-mediated normalization of elevated NTX and prolonged survival have also been reported in patients with prostate cancer, lung cancer, and a range of other solid tumors ($P \leq 0.01$ for all).[72]

Optimum Use of Bisphosphonates and Potential Complications

There remains uncertainty regarding the most appropriate duration and schedule of treatment. Factors that need to be taken into consideration include life expectancy, disease extent and the risk of developing a SRE, the logistics and accessibility of treatment for the patient, and treatment cost. The measurement of metabolic bone markers in serum, such as bone alkaline phosphatase (bALP), propeptides of procollagen type 1 (all markers of bone formation), and markers of bone resorption and the breakdown of type I collagen, such as serum C-telopeptide (CTX) and NTX in urine, are of value in predicting skeletal morbidity and clinical outcome and may be useful in selecting patients at highest priority for treatment.[73,74]

Bisphosphonates should certainly not be stopped following the development of a first SRE while on treatment; this should not be considered a failure of treatment, as bisphosphonates show a significant reduction in second and subsequent complications. The clinical trial experience confirms ongoing benefits from treatment up to two years. Beyond this point there are no randomized data on which to base continued treatment. However, further progression of disease would be expected to result in an increase in bone turnover and destruction, so the clinical consensus is that treatment should continue indefinitely.[17,63]

The role of bone-marker–directed therapy to tailor the schedule of administration according to the rate of bone turnover, using markers such as the collagen breakdown product NTX, is currently under investigation in a large prospective randomized Phase III trial known as BISMARK (EudraCT number 2005–001376-12).

Osteonecrosis of the jaw (ONJ) is an emerging problem characterized by the appearance of exposed bone in the maxillofacial region, with failure of healing after six to eight weeks. It is increasingly suspected to be a complication of bisphosphonate therapy, although causation has not been definitively proved,[75] and seems to be particularly related to potency, use of intravenous formulations, and increasing duration of treatment, coincident with the occurrence of a dental intervention, such as a tooth extraction. The risk appears to be approximately 1 percent per year on monthly intravenous therapy. It is therefore recommended, before starting bisphosphonate therapy, that patients be reviewed by a dentist and any preexisting dental problems treated.[76] Simple preventive dental measures have been shown to significantly reduce the risk of ONJ.[77,78]

Bisphosphonates may occasionally result in impaired renal function. This typically takes the form of interstitial glomerulosclerosis with pamidronate and tubular damage with zoledronic acid.[79] It is therefore important that the recommended dose and infusion schedule be carefully followed to minimize the risk of renal damage. Elderly patients may be at higher risk of developing renal impairment owing to reduced hydration and the use of concomitant nephrotoxic drugs, such as NSAIDs and antihypertensives.

The use of concomitant nephrotoxic agents with bisphosphonates should be limited if possible. An International Society of Geriatric Oncology task force[80] recommends that in patients being treated with pamidronate or zoledronic acid, creatinine clearance should be monitored in every patient, even when serum creatinine is within the normal range, with evaluation and optimization of hydration status and review of concomitant medications.

OTHER POTENTIAL TARGETS IN TREATMENT OF METASTATIC BONE DISEASE

We now have a greater understanding of the complex cellular and molecular signaling pathways that occur between bone cells, and between bone cells and tumor cells in the establishment of the vicious cycle of malignant bone destruction. Consequently, drugs inhibiting these targets in the treatment of bone metastases are in development. Osteoclast-mediated bone resorption is regulated by RANKL, and denosumab is a highly promising new agent in the treatment of bone metastases. A four-weekly dose of 120 mg by subcutaneous injection has been defined in a recent Phase II trial in breast cancer patients with bone metastases, and taken through to Phase III studies, in which superiority to zoledronic acid for the prevention of skeletal morbidity has been demonstrated.[81,82] Other potential targets include the inhibition of cathepsin K, a proteinase secreted by osteoclasts that is essential for osteolysis, and PTHrP, an abundant and important osteoclast-activating factor.

SUMMARY

In early cancer, an appreciation of the effects of cancer treatments on bone health and appropriate assessment and intervention in patients at high risk for skeletal complications is required to ensure that cancer survivors can enjoy a high quality of life and an active lifestyle.

Patients with bone metastases from cancer are at significant risk of skeletal morbidity associated with debilitating consequences that complicate the clinical course and contribute to reduced survival. There are important considerations specific to this population, and the optimal management of bone metastases requires an experienced multidisciplinary input to ensure appropriate and timely diagnosis and the coordination of both local and systemic therapeutic strategies.

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48 Role of Platelets and Thrombin in Metastasis

Boris Kobrinsky, Simon Karpatkin, and David L. Green

In response to vascular injury, platelets become adherent and undergo activation and aggregation. The formation of the primary hemostatic platelet plug occurs simultaneously with surface activation of coagulation, leading to thrombin generation and fibrin formation. Platelet function also contributes to thrombus formation in pathologic settings, leading to vascular occlusion, usually in the setting of underlying vascular disease. Platelets contain many biologically active mediators that are released upon activation, including growth factors, coagulation factors, adhesive ligands, proteases, heparanase, cytokines, chemokines, and vasoactive lipids. Other functions of platelets include a supportive role in vascular maintenance and regulation of angiogenesis, as well as putative roles in inflammation and immunity. Indeed, platelets are now linked to such diverse physiologic and pathologic processes as wound healing and tissue regeneration, response to microbial infection, inflammatory diseases, atherogenesis, tumorigenesis, and metastasis.

Platelets contribute to tumor cell metastasis by mediating tumor cell adhesion and subsequent extravasation, stabilizing platelet–tumor cell emboli in the circulation, and protecting tumor cells from the host immune system. In the tumor microenvironment, platelets become activated and may release growth factors, chemokines, matrix metalloproteinases (MMPs), and inflammatory mediators, with resulting production and remodeling of the extracellular matrix and tumor angiogenesis.[1] In this chapter, we review the role of platelets and thrombin in metastasis. We review clinical trial data with aspirin and anticoagulants in cancer and cancer prevention, and speculate on the potential effect of thrombin in unmasking tumor dormancy.

THROMBOSIS IN CANCER PATIENTS

The association of thrombosis with cancer is well recognized; thrombosis is an important cause of mortality and morbidity in cancer patients.[2] A causal connection between thrombosis and cancer[3] is suggested by an observed increased risk of cancer in individuals who present with idiopathic venous thromboembolism (VTE), the majority of whom have clinical manifestations of malignancy within six months of the thrombotic event.[4,5] The prognosis for cancer patients with VTE is significantly worse than that for patients without thrombosis. This does not appear to be accounted for by excess mortality attributable directly to VTE[5,6] but could be explained by the association of hypercoagulability with more aggressive malignancies.[7]

HISTORICAL PERSPECTIVE

Trousseau syndrome, or the hypercoagulability associated with malignancy, was initially described more than 140 years ago.[7] Trousseau discovered an association between migratory thrombophlebitis and occult malignancy and later made the diagnosis on himself. Billroth observed cancer cells within a thrombus[8] and associated this finding with tumor progression. Many studies have confirmed these earlier observations. Thrombosis is a recognized complication of all advanced cancers. The incidence of VTE is highest in cancers of the pancreas, ovary, and brain.[9] Thrombotic complications include thrombophlebitis (often migratory), VTE, stroke, arterial embolism, and nonbacterial thrombotic (marantic) endocarditis.[10] Hemorrhagic complications are also common, especially in cancer patients with associated hypofibrinogenemia, thrombocytopenia, and hyperfibrinolysis.[10] The hypercoagulability of malignancy not only is a function of tumor burden but also varies depending on tumor type.

LABORATORY FINDINGS IN CANCER HYPERCOAGULATION

Activation markers of coagulation are detected in most patients with advanced cancer.[11] Hemostatic

derangements include thrombocytosis,[12] hyperfibrinogenemia, elevated fibrin degradation products,[13] and disseminated intravascular coagulation (DIC). Chronic DIC, which in some cases precedes the cancer diagnosis itself, is a characteristic feature of Trousseau syndrome.[10] Thrombocytosis is an adverse prognostic factor in renal, prostatic, cervical, endometrial, ovarian, gastric, and lung cancer.[14] In contrast, in pancreatic cancer, a low platelet count is an adverse prognostic factor.[14] Increased platelet turnover and reduced platelet survival have also been reported in gastrointestinal, gynecological, lung, lymphoma, and bladder cancers.[15] Thrombocytopenia may be caused by DIC, marrow infiltration, or immune destruction, and commonly is caused by treatment with radiation or chemotherapy. Fibrinogen turnover is increased[16] as are coagulation activation markers fibrinopeptide-A,[17] derived by thrombin cleavage of fibrinogen A chain and prothrombin activation fragment F1+2. One mechanism leading to hypercoagulation in malignancy is the constitutive expression of tissue factor (TF) on most tumor cells. A combination of increased platelet activation with decreased physiologic anticoagulant mechanisms such as tissue factor pathway inhibitor (TFPI), protein C, and antithrombin[18] have also been proposed.

PLATELETS IN METASTASIS

Invasion and metastasis are defining hallmarks of cancer[19] that result in cancer mortality.[20] Cells are shed from the primary tumor and released into the lymphatics or the bloodstream to spawn new colonies. In the case of bloodborne metastasis, tumor cells gain access to the circulation (intravasation), lodge in the microcirculation, and subsequently extravasate into tissues. Tumor cells are able to coopt normal stromal elements in the process. Tumor cells proliferate and induce an angiogenic response, which further accelerates tumor growth.

Metastasis is extremely inefficient, as the vast majority (>98%) of intravenously administered labeled tumor cells are rapidly eliminated.[21] A large body of experimental evidence points to a role for platelets in promoting bloodborne tumor metastasis. Initial observations by Gasic in 1968,[22] confirmed in subsequent studies,[23] demonstrated the importance of platelets and platelet–tumor emboli in experimental tail vein metastasis. In the experimental bloodborne metastasis, tumor cells rapidly become trapped within a platelet-rich thrombus.[24,25] This mechanism may explain tethering and adhesion of platelet–tumor emboli to vascular endothelium,[26] as well as protection from intravascular shear forces. Tumor cells were demonstrated to aggregate platelets in vitro.[27,28] Some tumor cell lines are able to cause transient thrombocytopenia in animals.[27] In some studies, the platelet-aggregating activity was associated with metastatic potential.[27,28] A number of tumor cell lines exhibit a platelet requirement for metastasis.[27–29] Gasic and coworkers first reported that induction of thrombocytopenia significantly reduced the number of lung metastasis found after intravenous infusion of TA3 ascites tumor cells in mice. This effect was negated by reinfusion of platelets.[22] Experiments with intravenous injection revealed tumor cells trapped in the platelet thrombus in the arterioles.[30] The subsequent steps leading to metastasis include tumor cell penetration of endothelial cell tight junctions, interaction with subendothelial matrix, tumor–thrombus evolution, and tumor cell invasion through the subendothelial matrix.

Platelets in cooperation with leukocytes may help to stabilize tumor microemboli and delay their clearance from the blood. In addition, platelets may contribute to metastasis by mediating adhesion to the vascular endothelium, thus facilitating subsequent tumor cell extravasation. Adhesive ligands, chemokines, platelet-associated growth factors, and coagulation factors potentially mediate this process. Provisional matrix can arise as a consequence of local thrombin production and fibrin deposition. Platelets bind tumor cells and protect them from destruction by natural killer (NK) cells.[31,32]

EFFECT OF ANTIPLATELET AGENTS

Numerous studies evaluated antiplatelet agents as a potential therapy to impede cancer growth and metastasis. Results are disappointing, however, with negative studies also reported for aggregation, cyclooxygenase, and phosphodiesterase inhibitors, as well as for prostacyclin.[33–35] More recent studies on tumor cell adhesion using specific αIIβ3 inhibitors have shown promising antimetastatic activity in some tumor models, confirming earlier observations.[35,36] A combined targeting of integrins αIIβ3 and αvβ3 reduces angiogenesis, tumor growth, and tumor metastasis.[36] β3 integrin null mice did not develop osteolytic bone metastases from B16–F10 melanoma cells.[37] Thus, targeting activated αIIβ3 and subsequent platelet aggregation reduces B16 melanoma internal organ metastases in an arterial-based metastasis model.[37]

TUMOR CELL ADHESION

A number of integrins, adhesive ligands, and cell adhesion molecules mediate tumor cell adhesion to platelets and to endothelial cells (Figure 48.1). Under static conditions, CT26 colon carcinoma, B16a melanoma, and murine fibrosarcoma T241 tumor cells bind to platelet αIIβ3 receptor with the help of fibronectin and von

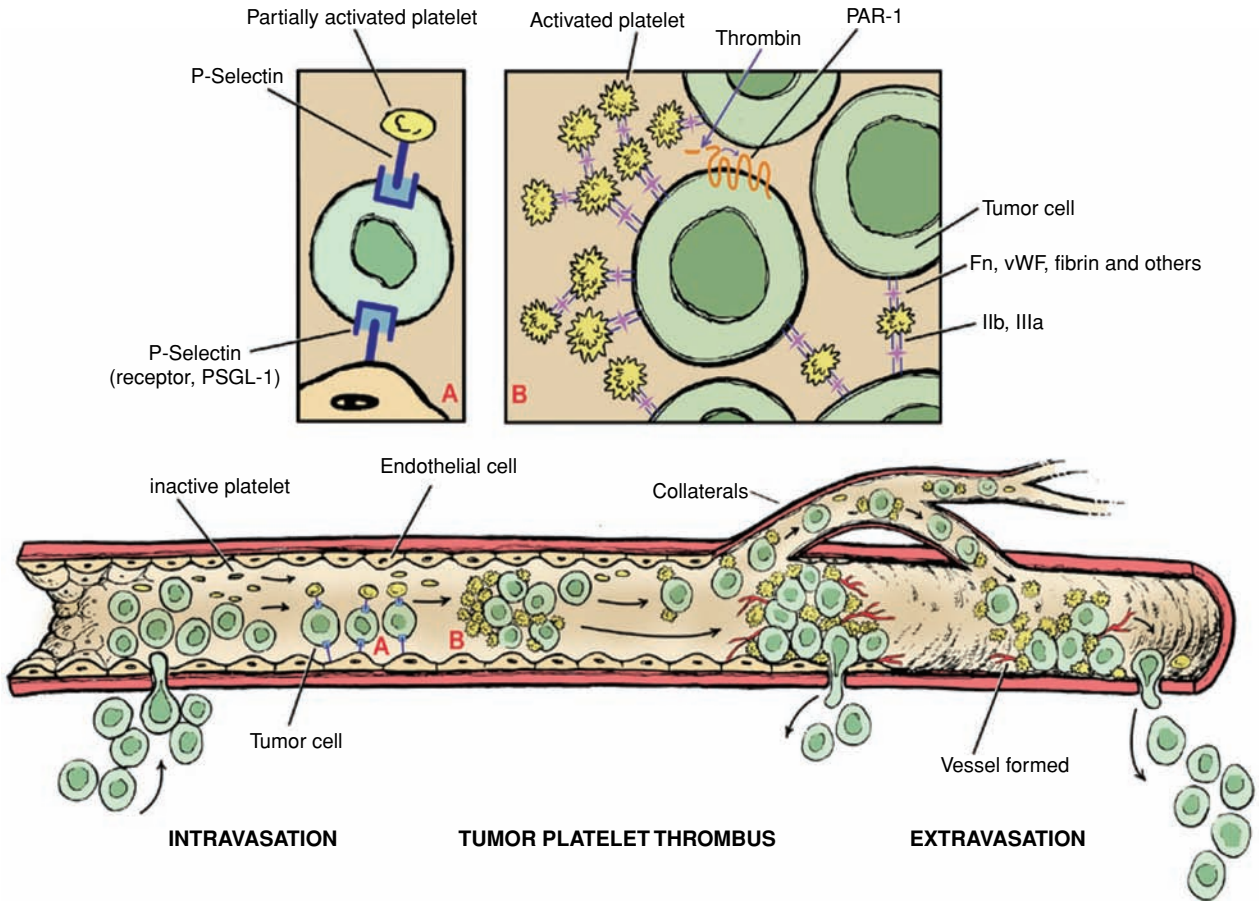

Figure 48.1. Events occurring following tumor cell intravasation, tumor cell–endothelial attachment, thrombus generation, tumor cell–platelet embolization, increased angiogenesis, and tumor cell extravasation. (1) Tumor cells have P-selectin (ligand) on their sur-(2) Thrombin upregulates P-selectin receptor on the surface of endothelial cells. (3) This triggers a weak P-selectin–mediated binding between tumor cells and endothelial cells. (4) Weakly activated platelets also bind tumor cells via P-selectin on their surface. This triggers weak tethering of tumor to endothelium and platelets. A tumor cell–platelet endothelial cell interaction producesthrombin at a more rapid rate, as platelets provide an ideal catalytic surface for thrombin generation. (5) A strong bond between platelets and tumor cellsdevelops, mediated by platelet integrin IIbIIIa binding to tumor integrins via VWF, fibronectin, and other RGDS ligands. (6) Angiogenesis is upregulated via thrombinstimulated synthesis and secretion of VEGF and GRO-α from tumor cells – as well as release of PDGF, VEGF, and ANG-1 from platelets, and increased ANG-2 and KDR in endothelial cells. Activated platelets and tumor cells produce more proangiogenic growth factors than antiangiogenic factors. (7) Platelets protect tumor cells from NK cells, leading to distal embolization and to ischemic and mechanical endothelial wall damage. (8) Tumor cells and platelets bind more readily to a now-damaged exposed subendothelial basement membrane and matrix. (9) Multiple distal tumor emboli result in tumor cellextravasation into the distal organs and neoangiogenesis. Reprinted with permission from Elsevier [3].

Willebrand factor (VWF).[35] Specific targeting of VWF and platelet αIIbβ3 reduces pulmonary metastasis using these tumor cell lines.[35] Immunoglobulinlike receptor Necl-5 facilitates CT26 colon carcinoma cells' interaction with platelets.[38] Necl-5 and αvβ3 are detected in the advancing edges of moving cells. Necl-5 interacts with CD226, a receptor on platelets. In addition, CD226 promotes interaction of thrombin-stimulated platelets with vessel wall.[39]

P-selectin plays an important role in tumor metastasis. Experiments with P-selectin–deficient mice demonstrated a reduction in tumor growth and metastasis.[40] Human colon tumor cell lines LS174HT and COLO205 were shown to gain stable adhesion through initial tethering using P-selectin followed by final adhesion via the αIIbβ3 in the experimental model based on dynamic flow.[41] Tumor cells use selectin ligands to attach to P-selectin on platelets. One mechanism by which heparin inhibits metastasis of human carcinoma is via interruption of P-selectin–dependent adhesion,[42] distinct from its antithrombin effect. Reduction in thrombin generation has been implicated as mediating the antitumor effect of heparin. However, the aforementioned finding points out that another possible mechanism for the heparin antitumor action is its inhibition of adhesion. The importance of platelet leukocyte

interactions for tumor cell adhesion is manifested by the finding that both leukocyte L- and platelet P-selectin are able to promote tumor metastasis.[42]

Soluble fibrin monomer enhances platelet–tumor cell adhesion.[43] Activated platelets stimulate human melanoma cells' $\beta 3$ integrin-dependent binding to collagen I matrix under dynamic flow conditions.[44] The previously described multiple mechanisms underlie the complexity of tumor cell adhesion. In this regard, other adhesive ligands that contribute include laminin,[45] vitronectin,[46] type IV collagen,[47] and thrombospondin,[48] and the numerous integrin receptors, such as $\alpha 3\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$,[46,49] which facilitate attachment to extracellular matrix components and thereby orchestrate adhesion, platelet–tumor interaction, and metastasis.

ROLE OF THROMBIN IN METASTASIS

Among its many actions, thrombin is a potent growth factor for mesenchymal cells[50–52] and a proangiogenic factor[53] that stimulates endothelial cell mitogenesis and migration. The cellular effects of thrombin are mediated by the G-protein–coupled seven-transmembrane-spanning protease-activated receptors (PARs)-1,-3, and -4 (PAR-2 is activated by other proteases but not by thrombin). Thrombin mediates platelet–tumor adhesion in vitro and enhances experimental pulmonary metastasis in vivo.[54] Thrombin activates GPIIb-IIIa and facilitates surface deposition of VWF and fibronectin, linking tumor cells to platelets, and results in their tethering to the vessel wall. Thrombin also exerts effects on tumor cells, which are stimulated to bind to platelets[54] and endothelial cells.[49] Under flow conditions, adhesion of thrombin-exposed human melanoma 397 cells is mediated by P-selectin and GPIIb-IIIa.[55] Thrombin-activated platelets increased HeLa cell migration and invasion. Eptifibatide, a GPIIb-IIIa inhibitor, decreased this effect.[56] Thrombin receptor activating peptide (TRAP)-activated platelets increased the invasiveness of human ovarian cancer cell line SKOV3 cells, which were blocked by prostaglandin E1.[57] Many tumor cell lines express PAR-1.[58] PAR-1 mediates thrombin-stimulated tumor cell motility in metastatic breast cancer.[59,60] Overexpression of PAR-1 in B16 melanoma cells increases experimental pulmonary metastasis fivefold.[61] Thrombin action on tumor cells triggers changes in gene expression that drive a more malignant phenotype. Thrombin promotes GRO-α[62] and Twist[63] gene expression in tumor cell lines B16F10 and UMCL. Gro-α is an important mediator of thrombin-stimulated angiogenesis.[62] Twist promotes tumor growth and angiogenesis.[64] Twist is a transcription factor that regulates embryonic morphogenesis and plays an essential role in murine breast tumor metastasis[65] by increasing cell motility and causing loss of E-cadherin–mediated cell–cell adhesion.[65] Cathepsin D is elevated and secreted by many cancers, particularly breast cancer, and is associated with poor prognosis. It has recently been shown to increase angiogenesis by activating MMP-9 to enhance cancer growth.[66]

The experimental tail vein tumor pulmonary metastasis model has obvious limitations, as it represents a highly artificial system in which a relatively large number of tumor cells are injected as a bolus into the tail vein of a mouse. Metastatic disease in humans, on the other hand, represents the dissemination of an unknown, but likely considerably smaller, cell number continuously into the circulation, with an unknown fraction of these cells actually able to implant at the target organ. In addition, the injection of thrombin does not accurately mimic local endogenous thrombin production at the host–tumor interface. In experiments with spontaneously metastasizing murine breast tumor 4T1, specific direct inhibition of thrombin by hirudin led to a reduction in tumor growth, circulating tumor cells, metastatic potential, and prolongation of survival in tumor-bearing mice.[67]

Recently, the tumor microenvironment has attracted considerable interest because of its great importance for tumor growth and metastases. In this respect, thrombin generation within the tumor microenvironment may lead to more aggressive tumor biology. Indeed, thrombin generation is promoted by tumor cells through significant expression of TF and activation of platelets. TF expression mediates both hematogenous metastasis of melanoma[68] and generation of thrombin. TF and factor VIIa may contribute to tumor growth and metastasis via signaling through the thrombin receptor.[69] Multiple surgical tumor specimens have clearly demonstrated significant surface thrombin activity, as evaluated by hirudin binding.[70]

In summary, local thrombin generation may alter cancer cell gene expression, leading to a more malignant phenotype; this in turn can stimulate a more hypercoagulable state, perpetuating a vicious cycle leading to tumor progression and metastasis. Thrombin is an important mediator in tumor cells' adhesion to endothelium, and activates a proangiogenic switch with a subsequent release of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin (ANG)-1,2 from activated platelets and tumors (VEGF, ANG-2, Gro-α, Twist, cathepsin D).

PLASMINOGEN ACTIVATOR INHIBITOR 1

Plasminogen activator inhibitor 1 (PAI-1), a serine protease inhibitor, regulates fibrinolysis. In addition, it is a regulator of cell attachment, detachment, and migration. As such, it is not surprising that it may play an

important role in a number of disease states, such as cancer.[71] Protease activity is increased in malignant cells. PAI-1 is associated with poor prognosis in breast cancer, as well as in a number of other cancers.[71] The mechanism is unclear and seemingly paradoxical; however, PAI-1 is produced by the stromal cells and may be increased in response to the tumor-cell–associated protease activity. PAI-1 may exert complex effects on tumor angiogenesis, in addition to tumor cell migration. PAI-1 complexes with urokinase-type plasminogen activator (uPA) and its cellular receptor (uPAR). This system initiates signaling events at the cell surface by interaction with other receptors, which may affect cancer growth, invasion, and metastasis.

GENETIC APPROACHES

Experiments with knockout mice have confirmed the importance of platelets in experimental hematogenous metastasis.[29,72] Lung metastasis is markedly reduced in nuclear factor-erythroid 2 (NF-E2) (-/-) platelet-deficient mice.[29] Similar results were published for fibrinogen (-/-) mice and in PAR-4 (-/-) mice with thrombin-unresponsive platelets.[29] Gαq (-/-) mice are deficient in platelet signaling and fail to respond to agonists in vitro.[73] These mice demonstrated protection from both experimental and spontaneous metastasis.[32] Interestingly, neither fibrinogen[72] nor Gαq[32] depletion affects the growth of primary tumors. Hirudin treatment results in an additional reduction in metastasis in PAR-4 (-/-)[29] and fibrinogen (-/-)[72] mice. These findings suggest that thrombin may enhance metastasis by a mechanism that is not completely dependent on platelet interaction and fibrin generation. Mice rendered genetically deficient in VWF develop increased tumor metastasis.[74] Restoration of VWF reduces metastasis in vivo and causes tumor cell apoptosis in vitro.[75] It is therefore possible that VWF may have opposing effects with respect to metastasis, as it has also been shown to promote tumor cell adhesion.

PLATELETS AS REGULATORS OF ANGIOGENESIS

Preservation of vascular endothelial integrity is dependent on platelets.[76] Vascular-dependent organ preservation is improved by infusion of platelet-rich plasma.[77] Also, platelets enhance proliferation of endothelial cells in vitro[78] by release of FGF and VEGF. Platelets serve as a reservoir of angiogenic growth factors, such as ANG-1 and platelet-derived growth factor (PDGF). Platelets enhance endothelial cell sprouting and promote tube formation in Matrigel.[79] Platelets are a major repository for multiple growth factors. Activated platelets are able to provide delivery of growth factors in the local vasculature. Multiple other growth factors were found within platelets; these include hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulinlike growth factor (IGF)-1 and -2, platelet-derived endothelial cell growth factor (PD-ECGF), and TGF-β. Platelets release mediators with complex, and sometimes opposing, effects. Thus, platelets may inhibit angiogenesis by releasing inhibitors of angiogenesis, such as angiostatin, thrombospondin-1, PAI-1, platelet factor-4, and endostatin. In addition, platelets are the important reservoir of bioactive lipids, such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA). Vascular maturation depends on the endothelial differentiation gene (Edg)-1, a G-protein–coupled receptor for S1P.[80] Edg-1 knockout mice are found to be devoid of vascular smooth muscle cells and pericytes, and die in utero secondary to hemorrhage.[80] S1P promotes vascular maturation by mediating N-cadherin function.[81] LPA receptors are found in human breast cancer.[82] In addition, platelet LPA stimulates breast cancer cell line MDA-BO2 osteolytic bone metastases.[82]

The mechanism of regulated release of platelet-derived proangiogenic versus antiangiogenic factors is unknown. In the aggregate, the platelet releasate is proangiogenic.[83,84] Selective PAR-1 agonists exert a proangiogenic effect by inducing VEGF release from platelets while inhibiting endostatin.[85] On the other hand, selective agonist PAR-4 stimulation produces the opposite effects.[85] Thrombin activates PAR-1 and PAR-4 on platelets. The recent finding of the organization of pro- and antiangiogenic proteins into separate α-granules implies that selective platelet granule release is possible.[86] Platelets were found to contain regulators of angiogenic activity that are influenced by tumors and can be considered as a potential biomarker of early tumor growth.[87] Thus, platelets may be important regulators of physiologic and tumor angiogenesis.[88] Platelets are a major source of VEGF,[85] which can enhance vascular permeability and stimulate angiogenesis.

Platelets circulate in close proximity to vascular endothelium in flowing blood but do not adhere to the vascular endothelial cells under normal conditions. Intact endothelial resistance to thrombosis is supported by prostacyclins, heparin-like mucopolysaccharides, and CD39 (ecto-ADPase). Platelets, similar to leukocytes, roll on a stimulated venular endothelial surface in a manner that is dependent on endothelial P-selectin expression.[89] Platelets express P-selectin glycoprotein ligand 1 (PSGL-1) and mediate platelet-endothelial interactions in vivo.[90]

In summary, a large body of preclinical evidence strongly supports the important role of platelets in metastasis through promoting cancer cell adhesion to endothelium at an extravasation site, escape from immune surveillance, and protection from high shear forces in the circulation, and by releasing nutrient growth factors that can support tumor growth.[91]

Thrombin is able to (1) enhance cancer cell adhesion to platelets, endothelial cells, and subendothelial matrix proteins, (2) stimulate tumor cell growth, (3) increase metastasis, and (4) stimulate tumor angiogenesis. These observations form the rationale for clinical trials of anticoagulants in cancer patients.

CLINICAL TRIALS OF ANTIPLATELET AND ANTICOAGULANT AGENTS IN CANCER PATIENTS

The cancer prevention activity of aspirin has been demonstrated in numerous epidemiologic studies.[92–94] Up to a 50 percent reduction in colon cancer was found in aspirin users.[92] Other studies suggest benefit from aspirin in the prevention of cancers of the esophagus, breast, ovary, and lung.[94] The correlation is stronger for prolonged and regular usage of aspirin. However, as of now, aspirin as a single agent has not demonstrated any antitumor activity in clinical trials in cancer patients, nor did it demonstrate any additional benefit as an adjunct to chemotherapy, as was seen in a randomized controlled study of 303 patients with small-cell lung cancer.[95]

On the other hand, there is considerable evidence that various anticoagulants may have antitumor activity in cancer patients with or without thrombosis. In 1984, Zacharski and colleagues[96] were the first to conduct a study on 441 patients with lung, colon, head and neck, and prostate cancer who were treated with warfarin (Coumadin) for a median of 26 weeks. The investigators reported prolonged survival in a subset of patients (50 patients total) with small-cell lung cancer (median survival 23 weeks vs. 49.5 weeks, $P = 0.018$). Chahinian et al. corroborated these findings in a randomized controlled trial with 328 patients with small-cell lung cancer.[97] Warfarin in combination with chemotherapy demonstrated an improved response rate (67% vs. 51%, $P = 0.027$) and a trend toward improved overall survival. Major bleeding complications, however, were more prevalent in the warfarin arm, with four life-threatening and two fatal bleeding events.

Other investigators demonstrated a survival benefit of unfractionated and low-molecular-weight heparin (LMWH) in small-cell lung cancer patients. Improved survival (317 days vs. 261 days, $P = 0.004$) was reported with the use of five weeks of heparin therapy in a trial of 277 patients with small-cell lung cancer.[98] In subgroup analysis, the survival effect was significant for limited-stage disease only. Chemotherapy with or without LMWH in patients with small cell lung cancer[99] demonstrated an improved median overall survival of thirteen months versus eight months ($P = 0.01$) in favor of the LMWH arm.

The Fragmin Advanced Malignancy Outcome Study (FAMOUS) trial randomized 385 patients with advanced cancer to daily LMWH-dalteparin injection or placebo for one year.[100] Although there was no difference in overall survival, a post hoc subgroup analysis revealed a significant survival advantage in favor of LMWH (43.5 months vs. 24 months, $P = 0.03$) for patients with more favorable prognosis who survived more than seventeen months.[100] The Malignancy and Low Molecular Weight Heparin Therapy (MALT) trial was a randomized controlled study with 302 cancer patients treated with six weeks of nadroparin versus placebo.[101] The MALT study demonstrated an overall significant survival benefit of 8 months versus 6.6 months for the LMWH arm ($P = 0.021$). Similarly to the FAMOUS trial, the survival benefit was greater in patients with a life expectancy of six months or more (15.4 months vs. 9.4 months, $P = 0.01$).[101]

Another trial explored the difference between warfarin and LMWH-dalteparin in 602 cancer patients with venous thromboembolism.[102] There was no difference in one-year survival. A subgroup analysis showed a survival advantage in patients without metastatic disease at the time of their venous thromboembolism (80% vs. 64%, $P = 0.03$). A study of 138 patients with advanced malignancy showed no survival benefit from LMWH.[103]

In a recent meta-analysis of eleven studies, anticoagulation demonstrated a reduction in one-year overall survival, with an absolute risk reduction of 8 percent for LMWH and 3 percent for warfarin.[104] In summary, the results of clinical trials in the aggregate suggest a modest antitumor effect of anticoagulants, especially LMWH, in patients with less advanced malignancies.

THROMBIN AND TUMOR CELL DORMANCY

A substantial number of microscopic or in situ clinically silent cancers of the prostate, thyroid, and breast, among others, have been reported at autopsies, implying that cancer cells can exist in a dormant state. Shulman and Lindmarker[6] studied patients with deep vein thrombosis treated for either six weeks or six months with warfarin and found cancer in 66 of 419 patients in the six-week group versus 45 of 435 patients in the six-month group (odds ratio 1.6, 95% CI 1.1–24, $P = 0.02$). The most striking difference was found in the incidence of urogenital cancers. We speculate that the inhibition of thrombin deters cancer from becoming clinically significant. In the second Northwick Park Heart Study, 3052 middle-aged men were examined for correlation between hypercoagulability and coronary artery disease.[105] Although the above association was not reported, the surprising finding was a large incidence of cancer-related death (11.3 vs. 5.1 per thousand person-years [$P = 0.01$]) in the group of patients with persistent activation of coagulation manifested by fibrinopeptide A and prothrombin activation fragments 1 + 2. The risk of cancer-related death was highest

for cancers of the digestive tract (relative risk 3.26, $P < 0.001$). Interestingly, in this study, the median time interval between onset of hypercoagulation and diagnosis of malignancy was approximately five years.

These studies suggest an association between thrombin activation and tumor cell dormancy. We hypothesize that persistent activation of coagulation may convert otherwise dormant tumor cells to a more biologically aggressive phenotype. The reason for persistent thrombin activation remains elusive at this time, although age and genetic factors may contribute.

CONCLUSION

Primary anticoagulant therapy has not been adopted as standard of care because of variability in the clinical trial data and legitimate concerns regarding the risk of major bleeding complications. The antineoplastic activity of novel anticoagulants should also be examined. To address the limitations of earlier clinical studies, activity in single tumor types and effects of treatment in the early-stage or minimal residual disease setting should be evaluated. Still another approach under development is targeted inhibition of PARs.

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49 Cancer Nanotechnology Offers Great Promise for Cancer Research and Therapy

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NANOTECHNOLOGY

Nanotechnology, sometimes shortened to "nanotech," refers to a field of applied science whose goal is to control matter on an atomic and molecular scale. Nanotechnology is an extremely diverse and multidisciplinary field, ranging from novel extensions of conventional-device physics to completely new approaches based on molecular self-assembly and to developing new materials with sizes ranging from 0.1 to hundreds of nanometers. A nanometer is one-billionth of a meter (10^{-9} m), which is about ten times the size of the smallest atom, hydrogen, and approximately 1/80,000 the width of a human hair. As Richard Feynman's famous statement that "there is plenty of room at the bottom" [1] portends, nanotechnology has the potential to create new materials and devices in the nanoscale range with wide-ranging applications in medicine, electronics, and energy production.

The human cell is 10,000 to 20,000 nm in diameter. Cellular proliferation and replication operate at the nanometer scale, thus demonstrating the need to translate molecular-based science into machines or devices matching the size of molecules in biology. There are several advantages to designing devices of this size in every industry imaginable. The computer chip industry has vastly expanded computational speed by decreasing the size and increasing the number of transistors per chip. The reduction in the size of key elements, down to about 100 nm, is possible because of improvements in photolithography that characteristically reduce the cost of production. Controlled chemical etching has aided in the development of nanopore filters, sheets of polycarbonate with pore sizes ranging from 15 nm to 12,000 nm. The ability to manipulate the size and structure of pores produces filters that are robust, uniform, and capable of blocking the passage of bacteria and perhaps even viruses. The discovery of quantum dots and the ability to manipulate their light emission by changing their size are key proponents of nanotechnology. These nanocrystals are used not only in biological research as markers for particular kinds of cells, but also in the development of the next generation of white-light–emitting diodes [2].

NANOPARTICLES AND PLATFORMS

Nanoparticles are prepared with organic polymers or inorganic elements and show unique physical and chemical properties depending on their specific composition and shape. (Figure 49.1 and Table 49.1). At present, there are five distinct classes of nanoparticles: liposomes, dendrimers, carbon nanotubes, quantum dots, magnetic nanoparticles, and metallic nanoparticles [3].

Liposomes are nanosized lamellar phospholipid bilayer vesicles and to date represent the most extensively studied nanoparticle platform. Liposomes are classified according to size and number of layers into multi-, oligo-, or unilamellar structures. Their amphiphilic nature enables them to transport hydrophilic drugs encapsulated within their aqueous interior, as well as hydrophobic drugs dissolved into their membrane. Liposomes have excellent circulation, penetration, and diffusion properties and are made of biologically inert materials that do not cause unwanted toxic or antigenic reactions. Liposome surfaces can be modified with ligands and/or polymers to increase delivery specificity, thus providing a delivery advantage by increasing interaction with the target cell population [4].

Dendrimers are well-defined highly branched synthetic polymer macromolecules with an architecture that consists of a central core, an internal region, and numerous terminal groups that determine their characteristics. A dendrimer can be prepared using multiple types of chemistry that define their nanoscale spherical architecture, polydispersity, and surface

TABLE 49.1. Examples of commercial applications of nanoparticles

Nanoparticle component	Application	Indication	Company
Liposomes	Drug delivery	Cancer	Liplasome Pharma (Lyngby, Denmark), Schering-Plough Corp (Kenilworth, NJ)
	Drug delivery	Vaccines: influenza, hepatitis A	Berna Biotech AG (Basel, Switzerland)
	Drug delivery	Fungal infection	Enzon (Bridgewater, NJ), Gilead Science (Foster City, CA)
Dendrimers	Therapeutics	HIV, cancer, ophthalmology, inflammation	Starpharma (Melbourne, Australia)
Carbon nanotubes	In vitro diagnostics	Respiratory function monitoring	Nanomix (Emeryville, CA)
	Imaging	Atomic-force microscopy probe tip	Carbon Nanoprobes Inc (Seattle, WA)
Quantum dots	In vitro diagnostics, imaging	Labeling reagents: Western blotting, flow cytometry, biodetection	Evident Technologies (New York, NY), Quantum Dot Corp. (Hayward, CA), Nanoco Technologies Ltd (Manchester, UK)
Magnetic nanoparticles	In vitro diagnostics	Cancer	Immunicon (Huntingdon Valley, PA)
	Imaging, therapeutics	Liver tumors, cardiovascular disease, anemia	Advanced Magnetics (Cambridge, MA)
	Therapeutics	Cancer	Nanospectra Biosciences Inc (Houston, TX)
Gold nanoparticles	In vitro diagnostics	HIV	Amersham/GE (Little Chalfont, UK)
	In vitro diagnostics, imaging	Labeling reagents (PCR, RNA, Western blot), angiography and kidney imaging	Nanoprobes Inc. (Yaphank, NY)

functionality. Dendrimers can be synthesized by convergent and divergent methods, resulting in a complex array of dendritic architectures with varying solubility and biologic activity. Dendrimers, like other macromolecules, are transported into and across cells via the endocytic pathway and therefore can be made into excellent drug and imaging diagnostic-agent carriers by chemically modifying their multiple terminal groups [5].

Carbon nanotubes (CNTs) are formed of coaxial graphite sheets rolled into cylinders that are classified by their structure into either single-walled CNTs consisting of a single sheet of grapheme rolled into a cylindrical tube, or multiwalled CNTs consisting of multiple grapheme sheets. CNTs exhibit excellent mechanical and electrical properties. They are also efficient heat conductors, which is why they are often used in biosensor applications. CNTs can be rendered water-soluble

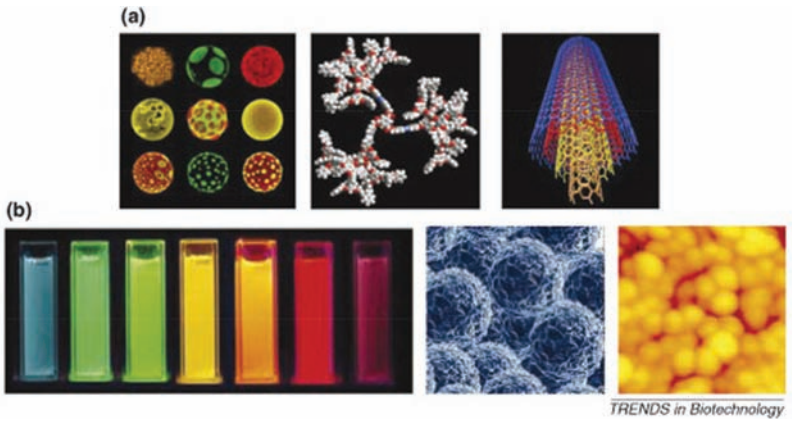

Figure 49.1. Current nanoparticle platforms (from left to right): (a) organic nanoparticles – liposomes, dendrimers, carbon nanotubes; (b) inorganic nanoparticles – quantum dots, magnetic FeOx nanoparticles, gold nanoparticles. Adapted from *TRENDs in Biotechnology*, with permission.

by surface functionalization, thus resulting in their use as drug carriers and tissue-repair scaffolds. CNTs are yet another platform for the simultaneous diagnosis, transport, and targeted delivery of drugs [6].

Quantum dots (QDs) are colloidal fluorescent semiconductor nanocrystals consisting of a central core composed of elements from groups II–VI or III–V of the periodic system. QDs are superior to organic fluorophores for biological imaging. Compared with conventional fluorophores, QDs offer a brighter signal, better photostability and resistance to chemical degradation, continuous absorption spectra spanning ultraviolet (UV) to near-infrared (NIR; 700–900 nm), long fluorescence lifetimes (>10 ns), narrow emission spectra (typically 20–30 nm full width at half maximum), large effective Stokes shifts, and the potential for integration into multifunctional nanoparticles. Recent developments in the surface modification of QDs allow for conjugation with biomolecules such as peptides and antibodies. This process enables the modified QD to be applied to cancer imaging and treatment. All these characteristics make QDs excellent contrast agents for imaging and labeling in bioassays [7].

Magnetic nanoparticles are composed of inorganic nanoparticle cores and biocompatible surfaces. Multiple magnetic materials have been investigated; however, most research focuses on superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs' physical properties make them excellent agents to label biomolecules in bioassays and to use as MRI contrast agents. The application of suitable surface functionalization on the biocompatible core allows for active targeting along with built-in imaging abilities. Magnetic nanoparticles have been evaluated extensively with agents such as Endorem, Lumiren, and Feridex IV. Next-generation magnetic nanoparticles are incorporating nanocrystalline cores, novel coating materials, and surface functionalization to improve specificity [8].

Metallic nanoparticles made of iron, gold, cobalt, nickel, and platinum can be prepared with different geometries, such as nanospheres, nanoshells, nanorods, or nanocages; however, these materials are often overlooked for biological applications because of their chemical instability. Biocompatible coatings, such as gold and silica, protect these materials from forming oxides in the presence of water and oxygen. Gold nanoparticles are excellent labels for biosensors because they can be detected by numerous techniques, such as optic absorption, fluorescence, and electrical conductivity. Bimetallic nanoparticles, the interaction between two chemical agents, are emerging, as this chemical alliance leads to greater stability. These nanoparticles are being investigated for their potential use in photothermal ablation as a therapeutic application [9].

CANCER NANOTECHNOLOGY

Cancer nanotechnology is an emerging new field combining biology, chemistry, engineering, and medicine. It seeks to design molecular-size tools capable of using cellular and molecular components to facilitate diagnosis and treatment. This field is expected to lead to major advances in cancer therapy. The ability to target nanoparticles greatly improves the prospects of detecting cancer cells with imaging agents, diagnosing cancer type with selective markers, and treating cancer by selectively delivering chemotherapeutics to tumor sites.

Nanoparticles for Cancer Diagnosis

The early detection of cancer is critical for an effective cancer therapy. As such, there is a focus on developing nanoscale biosensors for the purpose of detecting tumors. *Biosensors* are defined as analytical devices that combine a biological material (e.g., tissue, cell receptors, antibodies), a biologically derived material (e.g., recombinant antibodies, engineered proteins, aptamers), or a biomimic (e.g., synthetic catalysts, combinatorial ligands, imprinted polymers) that associates with a signal conversion unit called a *transducer*, which can be optical, electrochemical, thermometric, piezoelectric, magnetic, or micromechanical [10]. The application of biosensor technology in cancer testing has several potential advantages over other analysis methods. Increased assay speed, multitarget analysis, automation, reduced costs of diagnostics, and the incorporation of point-of-care (POC) testing give biosensors the enormous potential to deliver new, more efficient, molecular diagnostic strategies.

The ultimate goal in the designing of nanobiosensors is to recognize cancer at the earliest stage possible, ideally at the level of a few cells [11]. For this to occur, proteomic analysis, genetic analysis, and general research must be performed at each stage of the disease to identify and validate specific biomarkers. The other critical requirement is the ability to recognize the "targeted" biomarker within a heterogeneous population, which could be as complex as whole cells or as simple as a single molecule [12]. The merging of new biomarkers with nanotech platforms is aiding in the development of systems that will provide clinically relevant information about the presence of a tumor and the stage of tumorigenesis.

It is known that biologic entities such as antibodies, DNA, and proteins can be patterned on top of nanomaterials. As an example, novel antibody arrays are being developed in which antibodies are placed in an orderly arrangement on a solid support. Antigens are then identified by their binding at known positions. The reverse can also be done; nanomaterials can be coated with the antigen and studied for use as detectors of

antibodies [11]. Specific biomarkers bind to the biologic species, such as an antibody, on top of a nanocantilever, and this can change the electronic and optical properties of the material that can now be detected with great precision. An example of this was demonstrated by Panchapakesan using photolithography to build a biosensor in which multiple antibodies are attached to carbon nanowires (Figure 49.2). Upon antigen binding, the electrical properties, current flow change, can be measured through a gate transistor. This method provides real-time, rapid measurements of multiple cancer markers in a cost-effective manner for POC detection [11]. Another model biosensor proposed by Panchapakesan detects specific cancer biomarkers by binding specific targets on the surface of nanocantilevers (Figure 49.3). Slight changes in stress on the cantilever can be monitored by using position-sensitive lasers that reflect light off the cantilever. When a biomarker binds to the specific target on the cantilever, a change in free energy occurs, causing the cantilever to bend and change the angle of reflected light. Deflections on the nanometer scale can be detected by this method and are site-specific [11]. It is also possible to incorporate thousands of cantilevers with multiple tumor markers, which enables the simultaneous testing of expansive protein libraries on each sample, thus saving money and improving analysis time.

The nanowire and nanomechanical arrays of the cantilever can be used for massive, rapid multiplexing without labeling these biomolecules. In other words, these cantilevers have the ability to detect multiple biomolecules that have primary importance in cancer. Nanoarrays are capable of detecting multiple targets or large numbers of molecules simultaneously; they are used for proteomic profiling in cancer diagnoses and prognostics and also in the monitoring of therapeutic efficacies [12]. Cantilever sensors could be used to analyze body fluids in the nano- and picoliter range, offering the tantalizing potential of cancer diagnoses at the level of a single protein, DNA, or cell. In addition, nanoparticles created for imaging purposes may be used in the detection of cancer and are discussed in greater detail later.

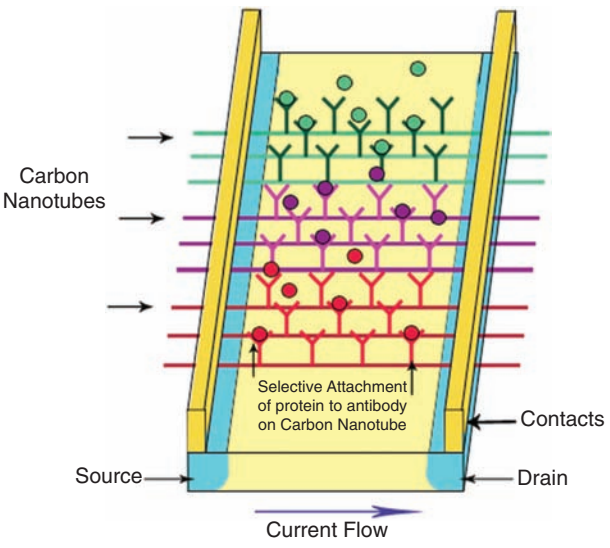

Figure 49.2. A nanotechnology-based chip for multicomponent detection of surface markers of cancer cells. Carbon nanotubes are attached to the contacts and labeled with antibodies specific to various cancer proteins (each color dot represents one type of protein). Upon selective attachment of the protein, a change in current can be measured from the device that allows the detection of particular cancer cells.

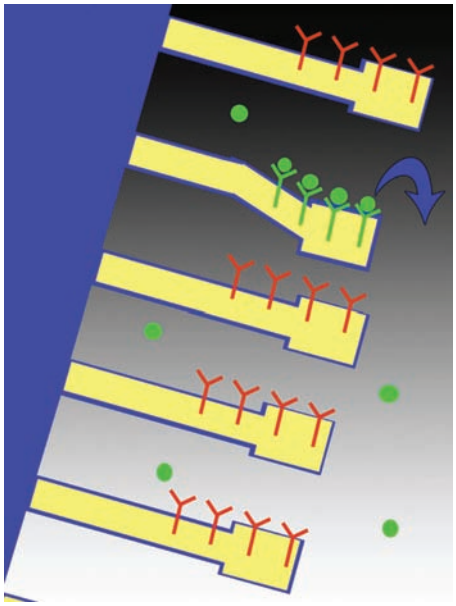

Figure 49.3. Nanoscale cantilever device having various antibodies labeled on the surface. When cancer proteins (green dots) interact with the surface, the cantilever bends under the weight and changes the free energy of the device, signaling detection of a specific protein.

Molecular Cancer Imaging

Molecular imaging typically includes two- or three-dimensional imaging as well as quantification over time. The techniques used include radiotracer imaging/nuclear medicine, magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), optical imaging, ultrasound, and the like. Targeted molecular imaging of receptors or proteins expressed on the surface of tumor cells is becoming a major field of research in clinical oncology, especially for the detection of cancer at its earliest stages, and for the determination of diagnosis and treatment methods. Traditional *in vivo* imaging probes and contrast agents are designed at the mesoscopic scale; however, recent advances in bioengineered nanoparticles provide many unique advantages to the traditional agents. Nanoparticles have large surface areas to accommodate both

large numbers and different types of functional groups that can be linked to multiple imaging agents [2]. These nanoparticles also have inherent properties, such as paramagnetism, fluorescence emission, and the ability to scatter visible light, that make them useful for traditional imaging modalities in clinical cancer screening.

Optical Imaging

Optical imaging (OIM) is a relatively low-cost method capable of evaluating a number of in vivo processes. A variety of fluorescently labeled probes has been developed that target cell surface receptors, enzyme biodistribution, protein function, and gene regulation [13]. Cancer research with OIM employs its ability to probe tissue with light for noninvasive detection of tumors. This process is practical because of the development of fluorescent probes that emit in the NIR spectrum, in which tissue has both low absorption and reduced scattering [13]. Owing to their superior fluorescent and physical properties, QD nanoparticles have recently become an alternative method for numerous *in vitro* and cell-based assays. Although optical imaging is not often used in clinical imaging, it provides a low-cost tool for proof-of-principle research.

Fluorescently labeled substrates have been designed that are quenched either because of the proximity of the fluorophores, or because a Förster resonance energy transfer (FRET) is used to quench the fluorescent signal that is then enhanced upon proteolytic cleavage [13]. Most FRET-based probes are built on polymer scaffolds, such as dendrimers, that improve circulation time, increase solubility, and allow for multiple fluorophore attachment. Scherer et al. have recently developed an MMP-7 QD probe that is both quantitative and sensitive, to detect tumor metastases in mouse models of cancer [14]. In these reagents, sensor fluorescence is "silenced" by FRET to a reference fluorophore with an absorption band that overlaps the sensor fluorophore emission. The sensor is linked to the dendrimer carrier through an MMP-7–cleavable peptide. Adjustments of the sensor–reference ratio and peptide conformation provide opportunities to modify the sensitivity and signal-to-background ratio of the reagent, thus optimizing its properties for *in vivo* imaging of clinically relevant levels of MMP expression [14].

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a powerful noninvasive imaging modality that is widely available in hospitals and clinical centers throughout the world. MRI is based on the interaction of certain nuclei with each other, and also with surrounding tissues of interest in an applied magnetic field. To improve contrast between tissues, novel contrast agents have been developed. The traditional contrast agents used are gadolinium (Gd)-based and are used both in clinical MRI to detect tumors and in research to assess the physical characteristics of tumor tissues [13]. Recently, new contrast agents, such as paramagnetic Gd-containing liposomes/micelles and SPIONs, have been developed [8]. These new contrast agents have significantly higher relaxivities. Also, better targeting efficacy may be achieved by coating the surface of these nanoparticles with ligands/antibodies/proteins. Because the major disadvantage of MRI is its inherent low sensitivity, future development of novel contrast agents with the additional capability of targeting cells may dramatically increase the MR signal and facilitate the biomedical applications of molecular MRI.

Ultrasound

Because of its safety, low cost, ease of use, and wide availability, ultrasonography is the most commonly used clinical imaging modality. The contrast of ultrasound depends on the sound speed, attenuation, backscatter, and an imaging algorithm [15]. Ultrasound contrast agents are generally in the form of small, acoustically active particles ranging from several hundred nanometers to a few micrometers in diameter.

With the introduction of microbubble contrast agents, diagnostic ultrasound has entered a new era that allows the dynamic detection of flow in both the macro- and microvasculature in tissues. Enhanced ultrasound contrast is made possible owing to the fact that tissue is almost incompressible, whereas gases are quite compressible, thus allowing the microbubbles to expand and contract in the alternating pressure waves of the ultrasound beam [15]. Targeting is accomplished either through manipulating the chemical properties of the microbubble shell or through conjugation of disease-specific ligands to the microbubble surface [15]. Both integrin $\alpha v\beta 3$ and vascular endothelial growth factor receptor 2 (VEGFR-2; Flk-1/KDR)-targeted microbubble ultrasound have been reported for functional cancer imaging [15]. Because these microbubbles are too large to extravasate, targeting must focus on the molecular changes in the vascular compartment to be imaged.

Nanoimaging of Tumor Metastasis

Cancer cells respond differently to their environment, and develop strategies to invade surrounding tissues. It is important to examine cancer cells carefully to understand what evokes these strategies, and how cancer cells adapt to them. The study of motility and of the invasion of cancer cells *in vivo* is hampered because of the difficulties involved in direct observation.

The superior sensitivity and physical properties of nanotechnology offer the possibility to detect a few cancer cells or even a single cell *in vivo*. Extravasation of cancer cells in live animals can be directly studied by using QD-labeled melanoma cells [16]. QDs fluoresce strongly and are photostable for weeks, thus allowing researchers to watch the cellular processes unfold. Multiple colors can be captured simultaneously using QDs, making it possible to track several molecules as they mingle [17]. In one study, tail-vein–injected tumor cells that were labeled with five different QDs were successfully visualized in the lungs using emission-spectrum scanning multiphoton microscopy [16]. In another study, again using five different QDs, simultaneous imaging of five different lymphatic basins was performed in mice [18]. These studies set the stage for researchers to investigate the interaction of various cells in the tumor microenvironment *in vivo*. As technology advances, new insights will enhance an understanding of cancer metastasis and will lead to better diagnosis and treatment.

Nanotechnology for Cancer Therapy

Targeting Therapeutics with Nanoparticles

Nanoparticles can be developed as vehicles to deliver anticancer drugs specifically to tumors. The use of nanoparticles for targeted drug delivery is the most exciting and clinically important application for cancer nanotechnology. Current therapies do not differentiate between cancerous and normal cells, which leads to systemic toxicity and adverse side effects. Nanoparticles designed to deliver anticancer drugs to the targeted tumor tissue will provide a platform to reach desired tumor tissues and will enable tumor cells to be killed without affecting normal cells. This strategy will improve the patient's survival and quality of life by increasing the effective dose of drug and reducing the systemic side effects.

The size and surface characteristics of nanoparticles control their ability to effectively deliver drugs to the targeted tumor tissue. Nanoparticles must have the ability to remain in the bloodstream for long periods of time without being eliminated from circulation by the reticuloendothelial system [19]. The nanoparticle design must take into account the size of tumor blood vessels, the tumor-infiltrating inflammatory cells, and the leaky tumor vasculature. The ability to tune their size, ranging from small enough to escape the reticuloendothelial system to large enough to prevent their rapid leakage into blood capillaries, gives them a major advantage. The capability to regulate surface groups on nanoparticles is also an important factor, as it improves circulation time and provides the ability to escape capture by macrophages [19]. Coating the surface of nanoparticles with hydrophilic polymers, such as PEG, or forming the nanoparticles from hydrophilic polymers, protects them from plasma protein adsorption and improves the solubility of hydrophobic drugs associated with the complex [19]. Because at least 40 percent of potential chemotherapeutic drugs in combinatorial screening programs are identified as poorly soluble, the ability to improve bioavailability with PEGylation may have a tremendous impact.

The ability to manipulate the size and surface characteristics of nanoparticles is leading to the development of highly specific and effective anticancer treatments. Passive and active targeting strategies are used for nanoscale drug delivery systems. In passive targeting, the pathophysiological characteristics of the tumor vasculature and microenvironment enable nanoparticles to selectively accumulate in tumor tissues (Figure 49.4a) [20]. Rapidly growing cancer cells release angiogenic regulators, such as growth factors and matrix metalloproteinases. The result is highly disorganized neovascularization, which leads to leaky defective architecture and impaired lymphatic drainage. The mechanism by which nanoparticle accumulation occurs is called the *enhanced permeability and retention* (EPR) effect. This effect describes the ability of macromolecules, including nanoparticles, with a molecular weight above 50 kDa, to selectively accumulate in the tumor interstitium [19].

The unique microenvironment surrounding rapidly growing cells also contributes to passive targeting. The high metabolic rate and resulting reduction in the supply of oxygen and other nutrients create an unsuitable environment for hyperproliferating cancer cells. Glycolysis is used by the tumor cells to obtain the required energy to maintain growth, and as a result, an acidic environment is created. pH-sensitive liposomes have been designed to be stable at physiological pH ~7.4, but break down and release their active drugs when the pH is less than physiologic values [19]. Furthermore, cancer cells release unique enzymes, such as matrix metalloproteinases, that have been associated with metastasis and cell survival mechanisms [13]. An alternative passive targeting strategy, tumor-activated prodrug therapy, makes use of these enzymes to deliver drugs to the tumor microenvironment by conjugating the drug to a nanoparticle with an enzyme-cleavable peptide. The drugs are inactive until divided at the targeted site by the enzymes produced by the tumor [20]. This strategy has been used to conjugate doxorubicin (DOX) to a MMP-2–specific peptide sequence that was observed to be efficiently and specifically cleaved by MMP-2 *in vivo* [19].

Drug delivery systems relying solely on passive targeting methods inevitably face limitations on specificity. These limitations can be overcome by the inclusion of active targeting agents that provide preferential

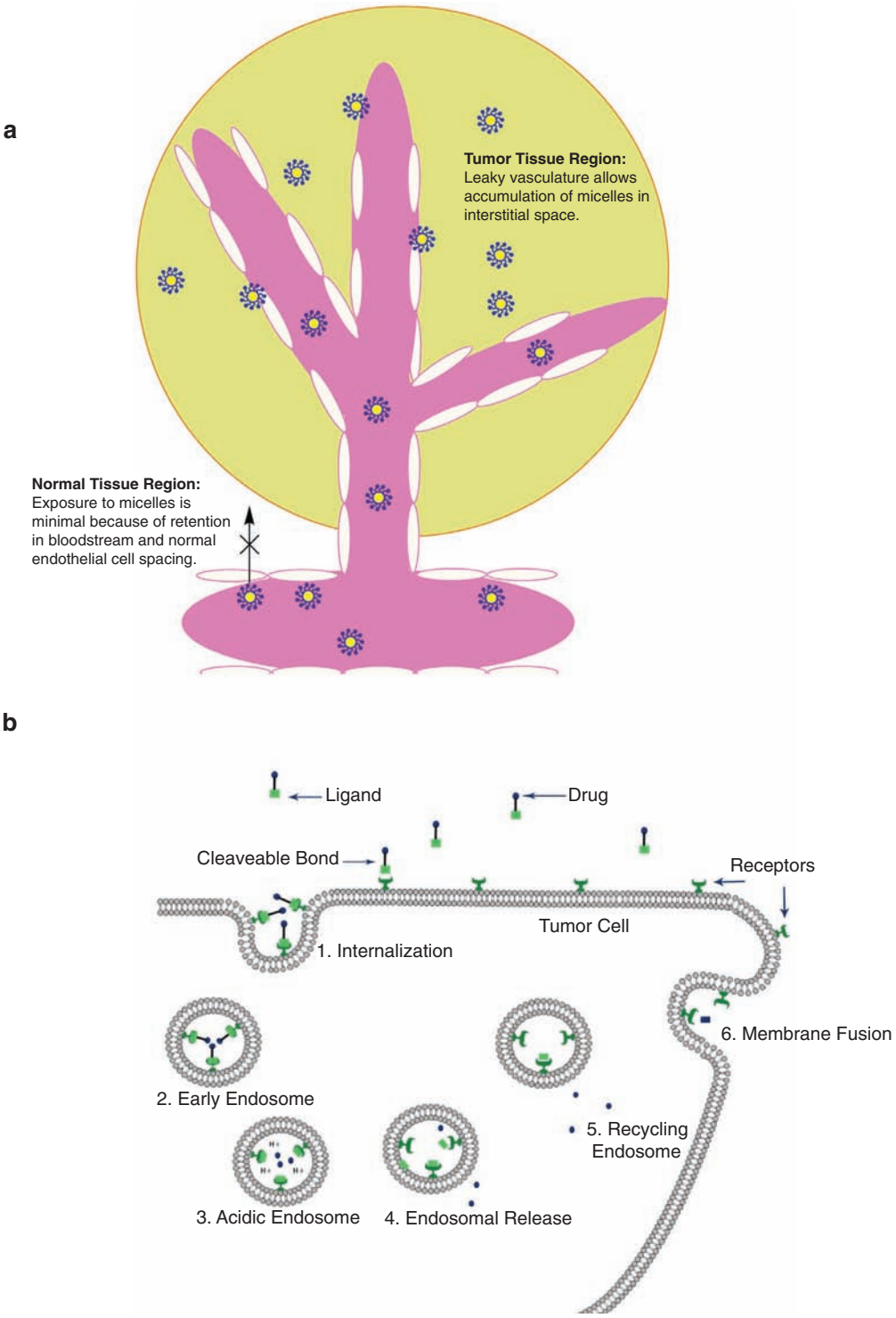

Figure 49.4. (a) Passive targeting: The overall size of the nanoparticle can affect its uptake to a specific organ. Leaky vasculature of tumors and the EPR effect allow for nonselective accumulation of nanoparticles at the tumor site, depending on the porosity of angiogenic tumor vessels as well as macrophage uptake. (b) Active targeting: Building on passive targeting, active targeting enables nanoparticles to recognize unique surface signatures of their target cells, allowing them access to enter the cells and target specific organelles. The active method should be more specific and selective toward the target cell and provide a means to actively deliver drugs to certain tissues.

accumulation of nanoparticles in the tumor-bearing organ, the tumor itself, individual cancer cells, or intracellular organelles inside the cancer cells. Active targeting is based on direct interactions of associated complexes, such as lectin with carbohydrates, antibody with antigen, and ligand with receptor (Figure 49.4b) [20]. Direct conjugation of an antibody to a drug was attempted but failed to show any advantages as a targeted delivery tool [19]. The inability to load large amounts of drug molecules to an antibody without affecting the immune recognition is one of the reasons that early conjugates were not very successful. The wide array of nanoparticle platforms discussed earlier has greatly improved the capability to increase the number of drugs effectively delivered without compromising the targeting moiety. Direct conjugation of antibodies to the surface of a nanoparticle allows greater specificity and increases the ability to load the number of drugs either within the nanoparticle or attached to its surface. Murphy et al. recently developed an integrin $\alpha\nu\beta3$-targeted nanoparticle loaded with the chemotherapeutic DOX. The integrin receptor is associated with internalization in many cellular processes and is also linked to high levels of expression on angiogenic endothelium of metastatic disease. The RGD-DOX-NP shows preferential delivery of drug and a fifteen-fold increase in efficacy as compared with nonspecific DOX [21].

The ideal targeting cell surface antigen or receptor should be homogenously expressed solely on tumor cells and should not be shed into blood circulation. After selecting a viable surface marker, it is also important to choose a targeted conjugate that can be internalized upon binding. Generally, internalization takes place via receptor-mediated endocytosis, which may provide a means to circumvent multiple drug resistance (MDR). It is believed that glycoproteins, such as P-glycoprotein, are unable to bind to polymer drug conjugates that enter cells via endocytosis, and that the drugs avoid recognition because of encapsulation in an endosome. This process was demonstrated using a folate receptor-targeted, pH-sensitive micelle containing DOX and transferrin-conjugated paclitaxel nanoparticles [19]. The use of nanotechnology in targeting drug therapy is very promising and should take cancer treatment to a new level.

Nanotechnology Improves Precision of Tumor Surgery

Oncologic imaging should provide an accurate staging of the tumor to aid in the decision for treatment, neoadjuvant therapy, or nodal dissection. It also should enhance tracking of the response to treatment after therapy. The detection of lymph node metastases using conventional MRI is a relatively insensitive procedure; thus, the use of nanoparticles in combination with MRI has been explored to enhance its sensitivity.

A major prognostic factor and determinant for adjuvant chemotherapy in breast cancer is the involvement of lymph nodes [22]. Axillary dissection (removal of all the axillary lymph nodes) was used for many years to detect lymph node metastasis before sentinel lymph node biopsy became a routine procedure. For a sentinel lymph node biopsy, a radioisotope and/or blue dye are injected into the tumor before surgical removal. Following the removal of the tumor mass, a Geiger counter, or simple visualization of the dye, is used to detect lymph nodes that have taken up the radioisotope and/or dye, and the sentinel lymph node is removed. If the sentinel lymph node is negative for cancer, there is no need to remove more lymph nodes, thus reducing the complications associated with surgical axillary dissection. However, sentinel lymph node biopsy has its limitations, such as poor spatial and temporal resolution, the use of a radioisotope, and the requirement for a handheld micro-gamma probe. To overcome these limitations, a nanosized MRI contrast agent, G6, for dynamic micro-MR mammolymphangiography was generated. The G6 contrast agent, administered directly into the mammary gland tissue, is large enough to be retained in the lymphatic system but not so large that it cannot be taken up efficiently. In a mouse mammary tumor model, lymphatic drainage and lymph nodes were visualized with G6 but not with the conventional FDA-approved MRI contrast agent, Gd-[DTPA]-dimegluine [23]. The G6 agent can be easily modified with an optical or fluorescent agent to make it more useful for surgeons to quickly and precisely locate a sentinel lymph node during surgery.

The detection of lymph node metastasis in prostate cancer was enhanced using MRI with lymphotropic superparamagnetic nanoparticles [23]. Intravenously injected superparamagnetic iron oxide is first extravasated into the interstitial space, and then is taken up by lymphatic vessels and transported to the lymph nodes, where it is internalized by macrophages and detected by MRI [24]. In a study using conventional MRI, 71 percent of the histologically detectable malignant lymph nodes were missed; however, with the addition of nanoparticles, the tumors were discovered. In a patient-by-patient analysis with an overall accuracy rate of 96 percent, the detection of lymph node metastasis increased to 100 percent sensitivity using nanoparticles, versus 46 percent with MRI alone [23].

Sentinel lymph node mapping was also performed using NIR type II QDs with an oligomeric phosphine coating that stabilizes QDs in the biological environment. The NIR QDs successfully located sentinel lymph nodes in mice and pigs using intraoperative NIR fluorescence imaging [26]. It was possible to visualize lymphatic node drainage in terminally ill dogs with urinary bladder carcinoma using near-infrared fluorescent (NIRF) albumin or NIRF QDs [27]. Interpatient

variation of lymphatic node drainage in the bladder makes it difficult to locate lymph nodes anatomically; however, this imaging enables real-time identification of patient-specific sentinel lymph nodes.

Multifunctional Nanoparticles

The development of novel materials and devices operating at the nanoscale range has led to major advancements in cancer imaging, diagnosis, and therapy. Multifunctional "smart" nanoparticles are being developed from a combination of drug delivery system technology and nanoscale imaging agents. The ability to diagnose, apply treatment, and assess a patient's response simultaneously with one agent could revolutionize the medical industry and personalize medicine.

Scientific literature is now reporting on multifunctional nanoparticles. These studies provide insight into the future of nanomedicine, and the possibilities nanotechnology is facilitating. Yang and associates have developed a multifunctional system with imaging capabilities using magnetic nanocrystals for MRI, therapeutic antibodies for active targeting, and DOX inclusion for synergistic chemotherapy [28]. NIR light-activated therapies, which are an encapsulation of iron oxide nanoparticles and the photosensitizer Photofrin, are under development by Ross and coworkers [29]. These particles also incorporate a vascular homing peptide that targets nucleolin, a surface marker on prostate cancer cells and angiogenic endothelial cells. Farokhzad et al. developed bioconjugated nanoparticles by using aptamers to recognize a prostate-specific antigen that specifically delivers docetaxel to localized prostate tumors [30].

Other researchers have modified paclitaxel, a standard treatment for metastatic breast cancer, to improve solvent-associated toxicity; a solvent-free 130-nm albumin-bound paclitaxel (ABI-007, nab-paclitaxel) has been generated [31]. Albumin is used as a transport vehicle employing albumin receptor (gp60)-mediated uptake by endothelial cells to deliver the drug to tumors [32,33]. The multicenter Phase II trial using nab-paclitaxel showed significant efficacy with less toxicity as compared with paclitaxel (Taxol, Bristol-Myers Squibb, Princeton, NJ) with the solvent Cremophor EL [31]. In a preclinical study of ovarian and mammary carcinoma, nab-paclitaxel was also effective as a radiosensitizer, and the combination of nab-paclitaxel and radiation provided a more significant antitumor effect than either treatment alone. Because of these results, it has been suggested that nab-paclitaxel be used in chemoradiotherapy [34].

It is conceivable that with the further advancement of nanotechnology, imaging agents, and the identification of tumor specific biomarkers, targeted multifunctional nanoparticles will provide not only an effective means to detect cancer at the earliest stages, but also the ability to kill the tumors while reporting tumor response simultaneously (Figure 49.5). Nanotechnology will help to turn the promise of personalized cancer therapy into reality in the near future.

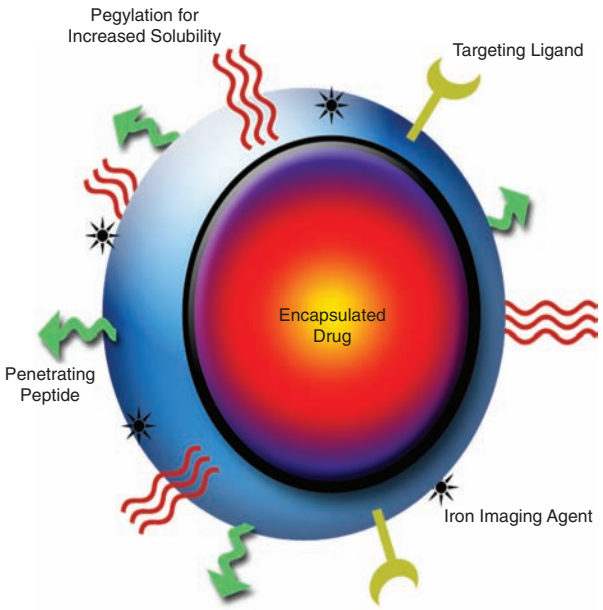

Figure 49.5. Multifunctional nanoparticles provide a viable means to perform *in vivo* imaging at targeted sites, selective delivery, and enhanced solubility of therapeutic drugs, as well as evaluation of the drug's efficacy over time. Multifunctional nanoparticles are in early stages of development but demonstrate the promising abilities of nanotechnology in cancer research and medicine.

CONCLUSION

Our knowledge of cancer biology has increased exponentially with advancements in material science and technology. Cancer nanotechnology is an emerging new field, combining biology, chemistry, engineering, and oncology. It seeks to design molecular-sized tools capable of using cellular and molecular components to facilitate diagnosis and treatment. This interdisciplinary collaboration will lead to a radical change in the treatment, diagnosis, and prevention of cancer. Further improvements in all the related fields, and a more efficient integration of nanotechnologies in cancer biology, are essential to meet the goal of eliminating suffering and death for cancer patients.

ACKNOWLEDGMENTS

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50 Metronomic Chemotherapy for Treatment of Metastatic Disease: From Preclinical Research to Clinical Trials

William Cruz-Munoz, Giulio Francia, and Robert S. Kerbel

Metastasis is the culmination of tumor progression and remains both the primary cause of mortality for cancer patients, as well as the most challenging aspect of cancer therapy. The main systemic treatment of metastatic disease – chemotherapy – was designed with the aim of killing as many tumor cells as possible by using cytotoxic agents at the maximum tolerated dose (MTD) [1, 2]. However, such regimens are associated with a number of inherent limitations. For instance, the administration of high dosages of chemotherapeutic agents results in toxicity, which is sometimes serious in nature (e.g., myelosuppression and damage to intestinal mucosa). As such, this requires the incorporation of prolonged breaks (often, three weeks) between treatments to allow recovery of depleted cells (e.g., neutrophils from bone marrow progenitors) [3]. Unfortunately, these breaks also allow for tumor regrowth to occur such that any regressions achieved by MTD therapy are usually only transitory [2]. In addition, due to the inherent ability of tumor cells to acquire resistance to cytotoxic agents, most MTD therapies eventually fail, resulting in resumption of disease progression. Overall, most MTD therapies have proven generally ineffective or of modest (mostly palliative) benefit in the treatment of advanced metastatic disease [3].

Clearly, a rethinking of approaches to treat metastatic disease is in order. This involves, at least in part, a reexamination of the dosing schedule regimens of chemotherapeutic agents that are best suited to treat this most intractable aspect of the pathology of cancer. We, and others, have been proponents of an alternative clinical strategy for chemotherapeutic dosing and scheduling that involves administering cytotoxic agents at lower doses, well below the MTD, at regular, close intervals over extended periods of time with no extended drug-free breaks [4–5]. The first obvious advantage of such "metronomic" regimens is reduced levels of toxicity such that it may decrease or even remove the need for growth-factor support to accelerate recovery from myelosuppression or supportive care drugs (e.g., antinausea agents), which are typically required with many MTD therapies [3].

Unlike MTD therapy, the primary target of metronomic chemotherapy schedules is thought to be the endothelial cell population of the growing neovasculature of the tumor. The impetus for this approach is based on the fundamental contribution that new blood vessel formation plays in controlling tumor growth, and, given this role, the importance of endothelial cells as targets for anticancer therapy [2, 6]. Because tumor-associated endothelial cells proliferate at a lower rate than tumor cells, they may not be effectively targeted by MTD regimens [7]. In this respect, the frequent administration of low doses of chemotherapeutic agents, which characterizes metronomic chemotherapy, is more effective in targeting the proliferation of these cells.

At one time, targeting endothelial cells was thought to offer an additional advantage, as these host cells were considered genetically stable, and thus less likely to acquire resistance to antiangiogenic drugs [8]. This may translate into sustained activity, even against drug-resistant cancer cells when using "direct-acting" antiangiogenic drugs that target host endothelial cells, at least in some instances [9, 10]. This aspect is particularly relevant to metastatic disease that is commonly refractory or poorly responsive to chemotherapeutic drug treatments or capable of rapidly acquiring resistance. However, it is now clear that resistance to antiangiogenic therapy can readily occur. This may be because of alterations in the tumor microenvironment mediated by such therapies, and those alterations in turn select tumor cells that are more resistant to hypoxia and thus the effects of antiangiogenic therapy [11]. Certainly, evidence has shown that (at least in primary tumor models) although metronomic chemotherapy is able to delay the growth of drug-resistant tumors, tumors eventually may escape from the control achieved by this

treatment and relapse [9]. The effectiveness of metronomic chemotherapy can be enhanced by combination with targeted biologic therapies, such as antiangiogenic drugs; such combinations can sometimes cause surprisingly robust and prolonged tumor responses in preclinical trials [3, 5, 12–14]. Indeed, Browder et al. [9] showed that the eventual relapse to metronomic chemotherapy, noted above, may be controlled by the concurrent addition of an angiogenic inhibitor, TNP-470. In our experience, we have also found that although metronomic chemotherapy using vinblastine is able to delay the growth of SK-NMC neuroblastomas (grown subcutaneously in immunodeprived mice), tumors eventually begin to relapse. However, this was not observed when an anti-VEGFR2 antibody (DC101) was combined with metronomic vinblastine, resulting in sustained tumor suppression for up to seven months [15].

Metronomic chemotherapy may also cause antitumor effects by mechanisms independent of angiogenesis inhibition [3]. For instance, daily oral low-dose metronomic cyclophosphamide (CTX) induces a decrease in the levels of circulating regulatory cells, leading to peripheral T-cell proliferation and elevated NK cell activity in end-stage tumor-bearing patients [16]. In addition, it is possible that metronomic chemotherapy may also cause direct tumor cell cytotoxic effects. The efficacy of metronomic chemotherapy in preclinical xenograft models has been demonstrated using various tumor types [9, 15, 17, 18]; in some instances, when MTD and metronomic chemotherapy have been compared, the latter has shown a superior anticancer effect [19].

Given that angiogenesis is an essential process in the growth of both primary tumors and metastases [20], it is reasonable to assume that the antiangiogenic activity mediated by metronomic chemotherapy would extend to the treatment of metastatic disease. Indeed, examination of the efficacy of this approach against advanced metastatic disease is a matter of ongoing evaluation in both preclinical and clinical settings. In this review, we describe both the relevance of metronomic chemotherapy for the treatment of advanced metastatic disease and the development of novel preclinical models that can be used as tools to test the efficacy of this strategy, including the use of other drugs or treatment protocols.

MODELS OF ECTOPICALLY TRANSPLANTED TUMORS AS PREDICTORS OF ANTICANCER ACTIVITY

The general inability to make significant gains in the development of effective treatments for metastatic disease is in great part due to the nature of the disease itself (its aggressiveness, acquired resistance, etc.). However, this has been compounded by the inappropriate use, or even the lack, of appropriate preclinical models of metastatic disease with which to test and examine the efficacy of novel therapeutic approaches [21]. Historically, examination of antitumor activity has been conducted mostly using subcutaneous mouse or human tumor xenograft models. More recently, orthotopically transplanted primary tumors or primary tumors arising in genetically engineered mouse models have been increasingly used. However, promising results obtained using all these models have generally not correlated well with results obtained against similar human cancer types in clinical trials in which patients have advanced metastatic disease [21, 22]. The same can be said of metastasis therapy experiments, which, in retrospect, have almost always involved easier-to-treat microscopic minimally residual disease at the time therapy was initiated [23, 24].

The use of transplanted or spontaneous primary tumors suffers from the fact that these tumors do not recapitulate treatment of advanced visceral metastatic disease. Thus, although these models may be useful in identifying potentially active antitumor agents, they need to be coupled to additional preclinical models involving advanced metastatic disease to confirm the therapeutic activity of the agent against the appropriate tumor type and in relevant sites of distant metastasis [23]. For example, with respect to the antiangiogenic effect mediated by metronomic chemotherapy, endothelial cells present in a primary tumor may not respond in a similar manner to those present in a metastatic growth. Such differences in sensitivity may be ascribed to the heterogeneity of endothelial cells present in different sites, as well as to differences in the microenvironment itself (e.g., presence of distinct growth factors or cytokines).

As noted by Fidler [25], the inhibition of angiogenesis in a subcutaneous tumor may not predict the effect seen in a metastatic organ site. Indeed, in our own experience, we have found that primary human xenograft tumors, even when grown in orthotopic sites, are not necessarily good models for predicting antimetastatic activity [12, 20, 26]. Thus, to minimize the disconnect between preclinical and a clinical outcomes, it is necessary to reexamine the validity of traditional tumor therapy models as tools to examine the efficacy of chemotherapeutic approaches (whether metronomic or MTD) against metastases and to consider the use of more appropriate preclinical models of metastatic disease [27, 28]. This view applies to all anticancer drugs and treatments, both old and new, but we discuss it from the perspective of the therapeutic modalities that we have been studying.

PRECLINICAL MODELS OF METASTASIS (SYNGENEIC VERSUS XENOGRAFT)

The early in vivo metastasis models were mostly syngeneic in nature and thus made use of transplantable

murine cell lines such as the B16 melanoma. The cells were generally introduced by means of intravenous injections to produce lung metastatic colonies [29]. The diverse number of syngeneic cell lines available for such purposes has been discussed extensively in a number of excellent reviews (see [29–31]). The advantage of the syngeneic model is that it allows the examination of the specific steps of the metastatic process in an immunocompetent host and thus allows the evaluation of the influence of host immune response on metastasis [30]. However, the use of murine cell lines introduces a number of disadvantages, resulting in models that are not necessarily representative of the biology of human tumors in a number of respects. Thus, murine cancer cell lines may not reflect the molecular alterations that occur in human tumors [30, 32]. For example, the spontaneous occurrence of cancers such as melanoma is rare in mice and, although activating mutations in the *b-raf* gene are present in 60 to 70 percent of malignant human melanomas [29], they have not been detected in mouse melanoma cell lines [33]. The early syngeneic metastasis models also lacked metastatic site-specificity that reflects the presentation of clinical disease [34]. In practical terms, syngeneic models also have the disadvantage that the large numbers of antibodies available against human-specific antigens often fail to cross-react with the murine tissue and thus may not be used for preclinical studies using such tumors [32].

To overcome some of the limitations that are associated with syngeneic models, human xenograft models have been developed for a large number of cancer types [32, 35, 36]. Xenografts have been generated through direct implantation of human tumor biopsy tissue or injection of established tumor cell lines. The former has the advantage of retaining the morphological and molecular markers present in the original tumor. However, the use of these human tumors often makes it difficult to establish cell lines that can serve as a continual resource for in vitro studies [21]. This is where established cell lines can be more useful as tools to conduct parallel in vitro (biochemical, molecular, pharmacological, and pharmacodynamic), as well as in vivo studies. Despite the advantages of xenograft models, they necessitate the use of immunologically privileged sites (e.g., cerebrum or the anterior chamber of the eye) or the more predominant use of immunocompromised (thymectomized/irradiated, nude, or SCID) mice to avoid tumor cell rejection [37]. However, this does not permit the examination of the potential role of immune responses in the metastatic turmor spread [30]. An additional disadvantage is that these immunocompromised mice may also show altered phenotypes with respect to processes, such as angiogenesis, that play a central role in the metastasis [38], as well as differences in the extent and pattern of metastatic dissemination associated with different cancer types in the various strains of mice available [39, 40]. Thus, the choice of model can clearly depend on the nature of the experimental aims of a given study.

EXPERIMENTAL AND SPONTANEOUS MODELS OF METASTASIS

The experimental models of metastasis (sometimes referred as "artificial" metastasis) have been the most widely used approach to study the biology and treatment of metastatic disease in mice. In these models, the tumor cells are delivered into the circulatory system by intravenous (tail vein) injections to generate distant metastases, usually in the lungs, or targeted to the central nervous system (CNS), or bone, by means of intraarterial injections [29]. Such metastasis models have a number of advantages, including controlled number of cells delivered that result in the formation of colonies of relatively uniform size, a defined location at which metastases form, and specific time course for the development of metastases [30]. All these aspects make such experimental metastasis models an important tool to examine the activity of drugs and biological agents. They have also been useful in improving our understanding of the mechanics, processes, and molecules that may be involved in the postintravasation phase of the metastatic cascade. However, because these models rely on the direct introduction of tumor cells into the circulation, they do not reflect the complete cascade of events, especially many of the early steps, involved in clinical metastatic disease [29, 41]. Another limiting aspect, which is inherent not to models but rather to the inappropriate design of preclinical studies, is their use to examine antimetastatic activity in which treatment is initiated shortly after, or even before, tumor cell inoculation.

In "spontaneous" metastasis models, the tumor cells spread spontaneously from a primary tumor site and follow the natural multistep route of cell dissemination leading to the formation of distant metastases, often (but not always) in relevant sites such as the liver and lungs that reflect the clinical presentation of the disease. Although they are highly relevant, these models have often been hampered by the limited extent of metastases that are generated [42]. Spontaneous metastasis is generally a rare event in models of subcutaneous implantation of human xenografts [35]. The inefficient metastatic spread from this primary ectopic site has been attributed to differences in terms of growth factors and cellular interactions that are essential for the metastatic process for a specific tumor type but are absent in the subcutaneous microenvironment [25, 43]. Also, subcutaneous tumors may be surrounded by a prominent fibrous capsule that acts to impede local invasion and, hence, distant metastatic spread. Indeed, as early as the nineteenth century, Paget noted that the outcome of metastatic cell dissemination is determined

TABLE 50.1. Preclinical models of spontaneous metastasis using orthotopical implantation of human xenografts

Model	Primary site of metastases	Reference
Melanoma (131/4–5B1, B2)	CNS, lung	[26]
Melanoma (113/6–4L)	Lung	[26]
Breast (231/LM2–4)	Lung	[12]
Gastric (St-4, St-40, H-111, Sc-1NU)	Lymph node, liver	[35, 45]
Ovarian (RMG-1)	Lymph nodes and distant organs, including the liver, kidney, pancreas, diaphragm	[46]
Pancreatic (PANC-4)	Liver and peritoneal	[47]
Colon (Co-3, Col-3-JCK, Col-5-JCK)	Liver	[35]
Lung (A549)	Lung, lymph node	[48]
Colon (KM12)	Lymph node, liver	[49]
Osteosarcoma	Lung, lymph node, liver	[50]
Bladder (RT10)	Lymph node, lung, liver, pancreas, spleen	[51]

not only by properties of the tumor cell but also by its successful interaction with the surrounding microenvironment [43, 44]. This has led to the increasing use of orthotopic implantation of tumor cells as a more physiologically relevant model for studying metastasis. Certainly, there is a growing number of such orthotopic spontaneous models of metastasis for various cancer types (see Table 50.1).

ORTHOTOPIC MODELS OF METASTATIC DISEASE

Melanoma

Melanoma is one of the most practical models to grow orthotopically, because the target site (the subdermis) is readily accessible for injection, and also because of the plethora of melanoma cell lines that can both grow and metastasize in immunodeficient mice [10]. A technical concern with implantation to this site is to avoid accidental injection of the cells below the dermis [10, 52], which would result in a subcutaneous tumor. The subsequent rapid growth of the primary tumor may limit the extent of metastatic spread, as the mice may succumb to the localized orthotopic tumor transplants before significant metastases have had a chance to develop [23]. Thus resection of primary tumor is often an important requirement for successful orthotopic melanoma metastasis models. However, this is also true for other tumor models, such as breast cancer. The requirement of primary tumor resection can also be understood in terms of concomitant antitumor resistance (CAR). As suggested by Folkman and others, the growth of distant metastases may be inhibited by endogenous angiogenesis inhibitors released by the primary tumor [53, 54]. In this respect, removal of the primary tumor would eliminate its distant, systemic antiangiogenic effect and allow or facilitate the progression of metastatic growth.

Typically, orthotopically grown melanomas show a propensity to metastasize to the lungs, and therefore most therapy experiments using metastatic melanoma models primarily involve targeting lung metastases. However, we recently reported the derivation of variants of the WM239A human melanoma cell line that spontaneously metastasize to the brain [26]. The generation of such cell lines was achieved by initially generating a highly metastatic variant of WM239, named 113/6–4L (as summarized in Figure 50.1A). Using this model, we were able to show that a combination of metronomic chemotherapy, using vinblastine and CTX, resulted in long-term survival of mice with visceral metastatic disease. Brain metastases were found in 20 percent of these long-term surviving mice, and from these metastases two cell lines were generated, 131/4–5B1 and 131/4–5B2. These cell lines were then found to spontaneously metastasize to brain parenchyma, with occasional localization to the leptomeninges, after orthotopic transplantation and subsequent removal of the primary tumor transplant (Figure 50.1B–E). As such, our findings represent the first report of spontaneous CNS metastases generated from primary tumors of any human cancer in mice, which heritably maintains this phenotype upon reinjection of the cells. This model should facilitate the study of therapies targeting melanoma metastases in the central nervous system, which is a common manifestation of malignant melanoma that is clinically associated with a very poor prognosis.

Breast Cancer

Orthotopic models of breast cancer are generated by the implantation of breast cancer cells into the inguinal mammary fatpad of female mice. Primary tumors can then readily be monitored by caliper measurements. One model that is predominantly used in this manner uses the human MDA-MB-231 breast cancer cell line. The preponderant use of this cell line is in part a reflection of the fact that there are few alternative human breast cancer cell lines that readily both grow and metastasize in immunodeficient mice (in contrast to melanoma, for example). One other cell line hitherto used extensively was MDA-MB-435.

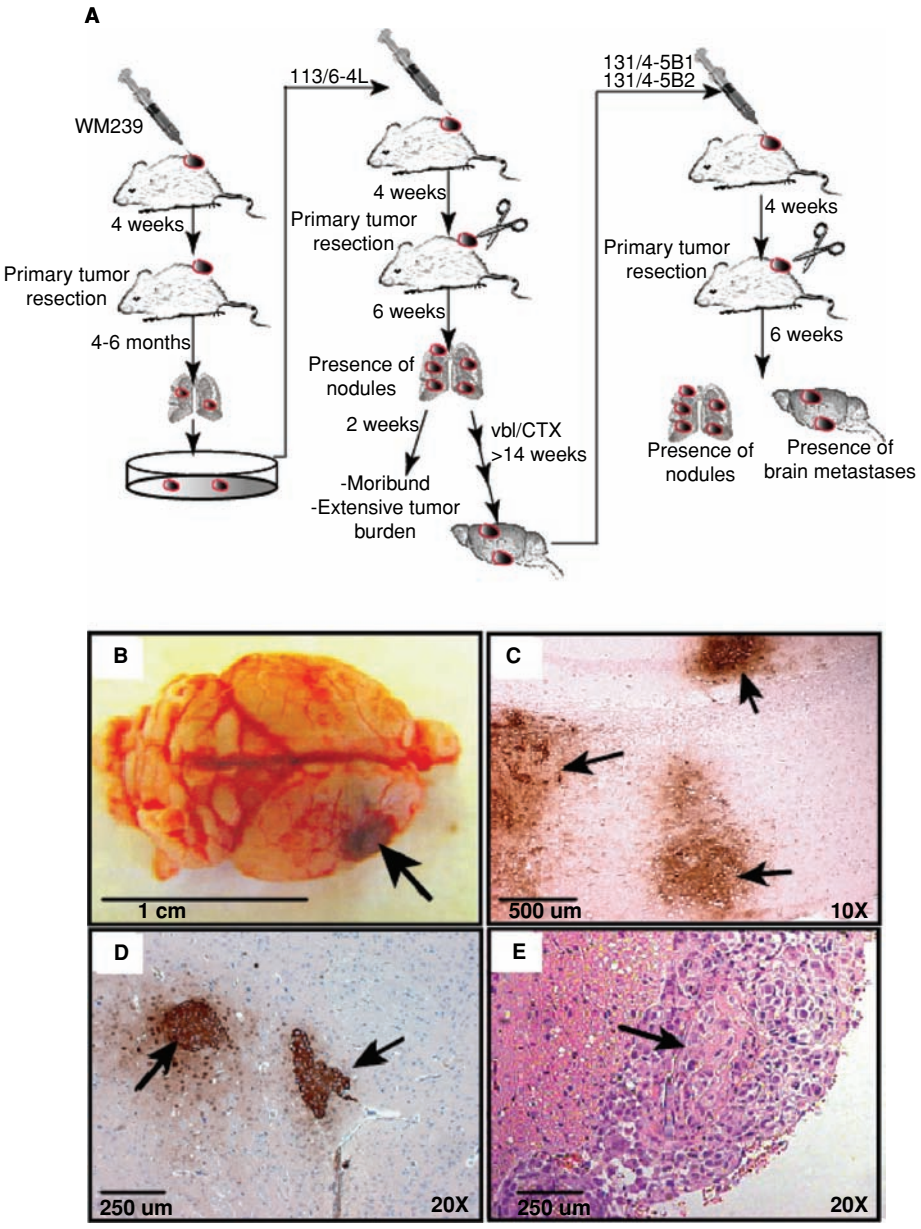

Figure 50.1. (A) Schematic representation of protocol used for isolation of highly metastatic variant 113/6–4L and brain metastatic 131/4–5B1 and 131/4–5B2. Parental unselected human WM239A melanoma cells were implanted subdermally; the primary tumors that developed were resected when they reached a size of approximately 400 mm^3. Four to six months following resection, lungs were excised from mice and adapted for cell culture and the 113/6–4L cell line derived. Implantation of 113/6–4L cells resulted in high metastatic load in lungs 6 weeks post-primary tumor resection. Orthotopic implantation of 131/4–5B1 and 131/4–5B2 cell lines results in spontaneous brain metastasis. (B) Twenty percent of mice that survived long-term CTX and vinblastine therapy in the 113/6–4L model of advanced metastatic disease showed the presence of brain metastases. From these metastases, 131/4–5B1 and 131/4–5B2 cell lines were then isolated. (C and D) Orthotopic implantation of 131/4–5B1 and 131/4–5B2 resulted in the formation of spontaneous melanoma metastases in brain parenchyma with (E) occasional presence of metastatic foci in the leptomeninges (H&E staining). (Adapted from Cruz-Munoz et al. [26].)

However, numerous reports have since shown that this cell line expresses melanoma markers; it is now classified as a melanoma derived from the M14 melanoma [55]. Other human breast cancer cell line models do grow in vivo, though some may require estrogen supplements (e.g., BT474) or display very slow growth (e.g., MDA-MB-361). Furthermore, the HER2-positive cancers (a major subclass of breast cancers) appear to be represented, counterintuitively, by cell lines that grow poorly in vivo (e.g., BT-474, MDA-MB-361). This fact

has hampered in vivo studies examining the efficacy of HER2 targeting in the treatment of metastatic disease. As a result of this limitation, our laboratory [56] and others [57] have generated HER2-positive variants of MDA-MB-231 (HER2 overexpression in this case was achieved via viral vector transduction).

Colorectal Cancer

Orthotopic colorectal cancer models involve the intracecal injection of colon cancer cells [58]. This is not very practical, however, principally because of the difficulty in preventing the collapse of the cecal wall as the cells are injected. When such injections are successful, the tumors readily grow and metastasize to other organs such as the liver (Man and Francia, unpublished observation). However, because of the difficulties involved, some investigators prefer to inject the cells in the spleen; this site strictly represents an ectopic implantation, but such protocols are erroneously referred to as orthotopic colorectal cancer models. Intrasplenically implanted human colorectal cancer cells readily (and rapidly) metastasize to the liver, which mirrors clinical observation on the spread of colorectal cancer [49].

In contrast to melanoma or breast cancer orthotopic models, colorectal tumors cannot be readily measured, as they grow inside the body of the host. To overcome this obstacle, a number of technologies have been developed, such as whole-body bioluminescent imaging using luciferase-tagged tumor cells [59] or measuring of secreted human choriogonadotropin protein in urine using transfected tumor cells [60, 61], which permit monitoring of relative systemic tumor burdens and their response to therapy [24].

Issues Regarding Appropriate Use

Orthotopic models of spontaneous metastasis would seem highly relevant tools to study the biology of the disease and to examine the efficacy of novel therapeutic approaches for cancer treatment. For practical reasons, however, the number of cell lines that can be used in these models is limited. In setting up therapy experiments involving such metastasis models, a number of aspects need to be considered. The first consideration is whether it is actually necessary to set up an orthotopic model of metastatic dissemination, which, in the case of certain tissues, such as the brain, can involve a substantial amount of additional materials and methods. The question in this case is whether information regarding the efficacy of a given therapy could be gained through the use of other models, such as direct intracranial implantation. Another issue is whether therapies are to be tested against a primary tumor, grown orthotopically, or tested against subsequent metastatic disease (see the example below of metronomic UFT, a 5-FU oral prodrug, and CTX combination therapy on advanced LM2–4 breast cancer metastases). Furthermore, it may also be useful to have a means of monitoring the progress of the disease under therapy. This is an area that has seen significant recent improvements with the development of fluorescent, luminescent, and secretable (e.g., hCG) markers [60]. Figure 50.2 shows the steps in a generic experiment using orthotopic and metastatic models, and some of the complications that may arise. Finally, consideration of the question of whether therapies are to be initiated at early (microscopic) or late (macroscopic) advanced disease, as detailed in Figure 50.2, should be considered. We have previously raised the point that patients in Phase I and II clinical trials typically have advanced metastatic disease, and as such preclinical examination of the efficacy of any given antimetastatic therapy should be examined against macroscopic well-established metastases, when possible, and if it can be done in a practical manner.

METRONOMIC CHEMOTHERAPY EVALUATED IN PRECLINICAL TUMOR MODELS

Efficacy of Metronomic Chemotherapy against Subcutaneously Grown Tumors

The initial preclinical report of a metronomic chemotherapy dosing was carried out using subcutaneously grown Lewis lung carcinoma. In this study, Browder et al. [9] showed that a once-every-six-day administration of approximately one-third of MTD for CTX could markedly suppress the growth of Lewis lung carcinomas for more than sixty days (MTD for CTX is 450 mg/kg every 21 days given intraperitoneally, where the drug is given at 150 mg/kg every 2 days for a 6-day cycle, whereas the metronomic regimen was 170 mg/kg every 6 days given intraperitoneally). Furthermore, the same low-dose regimen could inhibit the growth of variants of Lewis lung carcinoma previously selected for acquired resistance to MTD CTX in vivo. This study was accompanied by an independent report of marked and sustained suppression of SK-NMC human neuroblastomas in SCID mice by the administration of low-dose vinblastine given (about 1/10 to 1/20 of the MTD) every three days plus twice-weekly administration of the anti-VEGFR2 antibody DC101 (which would not target the tumor cells, but rather the VEGFR2-positive endothelium in the tumor microenvironment) [15]. This experiment incorporated an “up-front” higher cumulative dose of vinblastine (over 3 weeks by an infusion pump) at the start of the treatment; this was then followed by maintenance therapy of low-dose vinblastine.

In subsequent studies, the human PC3 prostate tumor grown subcutaneously was employed to show that although metronomic CTX chemotherapy was effective against this tumor type, further antitumor efficacy

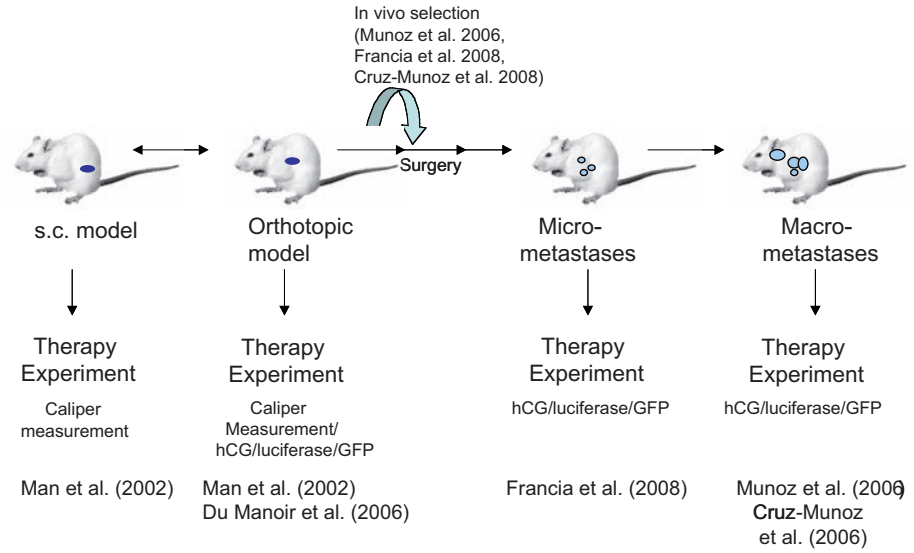

Figure 50.2. Schematic of a generic therapy experimental setup using orthotopic and/or metastatic models. One limiting factor in setting up therapy experiments is the availability of tumor cell lines that can grow and metastasize in mice. Another important factor is the feasibility of implanting models orthotopically (the small size of the mouse prostate makes orthotopic prostate models technically difficult, for example) as opposed to using simpler subcutaneous models. Assuming orthotopic implantation is feasible, it may be necessary to introduce artificial surrogate tumor markers (such as luciferase, GFP, or hCG) to eventually monitor the disease (e.g., as would be the case with colorectal cancer cells injected in the spleen). Furthermore, a number of studies have shown that metastases can respond to therapies that are ineffective on primary orthotopic tumors and vice versa (e.g., [12, 60]). To study the response to therapy of metastatic disease, it is often first necessary to select metastatically competent variants through a process of in vivo selection. Finally, a decision can be made on whether to initiate therapy on early microscopic metastatic disease, or on the later macroscopic metastases. All the models shown have proven to be useful in the evolution and development of the concept of metronomic chemotherapy.

could be obtained by the addition of an "up-front" bolus dose of CTX (150 mg/kg), which was repeated every three weeks. The benefits of this improved regimen were confirmed in subcutaneously injected EMT-6 mouse mammary carcinoma tumors and in spontaneously induced erythroleukemia models in which the bolus injection was repeated every three to six weeks, depending on the tumor model [62]. Such a regimen has been used in additional studies, including the RIP-Tag2 model of pancreatic cancer, but using only an up-front course of MTD therapy, which was not repeated [14].

Using a similar strategy, an up-front bolus CTX was then followed by low-dose CTX administration via the drinking water and used to treat subcutaneously growing Lewis lung carcinoma [63]. The experiment was carried out in both wild-type and TSP-1–deficient mice, revealing a less efficacious antitumor effect in the TSP-1 knockout mice. This study implicated TSP-1 expression by the cells as a mechanism contributing to the antiangiogenic effect of metronomic chemotherapy. These observations led to the suggestion, supported by some experimental evidence, of the potential benefit of combining metronomic chemotherapy with TSP-1 mimetics to improve the antitumor effects of metronomic chemotherapy [64].

Subcutaneously implanted PC3 tumors were also employed to demonstrate that the eventual development of resistance to metronomic CTX does not induce host alteration of CTX pharmacokinetics (as might be expected by a change in the expression or activity of liver detoxifying enzymes). Furthermore, the model was also employed to show that as tumors relapse while on metronomic CTX, a further growth delay could be obtained by the addition of tirapazamine, a bioreductive drug targeting hypoxic tumor cells. Collectively, these and other results defined the concept of what would come to be termed as "metronomic chemotherapy" [65] and were eventually followed by other studies confirming the validity of this treatment approach, even when it is tested against orthotopic and metastatic preclinical tumor models, as detailed in subsequent sections.

Use of Orthotopic and Transgenic Mouse Models to Study Aspects of Metronomic Chemotherapy

In 2002, Man et al. [18] reported that treatment of mice with continuous low-dose administration of CTX through the drinking water caused a growth delay of orthotopically implanted MDA-MB-231 human breast

cancer cells. Furthermore, the combination of metronomic CTX plus the anti-VEGFR2 antibody DC101 was found to produce sustained growth suppression for more than 100 days. This study was fundamentally important for several reasons. First the addition of CTX to the drinking water (whereby the mice would "dose" themselves), greatly simplified the feasibility of long-term preclinical metronomic chemotherapy experiments – at least when it is possible to use drugs such as CTX, which can be administered orally. Second, it confirmed that the results obtained by Browder et al. [9] could be reproduced in a more clinically relevant orthotopic model, using a modification (i.e., CTX administered in drinking water rather than injected intraperitoneally at 170 mg/kg every 6 days) of the original metronomic protocol. In this regard, as described later, many if not most clinical trials of metronomic chemotherapy involve oral drugs – especially CTX. Third, the therapy was found to be effective in a number of different preclinical models in addition to MDA-MB-231, including subcutaneously implanted human colorectal carcinoma HT29.hCG (discussed later), the subcutaneously implanted PC3 prostate cancer, and also in a spontaneous model of pancreatic islet cell carcinoma in Rip-Tag2 transgenic mice.

The report by Man et al. [18] was followed by a number of studies describing the effect of metronomic chemotherapy on different tumor types, as well as the testing of new drugs to evaluate their efficacy when administered in a metronomic schedule [3]. Efforts were also made to retest established and validated regimens on responsive tumor cell lines (e.g., metronomic CTX on MDA-MB-231), but in a setting of advanced orthotopic and metastatic disease. The difficulty with the latter models is that for certain tumor types such as prostate, the development of reliable orthotopic models is difficult or impractical. This is principally because of the limited number of prostrate cancer lines available that effectively metastasize in immunodeficient mice, and the small size of the mouse prostate that makes reliable orthotopic injection of these cells very difficult. Thus, in many instances, for studies to be carried out in advanced disease, the appropriate preclinical model first had to be developed. In the following sections, we detail the advances made in the identification of optimal metronomic chemotherapy regimens, and the application of this therapeutic strategy on models of advanced disease.

Metronomic Chemotherapy in the Preclinical Setting of Advanced Metastatic Disease

Breast Cancer

The human MDA-MB-231 breast cancer cell line was employed to develop a number of advanced-stage tumor models to study the impact of metronomic chemotherapy regimens in such settings. Thus du Manoir et al. [56] derived a HER2-positive variant, by retroviral transduction, that was termed H2N (Figure 50.3). Subsequently H2N cells were implanted orthotopically in the mammary fatpad of nude mice, and the model was used to test the effectiveness of combining metronomic CTX with the anti-HER2 antibody trastuzumab; the results showed that the combination could cause long-term growth delays in the absence of significant host toxicity. Furthermore the antitumor efficacy could be improved further by the inclusion of the anti-VEGF antibody bevacizumab when tumors began to relapse on CTX plus trastuzumab therapy. These results showed the potential benefit of the combination of metronomic CTX with targeted agents, including antiangiogenic agents such as bevacizumab, a finding that has been provisionally demonstrated in a Phase II clinical trial of metastatic breast cancer patients [66]. In a subsequent study, H2N cells were transfected with hCG and then selected in vivo to derive a metastatically competent variant, termed met2 [60]. Met2 cells were then allowed to grow as orthotopic tumors or as spontaneous metastases, and both were treated with trastuzumab. Whereas the orthotopic tumors responded to the antibody, the metastases appeared to be completely resistant. These results suggest that it may not be sufficient to study only the growth and response of orthotopic primary tumors, and that consideration should be given to studying the effects on established/advanced metastases [23, 60].

In a separate study, Munoz et al. [12] implanted the parent MDA-MB231 orthotopically and surgically removed the tumors when they reached an average

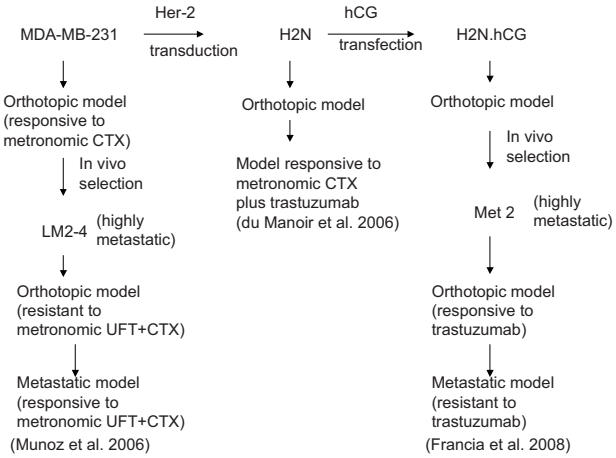

Figure 50.3. Schematic showing the development of orthotopic and metastatic preclinical breast cancer models based on use of the human MDA-MB231 cell line to study new metronomic chemotherapy regimens. HER2 and hCG gene insertion were used to make HER2-positive variants and to express hCG as an artificial marker for tumor growth burden that could be monitored in mouse urine. In vivo selection was used to generate metastatic variants. The response to therapy of primary orthotopic tumors to metastases may differ (as detailed for the highly metastatic variants LM2–4 and Met2).

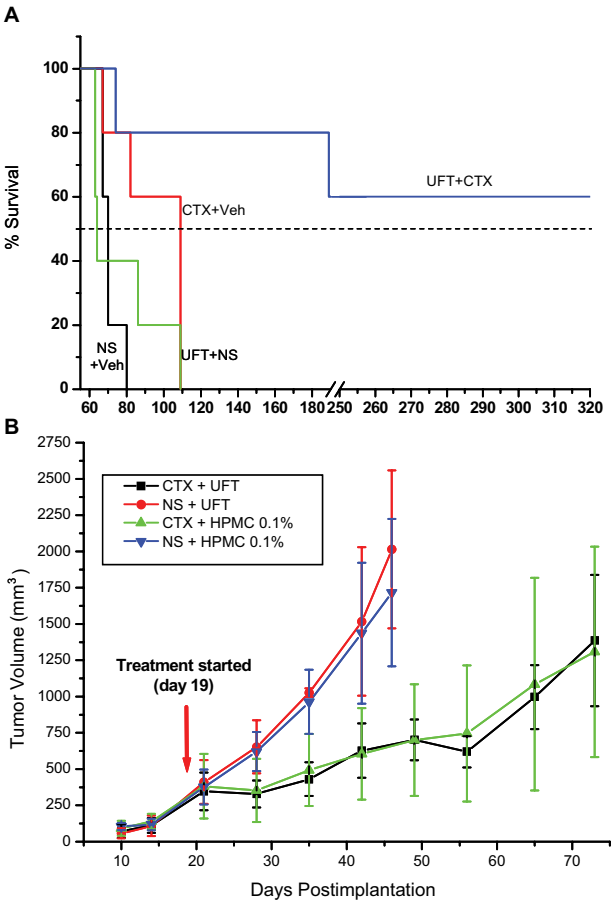

Figure 50.4. Response of the metastatic variant of human breast cancer cell line MDA-MB231, termed LM2–4 growing in SCID mice. (A) shows the survival of mice bearing metastases of LM2–4 (which develop following the surgical removal of the orthotopically implanted LM2–4 tumors) when treated with the metronomic combination of UFT and CTX. (B) shows that the same therapy does not significantly affect the growth of the orthotopically implanted LM2–4 tumor. Therefore, reliance solely on orthotopic tumors may cause the failure to discover new potentially beneficial regimens that may only be effective on metastatic disease (Adapted from Munoz et al. [12]).

size of 500 mm^3; this led to the development of lung metastases approximately six months later. Tumor cells were subcultured from the pooled metastases and used in a second round of in vivo selection to derive a highly metastatic variant, termed LM2–4 [12]. The LM2–4 model was then be used to test the response to metronomic therapies of a tumor population grown either orthotopically or (following surgery) as advanced metastatic disease. Munoz et al. [12] showed that a combination of two drugs, CTX and UFT, delivered in a metronomic fashion could significantly prolong survival of mice with established LM2–4 metastases [Figure 50.4A). Surprisingly, the remarkable benefits of this combination metronomic regimen were not observed when the therapy was initiated on established orthotopically implanted primary tumors (Figure 50.4B). A similar situation was also observed in our model of advanced spontaneous melanoma metastasis, described later [26]. This result, if repeated in additional systems, raises an obvious concern, as it is the norm in most preclinical therapy studies to start with, or restrict evaluation of therapies to, subcutaneous or orthotopic primary models. In addition, if the results obtained in primary tumor therapy experiments are used as a basis to decide whether to undertake further experiments in models of advanced metastatic disease, the aforementioned metastasis experiments would not have been done. By undertaking parallel experiments, however, the effects on metastatic disease were uncovered – results that were critical to the initiation of a Phase II clinical trial of daily metronomic capecitabine (another oral 5-FU prodrug), CTX, and bevacizumab every two weeks in metastatic breast cancer, which gave such promising results [66], that the protocol is now being evaluated in a randomized phase III trial in which the control arm is weekly paclitaxel plus bevacizumab (clinicaltrials.gov, under "metronomic chemotherapy").

Melanoma

Using the 113/6–4L spontaneous metastatic melanoma cell line in SCID mice, we recently reported the benefits of combining a metronomic doublet regimen of CTX and vinblastine. Researchers implanted 113/6–4L cells subdermally (an orthotopic site for melanoma) and the primary tumors generated by these implants were resected when they reached an average size of 500 mm^3. Previously these conditions were identified as permitting the development of extensive metastatic disease, as detailed in Table 50.2. Six weeks after the surgery, the mice were treated with metronomic CTX, vinblastine, or the combination of both drugs. Under these conditions, controls showed a median survival of 100 days, whereas the monotherapies of CTX and vinblastine showed a median survival of 110 and 130 days, respectively. The combination of CTX and vinblastine was found to significantly increase median survival to 190 days; moreover, this was observed in the absence of any overt toxicity. These results suggest that the metronomic CTX plus vinblastine treatment strategy may be promising for the treatment of melanoma metastases.

Further examination has now shown that the median survival achieved by this CTX/vinblastine combination compared favorably to MTD dacarbazine (the standard clinical treatment for melanoma metastasis). Additional studies show that metronomic regimen of low-dose dacarbazine plus vinblastine and DC101 results in effective suppression of advanced metastatic melanoma. In all these cases, the metronomic chemotherapy was associated with minimal toxicity. This was not the case with MTD dacarbazine, which was characterized by minimal effectiveness and higher levels of toxicity, especially when combined with other chemotherapeutic agents (CTX or vinblastine) or DC101 antibody [83].

TABLE 50.2. Clinical trials testing the efficacy of metronomic therapy for the treatment of advanced/metastatic disease

Cancer type	Regimen	Outcome/observation	Reference
Breast	Capecitabine (500 mg 3× daily) CTX (50 mg daily) Bevacizumab (10 mg/kg every 2 weeks)	2% CR, 46% PR, 41% SD, 11% PD	[66]
NSCLC	Docetaxel (25 mg/m^2), trofosfamide (50 mg/daily)	19% OR, 6.9 months OS, 2 year MPFS	[68]
Melanoma	Trofosfamide 50 mg 3× daily rofecoxib 25 mg day 1+ pioglitazone 60 mg day 1+	9% PFS	[72]
Colorectal	40 mg/m^2 CPT-11 doxifluridine 800 mg daily	36% OR, 452 days MOS	[73]
NSCLC	LDM (cisplatin 25 mg/m^2, docetaxel 25 mg/m^2) vs MTD (cisplatin 75 mg/m^2, docetaxel 75 mg/m^2)	MTD regimen induced significantly increased TSP1 and decreased VEGFR3	[74]
Colorectal	Day 1: Oxiplatin 85 mg/m^2 Leucovorin 200 mg/m^2 5-FU bolus (400 mg/m^2) and 600 mg/m^2 infusion Followed by 10-day daily UFT (200 mg/m^2) and leucovorin (30 mg/m^2)	35.7% PR, 5.2 months TTP, 13.4 months MOS	[75]
Breast	Trastuzumab (6 mg/kg) every 3 weeks Methotrexate 2.5 mg, bid day 1 & 4 Cyclophosphamide 50 mg daily	18% PR, 46% SD, 36% PD, 6-month MTP, 27% clinical benefit in patients with disease resistant to previous trastuzumab therapy	[67]
Breast	Methotrexate 2.5 mg daily (day 1, 2, or 4) and cyclophosphamide (50 mg daily)	3.2% total remission, 16% partial remission, 15.7% prolonged clinical benefit, 21-month median time to remission in patients with clinical benefit	[76]
NSCLC	Cisplatin (30 mg/m^2) day 1, 8,14, 28 Etoposide daily for 21 days out of 28-day cycle	45.2 OR, 58.1 disease control rate, 9-month TTP	[77]
Various cancer types	CTX (50 mg daily); rofecoxib (25 mg daily); vinblastine (3 mg/m^2)	4% CR, 9% PR, 17% SD ≥ 6 months, 30% clinical benefit	[78]
Breast	Arm A: CTX (50 mg daily); methotrexate (25 mg twice daily, days 1 and 4) Arm B: A+thalidomide (200 mg daily)	20.9% OR in arm A, 11.8% in arm B, 41.5 % clinical benefit for both arms	[71]
Prostate	CTX 100–150 mg daily, mesna 400 mg/day po	25% PR, 37.5% SD, 62.5% clinical benefit	[79]
Melanoma	Treosulfan (50 mg), rofecoxib (25 mg)	8% OR, 25% SD (36 weeks), 8% SD (24 weeks), median survival 13 months	[80]
Melanoma	Arm A: Bevacizumab (15 mg/kg intravenously every 2 weeks) Arm B: A+ low-dose IFN-alfa2b (1 MU/m^2 subcutaneously daily)	Arm A: 31% SD, 3 months PFS Arm B: 6% PR, 18% SD, 3 months PFS	[81]
Various cancer types	Cyclophosphamide (50 mg daily, oral) or etoposide at 50 mg daily. Celecoxib (400 mg twice daily)	16% SD (≥16 weeks)	[82]

CR: complete response; SD: stable disease; PR: partial response; PFS: progression-free survival; TTP: time to progression; MTP: median time to progression; OR: objective response rate; MOS: median overall survival

Colorectal Cancer

Preliminary results from colorectal cancer models have confirmed that metronomic CTX is effective in controlling the growth of orthotopically implanted HT29 cells (Francia and Kerbel, unpublished observation), confirming the earlier observation on this tumor model grown subcutaneously [18]. Future experiments will be aimed at determining whether metronomic

chemotherapy regimens are also effective when treating metastatic disease and whether the intrasplenic model will generate the same response as the (more complex) intracecal models.

Ovarian Cancer

A recent study from our group [84] and by Merritt et al. [85] showed that a potent anti-tumor effect in models of advanced ovarian cancer can be obtained by using treatment consisting of daily low-dose metronomic topotecan chemotherapy plus concurrent daily dosing with pazopanib, an antiangiogenic tyrosine kinase inhibitor. This regimen is now being considered for phase II clinical trial evaluation.

Summary

Important discoveries regarding the efficacious use of metronomic chemotherapy regimens have resulted from experiments using both subcutaneous and orthotopic primary tumor models [3]. Furthermore, the orthotopic models have proven important for the subsequent derivation of metastatic tumor models [10, 24], which in turn have revealed unexpected findings, such as the surprising efficacy of combining metronomic UFT and CTX for the treatment of advanced metastatic breast cancer, accompanied by minimal toxicity. Thus, we feel that it is important that more advanced orthotopic and metastatic models be developed. It is likely that the application of these new models for therapy experiments will enhance the frequency of successful translation of therapeutic concepts from the bench to the bedside.

CLINICAL TRIALS OF METRONOMIC CHEMOTHERAPY FOR TREATMENT OF ADVANCED/METASTATIC DISEASE

A number of clinical trials incorporating metronomic dosing and scheduling of chemotherapeutic agents have been undertaken to evaluate the efficacy of this strategy against advanced/metastatic disease of various cancer types [86] (see Table 50.2). Similar clinical trials are also under way, such as metronomic vinorelbine plus bevacizumab as salvage therapy for metastatic breast cancer; metronomic low-dose CTX and methotrexate with/without bevacizumab therapy for metastatic breast cancer; imatinib mesylate, bevacizumab, and metronomic CTX as antiangiogenic therapy for metastatic solid tumors, to name a few. (An up-to-date summary of many ongoing metronomic therapy trials can be found at http://clinicaltrials.gov under the search heading "metronomic chemotherapy." Among these, at least four randomized phase III trials are presently underway.) A large proportion of the clinical trials that have been reported in the literature incorporate low-dose CTX or trofosfamide (an oxazaphosphorine prodrug that is metabolized predominantly to the CTX analog ifosfamide) as the metronomic chemotherapy drug backbone of the regimen [67, 68].

Collectively, the use of metronomic therapy has shown effectiveness in the treatment of metastatic disease, achieving this effect with minimal toxicity. For instance, in one of the earlier studies examining the efficacy of this therapeutic regimen against advanced-stage solid tumors, Colleoni et al. [69] showed that a combination of metronomic CTX and methotrexate mediated an overall clinical benefit rate (including stable disease) of 31.7 percent in breast cancer metastasis patients. Among responding patients, the median duration of response was 6.8 months. This treatment was well tolerated (with grade I leukopenia being noted as the most common sign of toxicity) and did not show significant bone marrow suppression, mucositis, or hair loss, which are often associated with standard chemotherapy regimens [69].

The addition of "dedicated" antiangiogenic agents to metronomic chemotherapy is now becoming an important consideration as a means of amplifying the efficacy of this treatment concept. Preclinical evidence shows that the combination of metronomic chemotherapy and antiangiogenic agents, such as VEGFR-blocking antibody DC101, or small-molecule antiangiogenic drugs, results in an enhanced antitumor effect [3, 14, 15]. Equally, as discussed earlier, some of our preclinical studies have shown that in some combinations of metronomic chemotherapy and DC101 result in effective suppression of advanced-stage metastatic melanoma [83]. In the clinical setting, Burstein et al. [70] showed that the addition of bevacizumab to the doublet metronomic CTX and methotrexate regimen (described previously) resulted in enhanced activity when compared with the metronomic combination of these agents alone. In recent studies, Dellapasqua et al. [66] have shown that 68 percent of patients achieved disease control for at least six months with a combination of bevacizumab and daily oral low-dose CTX and capecitabine. However, an enhanced effect induced by the antiangiogenic agents is not universal. Colleoni et al. [71] noted that incorporation of thalidomide (an inhibitor of FGF2 and VEGF) did not enhance the response mediated by a regimen of metronomic low-dose CTX and methotrexate in patients with advanced breast cancer. Nevertheless, this regimen of CTX/methotrexate (with or without thalidomide) mediated a significant decrease in mean serum levels of VEGF and a relevant overall clinical benefit of 41.5 percent, while still noting minimal toxicity [71]. This response rate was even higher (55.6%) in a subgroup of in patients not pretreated with systemic chemotherapy for metastatic disease.

CONCLUSION

Standard MTD chemotherapy dosing regimes have often proven ineffective or of modest clinical benefit in the treatment of advanced metastatic disease, insofar as significantly prolonging survival times. Treatment has been largely seen largely as a palliative measure rather than a curative approach and is often accompanied by significant toxic side effects. Here we have suggested that a rethinking is necessary to develop more effective and less toxic treatment strategies involving chemotherapy drugs. The question initially raised regarding the efficacy of metronomic therapy against metastatic disease [20] is now being addressed by both preclinical and clinical studies. Evidence from many of these studies shows that metronomic therapy appears to be a promising alternative for the treatment of advanced metastatic disease.

Clearly, further examination of the efficacy of this approach is needed. We suggest that this effort should be conducted in a rational manner, in which the efficacy of chemotherapeutic combinations (with or without additional agents such as dedicated antiangiogenics) should be first assessed in relevant preclinical models of advanced metastatic disease that reflect the metastatic spread of tumor cells from an orthotopic primary tumor to distant, clinically relevant organ sites. This is an aspect of preclinical therapy models that has been absent in preclinical experimental therapeutic research over the past fifty years. Efforts from our laboratory have succeeded in generating such models for melanoma and breast cancer, but the development of additional models for other cancer types is needed. These models and approaches will help to address one of the major challenges of developing appropriate metronomic therapies – namely, determination of which cytotoxic agents or combinations of agents, including molecular targeted drugs, produce the most effective benefit [7]. Such efforts will benefit from a close interaction between preclinical and clinical researchers.

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51 Immunotherapy

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CANCER IMMUNOBIOLOGY

Tumors Are Recognized by the Immune System

The idea of fighting tumors with immunological weapons has been pursued empirically in modern medicine since the end of the nineteenth century. The birth of modern tumor immunology is usually credited to experiments in the 1940s and 1950s showing that inbred mice are protected by exposure to harmless tumor components (vaccination) from the growth of a subsequent inoculum of live tumor cells (challenge) (Gross 1943; Foley 1953; Prehn and Main 1957; Klein et al. 1960). Countless vaccination-challenge experiments were performed over the subsequent decades to dissect immune mechanisms that protect the vaccinated host, using either immunocompetent or immunodepressed hosts. The overall results are quite clear; however, they form the basis of a contradiction between preclinical and clinical tumor immunology that must be taken into account to understand current and future therapeutic developments.

Innate immunity is fundamental, both because its cells and molecules (e.g., phagocytes and interferons) directly attack tumors and because antigen presentation (dendritic and other cells) is required for the generation of adaptive immunity (Restifo and Wunderlich 2005). Myeloid-derived suppressor cells (MDSCs) play a negative role and inhibit antitumor immunity (Nagaraj and Gabrilovich 2008; Marigo et al. 2008). Natural killer (NK) cells are highly active against circulating tumor cells; hence they play a significant antimetastatic role: without NK cells, the experimental metastatic ability of tumor cells can increase a hundredfold (Ljunggren and Malmberg 2007).

T cells are the staple of tumor immunology; cytotoxic T cells (CTLs) directly kill tumor cells; helper T cells (Th) are needed to activate CTLs after antigen presentation by dendritic cells (DC), produce interferon (IFN)-γ and other cytokines that inhibit tumor cell growth, and enhance major histocompatibility complex (MHC) and antigen expression (Restifo and Wunderlich 2005). Regulatory T cells (Treg) inhibit antitumor immunity and can benefit both the afferent and the efferent phases (Colombo and Piconese 2007; Sakaguchi et al. 2008). Antimetastatic activity is also shown by NKT cells, which, however, also include subpopulations favoring tumors (Terabe and Berzofsky 2007).

B cells and antibodies were generally found to be irrelevant, or even detrimental, in vaccination-challenge experiments. A major determinant is that solid tumors are mostly resistant to complement-mediated antibody cytotoxicity, thanks to the expression of physiological complement inhibitors such as CD55 and CD59. The vaccination-challenge model, tends to downplay the protective role of antibodies with the sudden challenge of mice with a massive injection of fast-growing tumor cells in an ectopic anatomic site, such as the subcutis or the muscle. Furthermore, most tumor antigens involved in these experiments were not expressed on the cell surface; hence either they were unreachable by antibodies, or antibody binding did not directly inhibit mitogenic signaling, a major mechanism mediating the activity of current therapeutic antibodies, such as trastuzumab. Thus, tumor immunology was and remains focused on T cells, and translational efforts followed suit (Delves and Roitt 2000; Restifo and Wunderlich 2005). This is clearly at variance with the fact that monoclonal antibodies are the most effective immunotherapeutic tool and were approved worldwide by regulatory agencies, whereas T-cell–based therapeutic endeavors remain largely experimental.

Tumor Antigens

The study of tumor antigens, in particular those of human origin, relied for many years on xenogeneic antibodies raised in rodents, a technology that revealed

some interesting targets but also many molecules poorly immunogenic in the species of origin, and almost completely dismissed those recognized by T cells as MHC-associated peptides. The availability of tumor-specific T cell clones that could be used for library screening led in the 1980s to the discovery first in mouse, then in human tumors, of antigens targeted by CTL responses. Such antigens can mediate immune rejection of tumors and are suitable targets for T-cell–based immunotherapy.

The current database of human tumor antigens comprises more than one hundred molecules that can be classified into six classes (Mosolits et al. 2005; Novellino et al. 2005; Lollini 2009).

Cancer-testis antigens were the first to be discovered and include MAGE, BAGE, GAGE, and NY-ESO-1. They are expressed by human melanoma and several other tumors and some noncancerous tissues (testis, wound repair).

Differentiation or *lineage-specific antigens* include molecules expressed by normal tissues and by tumors of the same lineage, such as tyrosinase and related proteins, gp100 and MelanA/MART1 (melanocytes and melanoma) and prostate-specific antigen (PSA).

Shared antigens, such as carcinoembryonic antigen (CEA), HER-2/neu, MUC-1, survivin, and the catalytic subunit of telomerase (TERT), are expressed by multiple tumor types and also by some normal tissues.

The expression of tumor antigens by normal cells, a constant feature of the preceding classes, has two important consequences: (1) immune tolerance operates at multiple levels to prevent the generation of autoimmune responses, and a break of tolerance is required to generate effective antitumor immune responses; and (2) autoimmune responses unleashed by immunotherapy can destroy normal tissues expressing the target antigen.

Fusion proteins (e.g., BCR-ABL, PAX3-FKHR) and *mutant proteins* (e.g., RAS, p53) result from oncogenic mutations occurring during the carcinogenic process and tumor progression and are the only true tumor-specific antigens. Mutant proteins can be the consequence of random mutations and the antigenic sequence can differ from one patient to another; hence they are also called *unique tumor antigens* (Parmiani et al. 2007). Unfortunately, they are also poorly immunogenic, and the few clinical studies performed so far with vaccines targeting mutant RAS or p53 have been discouraging (Carbone et al. 2005).

A final class of operationally tumor specific antigens includes the *idiotypes* of T- and B-cell malignancies – the clonotypic and in itself antigenic part of immunoglobulins and T-cell receptors. Immunotherapy trials targeting idiotypes in B lymphoma yielded promising results (Timmerman and Levy 2004).

In addition, antigens expressed by tumor-associated endothelial, stromal, and leukocyte components can be usefully targeted by cancer immunotherapy (Hofmeister et al. 2008).

Immune Surveillance and Immunoediting

The existence of tumor antigens eliciting distinct antitumor immune responses means that the immune system should constantly protect the host from tumor onset, as it does from infections caused by bacteria or viruses. This hypothesis, called the *theory of immune surveillance*, was set forth in the 1950s, but its definitive demonstration had to wait until the beginning of the twenty-first century. Immune surveillance implies that immunodepressed hosts should have a higher incidence of tumors than immunocompetent hosts. Genetically engineered mouse (GEM) models completely lacking adaptive immunity and with severe impairments of innate response are indeed more prone to spontaneous and induced carcinogenesis than immunocompetent congenics (Shankaran et al. 2001). In humans the situation is more complex, because individuals with a level of immune depression similar to the aforementioned mice do not reach the age at which tumors commonly arise. Individuals with partial or transient depression of immunity, such as transplant recipients or AIDS patients, have a significant increase in the incidence of some tumor types, such as lymphomas and such viral tumors as Kaposi sarcoma (HHV8) and cervical carcinoma (HPV). Tumors arising in immunocompetent individuals must have found a way to escape immune destruction, so they should have a reduced immunogenicity. This phenomenon, now called *immunoediting*, was again clearly demonstrated by comparing the tumors of immunodepressed mice with those of immunocompetent mice. Tumors arising in the absence of a functioning immune system resulted in being more immunogenic than those that had found a way to escape from effective immune responses.

The latest incarnations of the theory of immune surveillance posit three successive phases (the three "Es"): elimination of cancer cells by the immune system; equilibrium between tumor growth and immune defenses (a likely mechanism for tumor dormancy), which can keep tumor growth in check for long periods of time; and escape of tumor cells from immune control, giving rise to progressive local or metastatic masses (Dunn et al. 2006; Koebel et al. 2007; Melief 2007; Ostrand-Rosenberg 2008).

Tumors adopt both passive and active strategies to escape immune recognition. Downmodulation of tumor antigens was found in human melanoma. Sampling of multiple cutaneous metastases showed that some lesions had downmodulated or completely lost expression of antigens expressed by the primary tumor. Activation of the T-cell antigen receptor requires

surface expression of the antigenic peptide bound to MHC molecules; however, 80 percent to 90 percent of all human tumors were found to express little or no MHC (Garrido and Algarra 2001; Campoli and Ferrone 2008). This probably makes the phenomenon of MHC downmodulation the most frequent immune escape mechanism, and also one of the most prevalent alterations of the tumor cell, on a par with telomerase activation, and much more frequent than p53 mutations. Additional escape strategies leading to the same consequences as MHC or antigen loss entail defects in the antigen processing machinery that degrades proteins (immunoproteasome) and transports peptides from the cytoplasm to the reticulum (TAP transporters), in which MHC-peptide complexes are assembled (Campoli and Ferrone 2008).

Tumor cells can also actively suppress antitumor immunity through the release of negative regulators, such as transforming growth factor β (TGF-β) or interleukin (IL)-10; the induction of suppressive myeloid (MDSC) or T (Treg) cells, and the expression of surface receptors of the TNF/Fas family that trigger apoptosis of helper and CTLs.

Oncoantigens

Knowledge of immunoediting mechanisms shows that not all tumor antigens are equally prone to the selection of escape tumor variants. *Oncoantigens* are defined as tumor antigens that persistently mediate immune recognition of tumors (Lollini et al. 2006). Members of this new category include tumor antigens that share the properties of being surface or soluble molecules and play an essential role in tumor growth. Surface or extracellular expression means that the oncoantigen remains recognizable by antibodies if MHC or antigen processing machinery are downmodulated, and the requirement for an essential role in tumorigenicity forbids growth of antigen loss tumor variants. Oncoantigens are attractive targets for cancer immunotherapy; however, only a few tumor antigens are known to fulfill the requirements, among them HER-2, MUC-1, and idiotypes. The suitability and antigenicity of many other potential members of this class, such as many growth factors and receptors, remain to be determined experimentally.

CANCER IMMUNOPREVENTION

Concept of Cancer Immunoprevention

The efficacy of immune surveillance is incomplete, as tumors do arise in immunocompetent humans and mice. The idea behind cancer immunoprevention is that treatments that enhance immune surveillance in healthy individuals should result in a lower tumor incidence later in life.

Immunoprevention of Viral Tumors

Prevention of viral tumors can be obtained with vaccines preventing viral infection. This was first obtained in humans with the hepatitis B vaccine, which significantly reduced the incidence of posthepatitic hepatocellular carcinoma (Chang et al. 1997). Excellent results were documented in clinical trials of vaccines against oncogenic papillomaviruses (such as human papillomavirus [HPV]), which led to their approval for mass vaccination in several countries. HPV vaccines are expected to significantly impact the incidence of cervical carcinoma and other HPV-related neoplasms (Koutsky et al. 2002; Chan and Berek 2007). A full-scale worldwide implementation of vaccines against oncogenic viruses, including vaccines not yet available against hepatitis C virus (HCV) and Epstein-Barr virus (Hepeng 2008), could prevent 10 percent of all human tumors.

Immunoprevention of Nonviral Tumors

The prevention of tumors unrelated to infectious agents was clearly demonstrated in several preclinical models of mice subject to spontaneous or carcinogen-induced tumor development. The best results were obtained with vaccines combining the target antigen with powerful immune adjuvants. Oncoantigens made the best targets, as the development of antigen- or MHC-loss tumor variants was minimized over time (the oncoantigen concept was first developed in the field of cancer immunoprevention) (Lollini and Forni 2003; Lollini et al. 2006).

Immunoprevention of non-infectious tumors is at a preclinical stage of development. The major issues that need to be addressed in view of human translation are the definition of suitable populations at risk for which target oncoantigens exist, and the safety of eliciting long-term immunity against autoantigens potentially conducive to autoimmune disease. The vaccines and strategies developed for immunoprevention are of immediate interest also for immunotherapy, as vaccines effective in preventing primary tumor onset were also found to prevent the outgrowth of distant metastases, because target lesions share the properties of being small in size and easily accessible to soluble and cellular immune effectors (Nanni et al., 2007).

CANCER AND METASTASIS IMMUNOTHERAPY

General Translational and Clinical Aspects of Cancer Immunotherapy

We address here some specific differences between cancer immunotherapy and cytotoxic chemotherapy, which are common to all cancer therapies making use

of biological agents and extend in part also to targeted therapy with noncytotoxic small molecules.

Therapeutic Agents

The range of molecules and cells that have been used for cancer immunotherapy is extremely wide. The major common issues here are the homogeneity and the repeatability of the preparation for clinical use. "Natural" preparations, such as animal antisera, which were used before the advent of monoclonal antibodies and recombinant DNA technologies, should be absolutely avoided whenever this is technically feasible. This is not always possible, however, as in the case of materials derived from individual patients. In such instances, strict procedures and algorithms should be enforced to minimize variability; the statistical potency of studies should be adjusted accordingly.

Species-specificity of the agent is an important translational difference between biotherapies and most small molecules. Preclinical studies of small molecules assume that the same agent will be tested first in animals, then in humans, and that the major differences will be in host responses. However, almost all biotherapeutic agents are species-specific to varying degrees, with human molecules inactive in mice and vice versa. In this case, it must be clear to both the translational and the clinical researcher that molecules going by the same name in different animal species are actually different, and there is no guarantee that they will elicit identical responses (Cheever et al. 2008). One must be particularly wary of *partial* cross-reactions, as it happened for example with tumor necrosis factor (TNF)-α, because the temptation to use the human molecule in preclinical animal studies could miss important therapeutic or adverse effects.

Finally, repeated administration of therapeutic macromolecules – proteins, in particular – can induce autoimmune responses against the therapeutic agent itself, as can happen with IFN-α, giving rise to peculiar mechanisms of resistance.

Study Design

Dose-finding trials of cytotoxic drugs assume a linear log dose–cell kill curve; thus they aim at finding the maximum tolerated dose (MTD), as the most effective drug dosage with acceptable side effects. With biological agents, linearity is not a given, and bell-shaped curves can occur, separating the optimal biological dose (OBD) from MTD. Hence Phase I trials could be split into two complementary studies, separately aimed at finding MTD (Phase Ia) and OBD (Phase Ib; Creekmore et al. 1991).

Similar to what happens in targeted therapy, patient eligibility for cancer immunotherapy can be more selective than in conventional Phase I studies – for example, by mandating a single homogeneous pathology, or the presence of intact lymph nodes.

When the therapeutic agent is potentially immunogenic, it is advisable to avoid recycling patients who received the lower doses at the beginning of the dose-finding study.

Both therapeutic and side effects caused by immunotherapy or biological agents can take unexpected forms, especially if the agent or therapy is tested for the first time in humans. The favorable outcome of early IFN-α trials in the 1980s kindled the hope that biological agents could be less toxic than traditional cytotoxic drugs, but the hope was soon extinguished by the severe toxicity profile exhibited by IL-2 and TNF-α. It must be kept in mind that, given the indirect mechanisms of action of many biological agents, not only the type but also the temporal kinetics of therapeutic and toxic effects can be markedly skewed, frequently giving rise to delayed responses. The evaluation of clinical responses in cancer immunotherapy is currently performed using standard criteria, such as those of the World Health Organization (WHO) or Response Evaluation Criteria in Solid Tumors (RECIST). Debate is now taking place on whether different criteria would be more appropriate, in particular to include long-term stabilizations observed during immunotherapy (Cheever et al. 2008; Tuma 2008; Hoos et al. 2010).

Immune Monitoring

Many immunotherapeutic agents do not affect tumor cells directly; rather, their effect is mediated by the immune system or other host's responses, which in turn kill tumor cells. Hence an insufficient clinical response can have two causes: either the agent fails to elicit an optimal biological response, or the biological response is indeed elicited, but it fails to effectively control tumor growth. Therefore clinical studies must include appropriate endpoints to evaluate the biological and immunological response in parallel with clinical response.

In cases in which biological predictors are scarce, a thorough comparison of clinical responders and nonresponders can shed some light on relevant immunological variables. In cytotoxic therapy, clinical responses are correlated to many other clinical parameters, and a posteriori analysis of survival in responders has the flavor of a self-fulfilling prophecy. The situation is conceptually different in cancer immunotherapy, however, because the evaluation of immunological responders is more akin to the situation of targeted therapy, in which separate evaluation of patients expressing the specific target is generally more informative than that of target-negative patients.

In practice, immune monitoring of cancer patients undergoing immunotherapy is difficult, given the local nature of several relevant immune responses. On the one side, it is obvious that the easier and more practical monitoring is based on peripheral blood sampling; however, clinically relevant and meaningful immune responses are those occurring within metastatic deposits and local lymph nodes, which in most instances can be sampled only by fine-needle aspiration or similarly cumbersome maneuvers. The reliability of peripheral blood indicators is maximal when the measured immune response is by nature systemic, such as circulating antitumor antibodies, and minimal for local cellular responses such as CTL activity, which entail immune cell extravasation, maturation, and activation. Cytokine levels in the blood are a mixed bag, because some phylogenetically ancient molecules, such as IFN-α, can reach meaningful systemic levels, whereas others, such as IFN-γ or TNF-α, operate physiologically to work at high local concentrations but become rapidly toxic if they reach high systemic levels.

As many antitumor immune responses are intrinsically autoimmune (as discussed previously), signs of autoimmunity in cancer patients undergoing immunotherapy can correlate with immunological and clinical antitumor responses and can be used for immune monitoring. The most conspicuous case is the autoimmune vitiligo that develops in some melanoma patients who are vaccinated or otherwise stimulated to react against differentiation antigens shared by melanocytes and melanoma cells. It was also recently shown that patients displaying generic laboratory findings of autoimmunity, such as antinuclear or antithyroid autoantibodies (Gogas et al. 2006), have markedly improved survival after IFN-α therapy.

Multiple Approaches to Cancer Immunotherapy

Over the years, experimental and clinical tumor immunologists devised literally hundreds of different immunotherapeutic approaches. In this chapter we will chiefly cover those endeavors that found at least some level of clinical application. Many attempts were made to classify immunotherapies, such as by therapeutic agent, type of immune response, and the like, but most classifications were unsatisfactory or became rapidly obsolete and did not stick. We partially use one of the oldest schemes, which is still popular (at least for what concerns terminology), with the caveat that some categories must be understood today merely as labels, because the underlying immunological concepts are no longer entirely sound.

The first dichotomy is between passive and active immunotherapy (Schuster et al. 2006). Passive immunotherapy is based on the administration of ready-made immunotherapeutic agents with intrinsic antitumor activity, such as monoclonal antibodies. Passive immunotherapy originally embedded the idea that the "passive" host did not need a functioning immune system to respond; however, we now know that most types of passive immunotherapy would not actually work without host responses (see, for example, the mechanism of action of monoclonal antibodies, discussed later in this chapter).

Active immunotherapy is based on agents that induce antitumor immune responses of the host. It is called *aspecific* (or nonspecific) when the agent stimulates immune defenses at large, regardless of the precise antigenic determinants of the tumor. Examples are generic immune stimulants such as BCG or cytokines such as IL-2 or GM-CSF. Specific immunotherapy includes tumor antigens in a vaccine formulation administered to immunize the host against the tumor. As vaccines include adjuvants that nonspecifically enhance antigen presentation and other immune responses, practically all types of specific immunotherapy incorporate aspecific elements, and most findings of aspecific immunotherapy can also contribute novel adjuvants to specific immunotherapy.

Active Aspecific Immunotherapy

Early medical observations (late nineteenth century) of neoplastic patients with concomitant infections showing evidence of tumor regression led to the idea that infection-related material of human or microbiological origin could be used for cancer therapy (Hall 1997).

Bacillus Calmette-Guérin (BCG) is a bovine mycobacterium used in tuberculosis vaccines, which has also been tested in cancer immunotherapy for more than fifty years. Early claims of efficacy against a wide variety of cancers upon systemic administration were dispelled by controlled clinical trials showing no effect. Today two therapeutic indications for local treatments remain valid: intralesional administration in metastatic melanoma, and endovesical treatment for recurring bladder and transitional kidney carcinomas. BCG stimulates strong local inflammatory responses that can destroy tumor deposits but fail to induce systemic immunity. BCG and other mycobacteria are effective adjuvants in combination with immunogenic antigen preparations in cancer vaccines (as discussed later).

Bacterial derivatives such as muramyltripeptide (MTP) and cytokines such as IFN-γ are potent macrophage activators. To treat cancer patients, MTP can be loaded into liposomes or other microparticles that are selectively phagocytosed by resident macrophages in the lungs or in the liver. Some positive clinical results were reported in the treatment of lung metastases of osteosarcoma in association with chemotherapy (Meyers et al. 2005)

Cytotoxic Chemotherapy as Immunotherapy

High-dose chemotherapy suppresses immune responses if it kills leukocytes or their precursors. However, it was found that "medium" dosages of some drugs, such as anthracyclines or cyclophosphamide, could actually enhance many types of antitumor immune responses by killing (more or less selectively) cell populations that negatively regulate antitumor immune responses, such as Treg cells. This did not result in significant clinical results per se; however, cyclophosphamide was recently used to enhance other types of immunotherapy (Brode and Cooke 2008).

Cytokines

The use of cytokines in cancer immunotherapy stems directly from the field of active aspecific immunotherapy. The therapeutic preparations made one century ago from infectious patients are now thought to have contained a cocktail of cytokines, including TNF-α. However, cytokines now comprise too many diverse molecules to allow classification under a single label such as active aspecific immunotherapy. Some cytokines are even used for purposes that transcend immunotherapy and the scope of this chapter, such as colony-stimulating factors (CSFs) in the prevention of leukopenia and other unwanted hematological effects of chemotherapy.

On the whole, the field of cytokines has contributed very few molecules to standard, approved cancer immunotherapy. The main reason is that most cytokines physiologically work at high local concentrations, whereas pharmacological use of systemic administration either produces severe side effects or fails to attain effective concentrations in target organs. In practice, only cytokines that physiologically reach high systemic concentrations, such as IFN-α or CSFs, are now approved for clinical use, whereas most other molecules gave rise to severe toxicities in early clinical trials and did not reach approval as therapeutic agents per se. However, a beneficial consequence was the development of various novel clever approaches to bypass systemic toxicity, ranging from adoptive immunotherapy to gene therapy. Here we deal with two paradigmatic cases, IFNs and IL-2, to examine important clinical and translational issues of general import for the whole field of cytokine immunotherapy. The reader is referred to chapters on individual tumor types for specific therapeutic indications.

Interferon α

IFN-α is a pleiotropic cytokine for which most normal and neoplastic cells express receptors. Its activity in cancer therapy can be regarded as a useful byproduct of its antiviral function. It has both direct antitumor activities, which include cytostatic and cytotoxic effects and the induction of MHC expression, and various activities mediated by the immune system, including the maturation of DCs, the induction of Fc receptors mediating ADCC, and the activation and differentiation of leukocytes, including macrophages and NK, T, and B cells. Early success in the therapy of hairy-cell leukemia led to widespread clinical trials in practically all tumor types. However, the extremely high response rates attained in various hematological malignancies were not replicated among solid tumors, even though IFN-α is now commonly used against melanoma, renal cell carcinoma, and bladder carcinoma (Moschos and Kirkwood 2007; Kujawski and Talpaz 2007).

In the era of targeted therapy it is clear that IFN-α is *not* a targeted drug, and the sheer multiplicity of target cells and mechanisms of action fostered a multitude of trials that demonstrated very low response rates in various solid tumors in which IFN-α was tried by analogy, without a solid and specific rationale, which in turn prevented further therapeutic developments.

The higher sensitivity of hematological malignancies to IFN-α can be attributed more to its antiproliferative and differentiative properties than to immunomodulation; under this respect, IFN-α pertains more to passive than to active immunotherapy. The low response rates of metastatic solid tumors – typically lower than 25 percent – are important, however, because they unmistakably indicate that a proportion of patients would derive a great clinical benefit from IFN-α therapy.

Basically, two classes of clinical correlates showed predictive value, those loosely related to disease extension, patient's status, and IFN responsivity and those related to immune response. The former include the number of IFN-α receptors in the primary tumor, enumeration of circulating tumor cells, patient's performance status, time from diagnosis, serum lactate dehydrogenase (LDH), hemoglobin, and calcium. Immune indicators include evaluation of DCs infiltrating the primary tumor; various parameters of cytokine and chemokine response, from circulating protein levels to genetic polymorphisms; and the activation of concomitant autoimmunity. The most important improvements of IFN-α therapy of solid tumors will probably come from the definition and validation of reliable predictive markers to circumscribe IFN-α administration only to responsive cases.

IFN-γ and TNF-α

The analogical reasoning that drove extensive IFN-α trials also led to clinical testing of other cytokines with similar activity profiles, such as IFN-γ and TNF-α. Unfortunately, neither demonstrated clinical activity, and they are not used in cancer therapy. IFN-γ was

tested in various clinical trials, up to Phase III; however, it did not demonstrate significant therapeutic activity, and there were also hints that it could have detrimental effects, possibly related to the prometastatic activity found in preclinical studies (Meyskens et al. 1995; Lollini et al. 1996).

TNF-α demonstrated promising antitumor activity in preclinical testing, but its toxicity in humans prevented systemic clinical use, allowing only local therapies, such as isolated limb perfusion in metastatic melanoma (van Horssen et al. 2006). Also, in the case of TNF-α, some experimental evidence suggested that the cytokine could have both protumor and antitumor activities (Balkwill and Mantovani 2001; Williams 2008). The demise of TNF-α as a drug fostered, in the 1980s, the advent of cytokine gene therapy, an important technological advancement that, however, so far has not improved the low response rate of cytokine therapy against solid tumors.

ADOPTIVE IMMUNOTHERAPY

IL-2 and Adoptive Immunotherapy

From a broad conceptual perspective, the activities of IL-2 are simpler than those of IFN-α, because most solid tumors are insensitive to IL-2; hence, any therapeutic effect must be mediated by immune activities, mainly the polyclonal activation of T and NK cells, which assume a cytotoxic phenotype collectively called lymphokine-activated killer (LAK), and become capable of lysing, with high efficiency, cells of diverse solid tumors. IL-2 demonstrated an unfavorable toxicity profile in early clinical trials, which led to the development of adoptive protocols based on the patient's lymphapheresis and in vitro exposure of lymphocytes to the cytokine, followed by reinfusion of large numbers of LAK cells (Rosenberg et al. 2008).

Response rates in the order of 20 percent to 30 percent were reported in metastatic melanoma and renal cell carcinoma, but other solid tumors were largely unresponsive. Interestingly, the proportion of responders was of the same order of magnitude as that obtained in the same pathologies with IFN-α, and combinations of IL-2/LAK and IFN-α did not yield synergistic effects, thus suggesting the existence of a population of advanced patients equally responsive to different immunotherapeutic modalities.

Logistic difficulties and costs of LAK generation, coupled with the paucity of clinical responses, prevented widespread acceptance of IL-2/LAK immunotherapy. However, the experimental and clinical development of adoptive immunotherapy has proceeded with the adoption of technological and immunological advancements. Recent improvements include nonmyeloablative lymphodepletion of the host and the use of drugs or depleting monoclonal antibodies to impair Treg cells, to improve the survival and enhance the activity of cytotoxic cells, and the use of more specific cytotoxic cells, such as tumor-infiltrating lymphocytes (TILs) or T cells either selected for tumor specificity or transduced with genes encoding T cell antigen receptors (TCR) specific for tumor antigens (Rosenberg et al. 2008).

Graft versus Tumor

The ability of adoptively transferred allogeneic T cells to destroy neoplastic cells is the most convincing proof of the therapeutic potential of cancer immunotherapy. Leukemic patients receiving an allogeneic bone marrow transplant are significantly more protected from relapse than patients receiving syngeneic grafts. T cell depletion abrogates the protective effect, thus effectively separating immunotherapeutic efficacy from the hemopoietic activity of bone marrow, and opening the way to adoptive immunotherapy with allogeneic T cells.

The main clinical problem is that graft-versus-host (GvH) reaction accompanies the therapeutic effect against leukemia (graft-versus-leukemia [GvL]), potentially giving rise to severe GvH disease (GvHD). Of the numerous attempts to obtain GvL without GvHD, the best results were with a suicide gene therapy approach, in which donor T cells were transduced in vitro with a gene conferring sensitivity to ganciclovir. Patients showing early signs of GvH reaction could then be treated with the drug to kill engineered T cells and prevent overt GvHD (Ciceri et al. 2007).

Attempts to extend the GvL approach to solid tumors (graft-versus-tumor) so far have not attained the clinical efficacy obtained in leukemic patients.

Active Specific Immunotherapy – Therapeutic Cancer Vaccines

The fundamental tenet of active specific immunotherapy is that the immune system could efficiently destroy tumor cells – as demonstrated, for example, by GvL – but under natural conditions it is insufficiently stimulated by tumor and metastasis growth, which in addition deploy evasive countermeasures. The remedy of active specific immunotherapy is to develop therapeutic vaccines to actively immunize the patient against antigens expressed by the tumor.

Countless vaccines have been designed and clinically tested over the years, particularly in metastatic melanoma and renal cell carcinoma. The reader is referred to tumor-specific chapters for details on individual tumor types; here we address mainly the general principles and outcomes of cancer vaccinology.

A general rule applying to all types of vaccine, sometimes cited as "the immunologist's dirty little secret," is

that specific antigens per se make ineffective vaccines unless they are combined with adjuvants that in a non–antigen-specific manner enhance antigen persistence, improve presentation, and attract and activate cells of the immune system. The range of adjuvants available for therapeutic cancer vaccines is much broader than that used for prophylactic vaccines against infectious diseases, because the latter are limited by the absolute requirement to be devoid of adverse effects in healthy populations, including newborns. Potent adjuvants can be used instead in cancer vaccines, even though they are not devoid of (mostly local) adverse effects (Belardelli et al. 2004). Table 51.1 is a list of popular adjuvants used in cancer vaccines. The field suffers from a lack of comparative studies allowing one to decide which adjuvant is actually the most effective for a given vaccine preparation.

The same wide range of options exists in the complementary field of tumor antigen preparations (Table 51.2), which over the years saw vaccinations administered with practically every conceivable antigenic preparation, including whole cells, proteins, peptides, RNA, DNA, modified/pulsed DC, and the like (Mosolits et al. 2005; de Gruijl et al. 2008; Melief 2008; Engell-Noerregaard et al. 2008). So far, however, active specific immunotherapy has not brought about large clinical benefits, and only recently the first DC-based vaccine was approved by the FDA (Kantoff et al. 2010). In general, the response rates of advanced cancer patients with most solid tumors have been very low (below 5%–10%), regardless of vaccine design, and active specific immunotherapy does not fare significantly better than aspecific immunotherapy (Rosenberg et al. 2004). Despite this dismal state of facts, research in this field is actively pursued, and all technological advancements are constantly implemented and clinically tested, in the unremitting hope of obtaining more effective therapeutic cancer vaccines.

TABLE 51.1 Adjuvants used in cancer vaccines

Adjuvant category	Examples
Physical treatments	Radiation, heat (induction of heat shock proteins)
Water–oil emulsions	Incomplete Freund's adjuvant (IFA), montanide
Cytokines	GM-CSF, IL-2, IL-12, IFN-α
Allogeneic MHC glycoproteins	Allogeneic cells
Antagonists of immune suppression	Cyclophosphamide, mAbs against CD25 or CTLA4
Viruses	Poxviruses, vesicular stomatitis virus (VSV), Newcastle disease virus (NDV)
Bacteria and bacterial components	BCG, detox PC (mycobacterial walls + *Salmonella* lypid A + squalene + detergents)
Plant components	Saponins
Animal components	Keyhole limpet hemocyanin (KLH)

TABLE 51.2. Therapeutic cancer vaccines

Antigen formulation	Implementations
Tumor cells	Autologous/allogeneic
	Whole cells/cell lysates/heat shock proteins
	Genetically modified cells (genes encoding cytokines, tumor antigens, etc.)
Dendritic cells pulsed with antigens	Cell lysates/heat shock proteins/apoptotic bodies/microvesicles
	Proteins/peptides
	Nucleic acids
Proteins	Recombinant antigenic proteins, anti-idiotype antibodies
Peptides	Synthetic peptides
Gangliosides	GD2, GD3, GM2
DNA vaccines	Plasmid DNA-encoding tumor antigens

Monoclonal Antibodies

Monoclonal antibodies (mAbs) are the most successful clinical application of tumor immunology (Table 51.3), thanks to decades of preclinical development, easy integration in standard clinical procedures and protocols, and multiple antitumor activities. The inclusion of antibodies in passive specific immunotherapy does not encompass various mechanisms of action that require an active immune response of the host; moreover, various therapeutic activities are actually independent of the immunological nature of antibodies and might be replaced in the future by different molecules not pertaining to immunotherapy.

The immunological antitumor activities include activation of the complement cascade, which can result in complement-mediated lysis of target cells and in complement-dependent cell-mediated cytotoxicity (CDCC) by macrophages expressing complement receptors; moreover NK cells and other cells with Fc receptors can perform antibody-dependent cell-mediated cytotoxicity (ADCC) (Figure 51.1). All immune mechanisms are activated via the Fc moiety of the antibody molecule, thus are also called "Fc-dependent" (Weiner 2007).

TABLE 51.3. Monoclonal antibodies approved for use in cancer patients

Antibody	Type	Target	Indications
Rituximab (Rituxan)	Chimeric IgG1	CD20	B-cell lymphoma
Ibritumomab tiuxetan (Zevalin)	Conjugate (mouse IgG1–^{90}Y or ^{111}In)	CD20	B-cell lymphoma
Tositumomab (Bexxar)	Conjugate (mouse IgG2a-^{131}I)	CD20	B-cell lymphoma
Gemtuzumab ozogamicin (Mylotarg)	Conjugate humanized IgG4-cytotoxic antibiotic	CD33	AML
Alemtuzumab (Campath)	Humanized IgG1	CD52	B-CLL
Trastuzumab (Herceptin)	Humanized IgG1	HER-2	Breast carcinoma
Cetuximab (Erbitux)	Chimeric IgG1	EGFR	Colorectal, head and neck, non–small-cell lung carcinoma
Panitumumab (Vectibix)	Human IgG2	EGFR	Colorectal carcinoma
Bevacizumab (Avastin)	Humanized IgG1	VEGF	Colorectal carcinoma
Arcitumomab (CEA-Scan)	Conjugate (mouse IgG1–^{99}Tc)	CEA	Colorectal carcinoma (diagnostic)
Capromab (ProstaScint)	Conjugate (mouse IgG1 – ^{111}In)	PSMA	Prostate carcinoma (diagnostic)
Nofetumomab (Verluma)	Conjugate (mouse IgG2b-^{99}Tc)	CAA	Small-cell lung carcinoma (diagnostic)

Nonimmune, or Fc-independent, mechanisms are based on neutralization of biological activities of the target molecule; thus, their antitumor potential is directly related to the relevance of the target antigen for proliferation, survival, and metastatic spread of the tumor. It can be said that nonimmune mechanisms are relevant when the target is an oncoantigen (as discussed previously). First, antibody binding can trigger internalization and recycling of the target, resulting in its absence from the cell surface, at least until a sufficient antibody concentration is maintained. Second, an antibody can block molecular interactions required to initiate signaling – for example, by inhibiting dimerization of tyrosine kinase receptors such as HER-2 and EGFR. In the case of HER-2, a further effective mechanism can be the protection of target from activating proteolytic cleavage that converts p185 into the constitutively active form p95 (Saez et al. 2006).

Individual neoplastic cell types and molecular targets can be more sensitive to one or another mechanism. For example, B cells are typically sensitive to immune lysis, whereas targets such as CD20 seem to play a minor role as oncoantigens; hence, immune mechanisms play a more important role in therapy

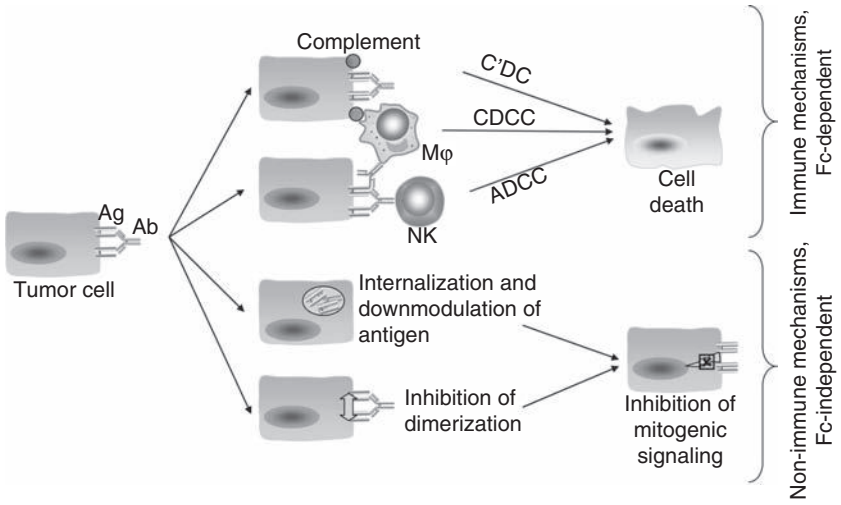

Figure 51.1. Mechanisms of action of monoclonal antibodies. (Abbreviations used are Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; Ag, antigen; CDCC, complement-dependent cell-mediated cytotoxicity; C'DC, complement-dependent cytotoxicity; Mφ, macrophage; NK, natural killer cell.)

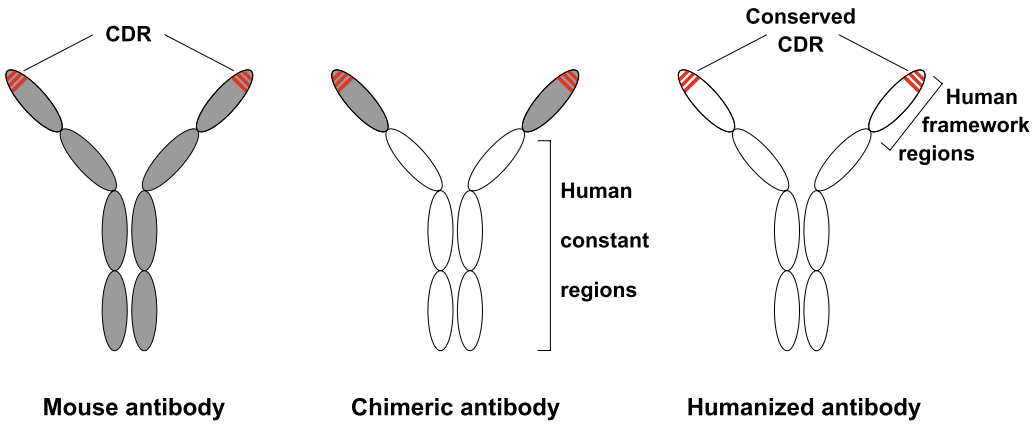

Figure 51.2. Humanization of murine monoclonal antibodies. Complementarity-determining regions (CDRs) comprise antigen-binding sites. Gray represents mouse sequences; white represents human sequences.

of B cell lymphomas with CD20 mAbs. On the contrary, metastatic solid tumors are frequently resistant to a varying degree to complement and other types of immune lysis, but their growth and survival can depend on functional expression of HER-2 or EGFR; thus, the balance of therapeutic activities can be more heavily loaded by nonimmune activities of mAbs.

An entirely different therapeutic approach is that of immunomodulatory antibodies aimed either at activating (agonistic mAbs) or inhibiting (antagonistic mAbs) the function of a target molecule expressed by cells of the immune system (Melero et al. 2007). The most clinically advanced immunomodulatory mAbs are directed against CTLA4 (CD152), a surface receptor that inhibits T-cell activation and is also constitutively expressed by Treg.

Most mAbs are of murine origin; thus, they can be recognized as foreign proteins by the human immune system and elicit neutralizing human antimouse antibodies (HAMAs). Moreover, some Fc-dependent effects can be hampered by the relative species-specificity of human Fc receptors. Such limitations are admissible for mAbs administered once for in vivo diagnostic purposes but not for repeated therapeutic administrations. The biotechnological solution is to genetically engineer mAb-producing hybridoma cells to replace murine coding sequences with human ones (Figure 51.2). This can be limited to constant regions of the heavy and light chains of the immunoglobulin molecule, resulting in chimeric antibody molecules that retain only murine variable domains or could be extended to framework regions (the invariant parts of variable domains, not directly involved in antigen binding), to obtain fully humanized mAbs. Given that the bulk of HAMAs are directed against constant regions, both solutions are acceptable for repeated human use, even though full humanization is more desirable in principle. In recent years, the technologies to produce human mAbs became more reliable, and we are now seeing a constant influx of these agents, which obviously do not require structural modifications for human use (Reichert and Valge-Archer 2007; Ledford 2008).

In addition to their use as drugs, the exquisite target-specificity of mAbs can be harnessed to home imaging and therapeutic agents in to neoplastic lesions. The large molecular mass of antibody molecules makes it easy to link a variety of small molecules through chemical reactions or genetic engineering, without alterations of antibody specificity and function. Labeling with isotopes such as ^{90}Y, ^{99}Tc, ^{111}In, or ^{131}I is used to generate mAbs for in vivo diagnostic purposes; several such preparations have been approved to detect lymphomas or metastatic solid tumors (Table 51.3). Radioisotopes, toxins, drugs, or prodrug activators are linked to mAbs for therapeutic purposes; however, this approach is fraught with problems related to antigenicity and nonspecific cytotoxicity of the toxic moiety and has so far failed to gain widespread use for metastatic solid tumors.

Therapeutic mAbs against B-cell antigens such as CD20 were the first to demonstrate clinical activity and to be approved in the United States, probably thanks to the frequency of target expression among B-cell lymphomas and the sensitivity of tumor cells to immune-mediated mechanisms (Li et al. 2008). Approved agents now include mAbs targeting CD33 in acute myeloid leukemia and CD52 (Campath) in B-cell chronic lymphocytic leukemia.

The development of mAbs against solid tumors has met many challenges, and even a now-standard mAb such as trastuzumab risked being abandoned in the pipeline (Bazell 1998). The demonstration of therapeutic activity of trastuzumab in metastatic breast cancer and, more recently, in an adjuvant setting (Burstein 2005) has paved the way to the development and approval of other mAbs against growth factor receptors expressed by tumor cells (EGFR) or by endothelial cells (VEGFR).

mAbs are a universal and manageable technology to target practically any conceivable surface or extracellular molecule relevant to cancer. The clinical success of the first few approved mAbs is thus paving the way to many more currently in the pipeline (Reichert and Valge-Archer 2007).

mAbs and Small-Molecule Inhibitors in Targeted Therapy

Significant oncoproteins can now be targeted using either mAbs or small-molecule inhibitors (SMIs); therefore, it is important to understand the distinct advantages and limitations of each type of therapeutic agent.

- mAbs combine both cytostatic nonimmune mechanisms of action and cytotoxic, immune-mediated activities, whereas SMIs cannot activate the latter.
- The most important limitation of mAbs is that immunoglobulins cannot penetrate inside tumor cells; hence, mAbs can target only surface or extracellular antigens, whereas SMIs can home in on intracellular domains of surface molecules, such as the catalytic domains of tyrosine kinases, and all types of intracellular proteins.
- "Wide-spectrum" SMIs are more easy to obtain, either by design or by chance. Actually many SMIs aimed at inhibiting one specific target actually affect more than one, which eventually can translate into additional clinical benefits, as was the case of imatinib and KIT in gastrointestinal stromal tumors (GIST). The exquisite specificity of mAbs can be detrimental under this respect; however, multiple mAbs can easily be combined to make a multitarget, wide-spectrum drug.
- Both mAbs and SMI therapies are prone to the emergence of resistant tumor cell variants; however, the molecular mechanisms of resistance can differ considerably. mAb-resistant tumors can display activation of "collateral" signaling circuits that make tumor cell survival and proliferation independent of the target molecule (Nahta et al. 2006). Tumor cells resistant to SMIs exhibit mutations that alter the structure of the target, such as mutations affecting the ATP-binding pocket of tyrosine kinases. mAbs are typically insensitive to mutations in intracellular catalytic domains that cause resistance to SMI. The reverse is probably also true; however, the field of mutation-dependent resistance has been thoroughly investigated only for what concerns SMIs.
- Unlike SMIs, mAbs targeting different functions or different molecules are a homogeneous class of molecules. The biological, immunological, and pharmacological properties are dependent mostly on constant regions of the immunoglobulin molecule, and independent of the antigenic specificity encoded by variable regions.
- The constant/variable region dualism extends to unwanted toxicities and side effects of mAb therapy. Generic side effects (e.g., flulike symptoms) are mostly short-term and mild, caused by the immunological interactions of constant regions and independent of target specificity. Target-specific toxicities of both mAbs and SMIs can be more or less severe, depending on the importance and function of each target molecule in normal cells, tissues, and organs.
- The molecular mass of antibodies is about a hundred times higher than that of the typical SMI; the consequences are manifold.
  - The traffic of mAbs within the body is more cumbersome than that of SMI. mAbs do not cross an intact blood–brain barrier and are mostly ineffective against brain metastases.
  - mAbs can be easily harnessed as carriers of small molecules of diagnostic or therapeutic significance.
  - The molecular mass of antibodies is of the same order of magnitude as that of their targets, which makes easier to design mAbs to inhibit macromolecular interactions, such as receptor dimerization, a feat definitely more difficult to attain with SMIs.
- The current generation of therapeutic mAbs is designed to work by parenteral administration and cannot be used orally, a definite advantage of many modern SMIs.
- Distinct and specific catabolic systems affect the half-life of mAbs and SMIs, which translates into different kinetics of therapeutic and side effects. Usually mAbs have longer half-lives, allowing for more relaxed schedules of administration.

In general, it can be argued that the lack of cross-resistance between mAbs and SMIs prompts for combination or sequential protocols using both mAbs and SMIs, which are only beginning to be explored. The same applies to the broader perspective of multimodality/combination therapies that could integrate all effective antimetastatic approaches, including immunotherapy, targeted therapy, hormone therapy, and cytotoxic agents (Begley and Ribas 2008; Andersen et al. 2008).

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52 Discovery and Development of Drugs Targeting Tumor Invasion and Metastasis

Rob J. Jones, Tim P. Green, and Paul Elvin

THE ERA OF MOLECULAR TARGETED THERAPY

Efforts to discover and develop novel molecular targeted cancer treatments that commenced in many pharmaceutical companies in the early 1990s have begun to make significant contributions to patient survival. Small-molecule and antibody therapeutics against targets such as EGFR (e.g. cetuximab, erlotinib, gefitinib), erbB2 (trastuzumab, lapatinib), VEGFR (bevacizumab, sorafenib, sunitinib, pazopanib, vandetanib), Abl (imatinib, nilotinib), and mTOR (sirolimus, temsirolimus) have delivered clinical efficacy in certain disease settings. The emergence of these therapies has led to an era of treatments increasingly targeted to selected patient populations, and this has implications for the development of future novel treatments [1–3]. As outcomes improve, increasingly demanding goals are set for new drugs to achieve, both preclinically and in clinical trials.

The majority of small-molecule and antibody agents to date have focused attention on proliferation, survival, and angiogenic pathways. Although there are examples of drugs targeting c-met, TGFβ, src, and FAK that have progressed to clinical trials (Table 52.1), there has been relatively little commercial activity specifically targeted against the molecules and pathways contributing to the invasive phenotype and the process of metastasis. Whereas key cell proliferation and survival pathways may ultimately have an impact on metastasis [4–6], it is also clear, from much that has been discussed elsewhere in this volume, that there are targets and pathways not currently exploited, and that disease progression could be modified by modulating the underlying mechanisms contributing to tumor invasion and metastasis. The clinical, health economic, and commercial importance of tumor invasion and metastasis cannot be overlooked. Although surgery and radiation treatment remain the two most effective means of dealing with the primary tumor, treatment failure, morbidity, and mortality can largely be attributed to progression of metastatic disease.

METASTASIS AND CHALLENGES FOR DRUG DISCOVERY

It has been estimated that more than 90 percent of cancer deaths may be attributed to metastatic disease progression, but only a relatively small proportion of research funding is focused on research into invasion and metastasis [7]. Although pharmaceutical research recognizes the unmet clinical need and challenge of treating metastatic disease, this is balanced by the commercial risk of developing antiinvasive or antimetastatic agents in the absence of any clear precedent for success. Disseminated late-stage disease implies tumor cell populations that are difficult to target and difficult to treat, set against a prevailing view that successful treatment is equated with tumor shrinkage and tumor cell death. In contrast, prevention of further metastatic spread and tissue damage has the potential to transform cancer into a less life-threatening chronic disease. Such a concept could be equally applicable to metastatic disease that is overt at time of first diagnosis, and to "dormant" micrometastases associated with emergence of disease following apparently successful treatment of the primary tumor.

In terms of drug discovery, clinical development, and the feasibility of targeting metastasis, it is helpful to make a distinction between metastasis per se and invasive tumor cell behavior. Prevention of metastatic disease implies an early therapeutic intervention coupled with potentially long clinical trials, with distant metastasis-free survival as a clinical endpoint. Anti-metastatic trials would require large numbers of patients to account for inherent variation

TABLE 52.1. Drugs in clinical trials targeting key regulators of the invasive phenotype

Target	Therapeutic	Company	Current stage	Status
FAK	PF04554878	Pfizer	Phase I	Ongoing
	PF562271		Phase I/II	Planned
	GSK2256098	GlaxoSmithKline	Phase I	Completed
Src	Saracatinib (AZD0530)	AstraZeneca	Phase II	Ongoing
	Dasatinib (BMS354825)	BMS	Phase II/III	Ongoing
	Bosutinib (SKI606)	Wyeth	Phase II	Ongoing
	KX-01 (KX2–391)	Kinex	Phase II	Ongoing
Src/IGF1R	XL-228	Exelixis	Phase I	Ongoing
IGF1R	BMS754807	BMS	Phase I/II	Ongoing
	OSI-906	OSI Pharmaceuticals	Phase I/III	Ongoing
c-Met/HGF	XL-184 (BMS907351)	Exelixis/BMS	Phase II/III	Ongoing
	MK8033	Merck	Phase I	Ongoing
	ARQ197	ArQule	Phase I/II	Ongoing
	AV299 (anti-HGF antibody)	Aveo/Merck	Phase I/II	Ongoing
	HuL2G7/TAK701 (anti-HGF antibody)	Takeda/Millenium (Galaxy Biotech)	Phase I	Ongoing
	E7050	Eisai	Phase I	Ongoing
	LY2801653	Eli Lilly	Phase I	Ongoing
	RG3638	Roche/Genentech	Phase II	Ongoing
	Riliotumumab (AMG102, anti-HGF antibody)	Amgen	Phase I/II	Ongoing
	PF4217903	Pfizer	Phase I	Closed
	JNJ38877605	Johnson & Johnson	Phase I	Closed
Cathepsin K/L	SB462795 (Relacatib)	GlaxoSmithKline	Phase I/II	Completed
	MIV701	Medivir	Phase I	Completed
	MK-0822 (Ondanacatib)	Celera/Merck	Phase I	Completed
Urokinase	Mesupron (Wx671/WxUK1)	Wilex	Phase II	Ongoing
TGFβ	TGFβ antibody	Eli Lilly	Phase I	Ongoing
	AP12009 (Trabedersen-antisense)	Antisense Pharma	Phase I	Ongoing
	Fresolimumab (GC1008, anti-TGFβ antibody)	Genzyme	Phase I/II	Completed
Hedgehog	XL139 (BMS833923)	Exelixis/BMS	Phase I	Ongoing
	GDC0449 (vismodegib)	Curis/Roche/Genentech	Phase I/II	Ongoing
	LDE225	Novartis	Phase I	Ongoing
Notch	MK-0752	Merck	Phase I/II	Ongoing
	RG-4733	Roche	Phase I/II	Ongoing
CXCR4	LY2510924	Eli Lilly	Phase I	Ongoing
	CTCE-99808	Chemokine	Phase I/II	Completed
CD44	A6 (peptide)	Angstrom	Phase II	Completed
Endothelin A	AZD4054 (zibotentan)	AstraZeneca	Phase II/III	Ongoing
	ABT-627 (atrasentan)	Abbott	Phase II/III	Ongoing

Based on clinical trial listings in Citeline TrialTrove (http://www.citeline.com) for clinical trials in solid tumor disease. Table is not exhaustive and is confined to primary mechanistic targets; all therapeutics are small molecules unless otherwise noted.

in the frequency and time-course of metastatic disease progression. Such trials are commercially unattractive, not only because of the huge costs involved but also from a consideration of patent lifetimes. In contrast, the approach of inhibiting invasive tumor cell behavior provides the possibility of inhibiting metastatic spread and local tissue destruction by invasive tumor cells, thereby improving morbidity and mortality outcomes in patients with more advanced disease.

As discussed elsewhere in this volume, metastasis is the outcome of a complex biological process in which there may be many potential points of intervention. However, for individual drug targets, pre-clinical screening may be limited by the ability to develop

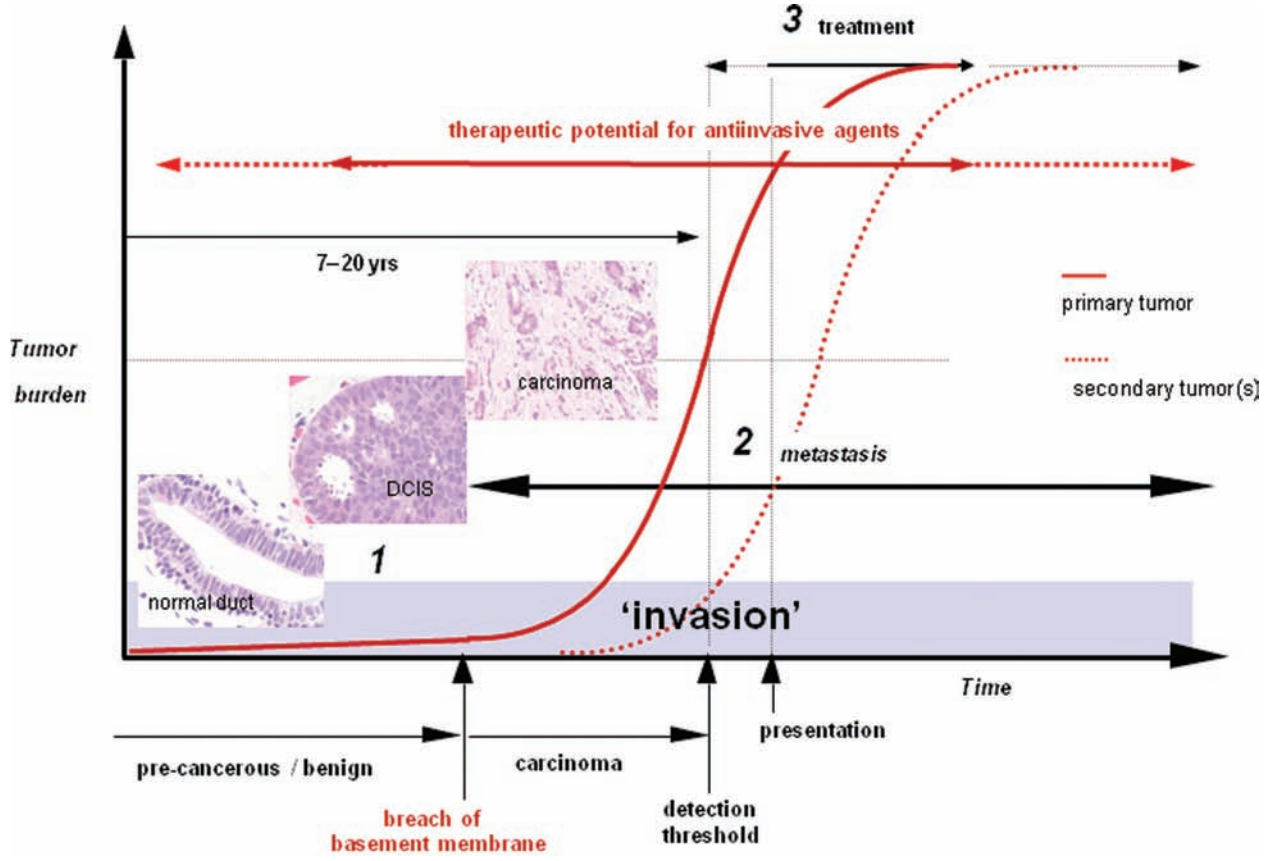

Figure 52.1. Therapeutic potential of anti-invasive agents in the context of tumor burden. (1) Loss of polarity, abnormal cell adhesion. (2) At diagnosis, a primary tumor mass of a few mm^3 is likely to be associated with undetectable, asymptomatic, metastatic disease. (3) Current treatment windows provide an opportunity to treat metastatic disease, but the real potential of anti-invasive agents will be realized through treatment of early disease. Time scale and the representation of disease progression using breast cancer as an example are for illustrative purposes only.

appropriate in vitro or in vivo assays, or it may be impossible to identify chemical starting points. By considering the underlying processes that contribute to the invasive behavior of metastatic tumor cells, it may be possible to develop a new paradigm of targeting invasion as a phenotype that is measurable both preclinically and clinically.

TARGETING TUMOR INVASION

In histopathology, invasion through the basement membrane is the event that distinguishes malignant from benign disease, and the transition to carcinoma in the case of epithelial tissues. The invasive phenotype encompasses aberrant cell behavior that contributes to dysplasia, hyperplasia, and growth of both primary and secondary tumors. Making the distinction between the process of invasive tumor growth/progression and metastasis provides the rationale to support a clinical hypothesis test for such agents at all stages of disease progression. Based on a consideration of human tumor growth rates and a tumor detection threshold of 0.5 to $1cm^3$, metastatic disease will most often emerge to be clinically evident at some time during the phase following first diagnosis of organ-confined disease [8] (Figure 52.1). Anti-invasive agents may be first used in an advanced disease setting – for example, to reduce the risk of progression after initial de-bulking surgery or radiotherapy. The ultimate goal of an anti-invasive treatment would be intervention in earlier disease settings, to prevent extensive metastasis. In early disease, rather than treating large tumor masses with associated issues of hypoxia, necrosis, drug penetration, and other efficacy-limiting issues, anti-invasive agents may target tumor cell populations wherein the mechanisms are of critical importance to tumor progression.

In terms of targets for drug discovery, the invasive phenotype (Figure 52.2) may be conveniently viewed as interdependent behaviors:

- Deregulated cell adhesion
- Acquisition of cell motility
- Modification of the extracellular microenvironment

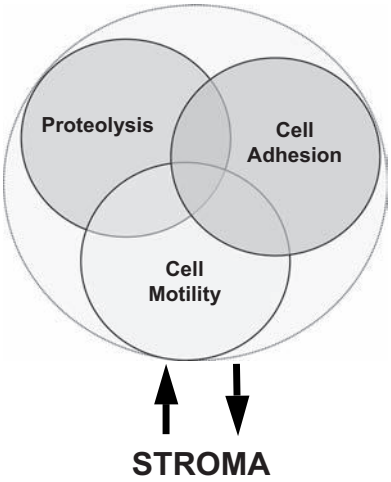

Figure 52.2. Interdependent features of the invasive phenotype. Cell adhesion, via the integrins to the extracellular matrix and through cadherins and other adhesion complexes to adjacent cells, regulates epithelial cell polarity and function. Deregulated cell adhesion is required for epithelial dysplastic and hyperplastic change and is necessary for the acquisition of a motile phenotype that facilitates invasion. Modification of the extracellular microenvironment, through degradation or aberrant synthesis of ECM, affects cell adhesion, motility, and invasion. The phenotype, and drug targets, are subject to changes in the genetic background of the tumor in the context of the host microenvironment.

Deregulated Cell Adhesion

Abundant literature evidence, summarized elsewhere in this volume, has identified a number of common changes in cell adhesion that may be useful therapeutic targets. Cell adhesion regulates both epithelial cell polarity [9] and epithelial cell function in the context of microenvironmental cues [10]. Mechanical sensing of the extracellular matrix (ECM) has been shown to dramatically alter cell polarity, intracellular signaling, and ultimately tumor cell behavior [11]. Loss of E-cadherin and deregulation of β-catenin signaling has a significant impact on tumor invasion [12]. Although P- or N-cadherin may compensate in part for the loss of E-cadherin, such a "switch" may have a significant impact on tumor progression and survival [13]. Abnormal integrin expression, function, and modification have all been implicated in tumor invasion and metastasis, and targeting individual integrins or matrix molecules may restrain further progression or restore normal responses to microenvironmental signals [14,15]. Aberrant interactions with the ECM may also be responsible for resistance to other targeted inhibitors of tumor progression [16].

Acquisition of Cell Motility

The pioneering work of Ann Chambers using intra vital microscopy to observe tumor cell motility in real-time [17], and later the description of amoeboid, mesenchymal, and collective tumor cell motility by Peter Friedl [18] and others [19], has increased our understanding of tumor cell motility. In terms of pathways and potential drug targets, cell motility has much in common with the pathways involved in the maintenance of a normal epithelium and are thus of relevance to the earliest changes in tumor development. The recovery of motile cells from primary human tumors, as demonstrated in pre-clinical models [20, 21], could identify key pathways and therapeutic targets for future anti-invasive treatments. However, there are few, if any, studies that have related in vitro motility, or the impact of treatment on motility, to clinical outcome.

Modification of the Extracellular Microenvironment

Many of the enzyme activities implicated in tumor invasion have similar roles in angiogenesis and so have the potential to target both host and tumor compartments. In addition to the matrix-metalloproteases (MMPs), serine (urokinase, matriptase, hepsin) and cysteine proteases (cathepsins B, D, L) are all the subject of drug discovery projects, with some agents in clinical trials (see Table 52.1). Many of these enzymes perform a normal physiological role, and the consequence of long-term inhibition of their activity is a key concern for clinical development, particularly as chronic inhibition may be key to therapeutic success. Proteolytic modification of the ECM has been shown to influence the cell migration phenotype of tumor cells, which may switch between amoeboid and mesenchymal-like migration [22]. The implication is that the simultaneous inhibition of more than one enzyme and both migration patterns may be necessary for an effective anti-proteolytic strategy.

Signaling Pathways

Cell surface receptors such as c-met [23], erbB2 [24], TGFβR1 and R2 [25], and Axl [26] have demonstrated key roles in the regulation of the invasive phenotype; however, clinical development programs have generally focused on these as proliferation targets. Intracellular regulation of cell adhesion by kinases that regulate scaffolding functions and turnover of cytokeletal proteins has also provided targets to regulate cell motility and invasion.

Inhibitors of Rho-associated coiled-coil-containing protein kinase (ROCK) showed pre-clinical promise as anti-invasives [27, 28] although clinical progress has been disappointing, with lack of efficacy and undesirable toxicity. Inhibitors of focal adhesion kinase (FAK) [29, 30] and src kinases [31–33] are currently in clinical trials and have shown encouraging activity in pre-clinical models that suggest promise as anti-invasive treatments. However, because researchers are

distracted by additional overlapping activities that will have an impact on tumor growth and the potential to reverse resistance to cytotoxic drugs, the anti-invasive capabilities of these drugs remain untested in clinical trials to date.

Stromal Factors and Invasion

The contribution of host tissues to the process of invasion and metastasis is the most challenging aspect of the area with implications for evaluating and validating responses in pre-clinical models. As recognized elsewhere in this volume, increases in our understanding of the key stromal interactions and drivers of invasion and metastasis have identified many targets, such as CXCR4 [34] RANKL [35], and endothelin A [36], that affect metastatic disease.

PRECLINICAL MODELS IN DRUG DISCOVERY

In Vitro

Reduction of complex disease processes such as invasion and metastasis to individual molecular targets, for the practical purpose of in vitro screening, introduces a considerable level of risk that potent in vitro inhibitors will lack potency or efficacy in vivo. Screening for activity at the cellular level is complex and generally addressed when the numbers of hits from primary screening have been reduced to molecules of real interest with potential for pharmacological activity in vivo. In terms of biology, the tumor cell cannot be divorced from interactions with the host stroma, and activity in cell culture may be a poor surrogate of activity in animal models, introducing a further tier of uncertainty in the translation from in vitro drug activity to clinical efficacy.

Proteolytic activity, cell adhesion, and cell motility are all accessible and measurable in vitro, and technological advances have improved the practicality of moderate throughput assay systems. Variations of the Boyden Chamber assay [37] using automated cell counting or confocal microscopy to measure the extent of invasion through Matrigel™ have improved the reproducibility and accuracy of a well-proven model system [38]. However, the Boyden Chamber model does not reproduce a tissue microenvironment, and recent developments using invasion of tumor cells into tissue fragments, or outgrowth of tissue fragments into Matrigel, provide some improvement in the context of in vitro assays. Measurement of cell motility, although only one component of the invasive phenotype is well served by new technology (e.g. Incucyte, Essen Instruments), and similar approaches to measuring cell adhesion are easily incorporated into screening cascades

In Vivo

Models of disease progression in vivo represent the greatest challenge and current weakness of pre-clinical anti-invasive drug research. The focus on molecular targeted treatments has highlighted the inadequacies of the majority of pre-clinical xenograft tumor models. In addition to the gross disparity in tumor growth kinetics between human cancers and subcutaneously transplanted tumors in mice, the majority of such models rarely exhibit local invasion or give rise to distant metastases [39]. Thus, the usefulness of subcutaneous models of tumor growth to evaluate anti-invasive approaches is somewhat limited and may require both surgical removal of the primary tumor and long observation periods to evaluate metastatic endpoints, as recently described for a model of prostate tumor progression [40].

Attempts to model the growth of human tumors through subcutaneous transplantation of primary human tumor fragments in breast cancer [41] or colon cancer [42, 43] have demonstrated the retention of tumor morphology and metastatic behavior. However, the application and impact of such models on the success of drug discovery remains to be demonstrated. Orthotopic transplantation of tumor cells into the tissue of origin has been shown to be an important determinant of tumor growth and progression and response to chemotherapeutic agents [44]. Recapitulation of the metastatic cascade in such models, essential for the evaluation of new agents targeting invasive tumor growth and metastasis, is highly desirable. However, the reproducible and reliable performance of such models is highly variable, and few models meet the demands of in vivo testing cascades. Whereas transgenic models have provided an opportunity to dissect tumorigenesis, it is rare for such models to give rise to a significant metastatic burden [45]. Although there are notable exceptions, such as the TRAMP model of prostate cancer [46], the relative lack of metastasis in transgenic models, and their longer time- course compared with orthotopic xenografts, restricts the usefulness of genetically engineered models [47].

Sophisticated advances on the pioneering work of Hoffman and Chambers [48, 49], using fluorescently tagged tumor cells and stromal elements together with high-resolution optics, have led to models in which invasion and resultant changes in tissue architecture can be monitored in real-time [50, 51]. Similarly, the use of luciferase-tagged tumor cells has enhanced the ability to monitor disease progression [52]. Such models offer the opportunity to redefine some of the principles of the metastatic cascade and refine the evaluation of potential anti-invasive agents. Models of bone and brain metastasis, though not suited to use in screening cascades, do provide the opportunity to investigate the

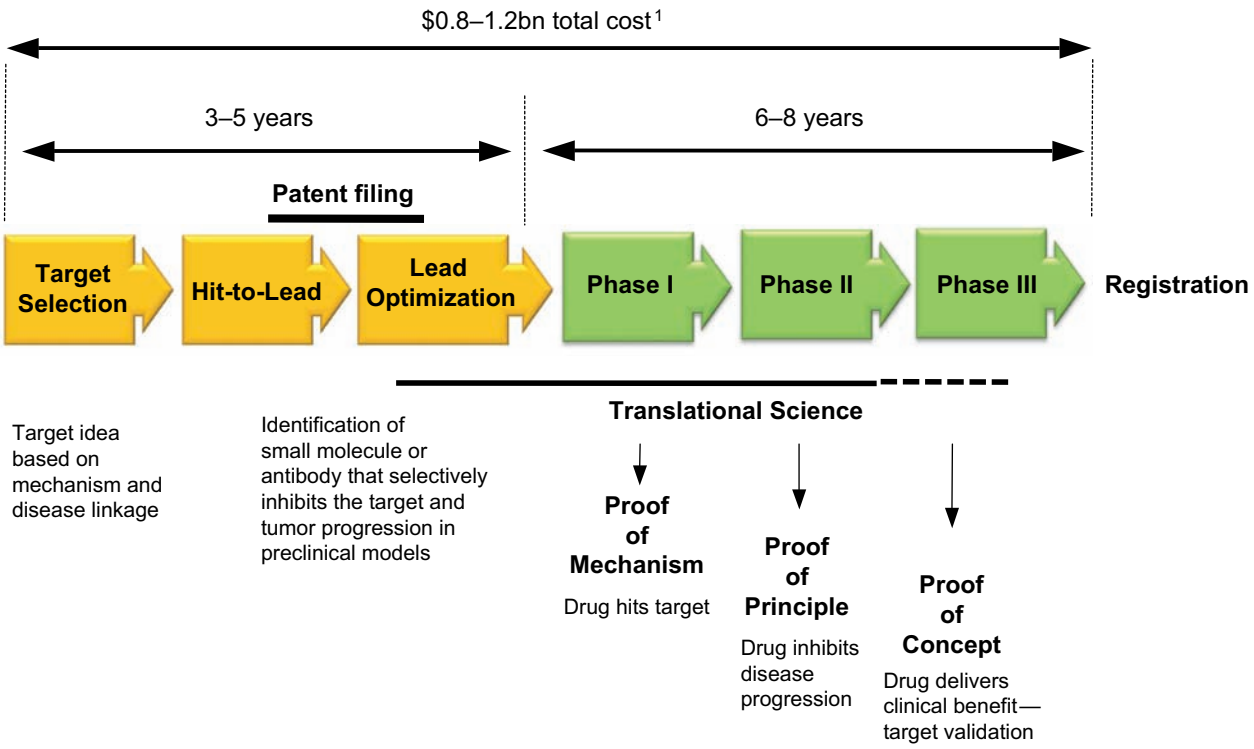

Figure 52.3. Drug discovery and development. Representative outline of oncology research and development steps with approximate time scale and costs. The time scale is aspirational and may be target- and disease–setting-dependent. Phase I/Phase II trials aim to establish drug safety and target inhibition at tolerable doses. Patent filing may be earlier than indicated for antibody based therapeutics. [1](DiMasi, JA et al. [2003] *J Health Econ.* 22: 151 and Di Masi JA, Grabowski, HG [2007] *Manage Decis Econ.* 28: 469.)

impact of novel agents on specific aspects of disease progression. In summary, although there are many in vivo models that have enabled a better understanding of invasion and metastasis, there remains a need for models that more closely mimic clinical disease, such as minimal residual disease or quiescent micrometastatic disease.

CLINICAL DEVELOPMENT CHALLENGES

Stages of Clinical Development

In addition to progress through Phase I–Phase III trials, sponsoring organizations measure progress against decision points that provide increased confidence in the likelihood of attaining a successful registration (Figure 52.3). For anti-invasive agents, early proof of mechanism (PoM), in parallel with tolerated dose selection in Phase I, would provide evidence that the drug has inhibited the desired target, ideally within tumor tissue, or a surrogate mechanism in normal tissue. PoM supports progress into Phase II, in which the use of biomarkers indicative of anti-invasive activity should be correlated with some measurable impact on disease progression (proof of principle, PoP). In turn, a successful PoP will mitigate some of the risk of the larger Phase III registration trials to demonstrate proof of concept (PoC), that an anti-invasive mechanism results in significant clinical benefit. Although the stages of development from PoM to PoC for an anti-invasive agent are in essence no different to the development of, say, antiproliferative agents, selection of appropriate patient populations will be critical to demonstrating clinical benefit.

Conventional Outcome Measures

Early clinical activity is largely assessed against established Response Evaluation in Solid Tumors (RECIST) criteria [53], in which tumor shrinkage is the primary treatment goal. Although RECIST criteria have been modified [54], clinical outcome measures remain focused on tumor shrinkage as a surrogate for patient survival. In the case of treatments targeting tumor invasion, clinical endpoints such as time to progression (TTP) may be a more relevant intermediate clinical response measure, particularly if no tumor shrinkage is anticipated based on pre-clinical data. As discussed later, TTP in certain disease settings may provide an opportunity to demonstrate benefit to patients that would support registration of drugs directed against invasive tumor cell behavior.

Legacy of Early Antiinvasive Clinical Trials

The resounding failure of the Phase III programs for the first-generation MMP inhibitors (MMPIs) that took place during the last years of the twentieth century has resulted in a high degree of nervousness surrounding clinical development of novel anti-invasive drugs. However, it is timely to consider some of the reasons that these programs failed, with particular regard to novel approaches that may have prevented these failures.

Poor Target Validation

The MMPs are a diverse group of at least twenty-three different proteases. Pre-clinical data now suggest that only some family members are likely to be cancer targets (notably MMP-1, -2, and -7), and that deregulation of MMP-14 alone may be a key determinant of invasion [55]. Furthermore, there is evidence to suggest that inhibition of some MMPs could enhance tumor progression [56–58]. The drugs previously developed to Phase III were largely non-selective, and this may be one reason for the lack of clinical benefit. It is also likely that some of the dose-limiting toxicities observed with these drugs were the result of inhibition of specific MMPs. More selective MMP inhibitors, lacking activity against MMPs having an essential role in normal physiology, may have delivered a clinically beneficial result whereas the tested drugs failed [59].

Failure to Demonstrate Proof of Mechanism in Humans

There are few data from clinical studies with second-generation MMPIs that attempt to demonstrate target inhibition in humans at clinically relevant doses. Those that have been published revealed disappointing activity [60–62]. It is therefore possible that doses chosen to minimize toxicity were insufficient to inhibit the therapeutic target in these trials.

Inappropriate Patient Selection

Of thirteen Phase III trials of MMPIs, eleven targeted patients with metastatic disease. Two "adjuvant" studies enrolled only patients with inevitable early disease recurrence [63, 64]. Although the therapeutic hypothesis for these drugs does not exclude a possible cytostatic effect in advanced disease, none of these trials satisfactorily addressed the primary hypothesis that they should have inhibited stage progression from early disease.

Absence of Target-Related Patient Selection Hypothesis

Differences in MMP expression and activity between tumor types and patients have been difficult to demonstrate. Despite the failure to demonstrate overall clinical benefit, it was clear that some individual patients gained clinically meaningful benefit from these drugs. Therefore, it is possible that the populations studied were simply too heterogeneous to demonstrate benefit overall.

Failure to Address the Primary Therapeutic Hypothesis

The most enticing preclinical data suggested that an MMPI that is effective at preventing progression from pre-invasive disease has no effect on progression after the tumor has become invasive [65]. Although clinical replication of such a study is challenging, none of the published Phase III MMPI trials has addressed patients with pre-invasive lesions.

TRANSLATIONAL SCIENCE AND ANTIINVASIVE DRUGS

Recognition of high attrition rates in early clinical development [66] has been one of the major drivers to establishing translational science as a discipline. One purpose of translational science is to match patient populations with the activity of new agents to reduce failure rates in early clinical development. The selection of clinical settings in which to demonstrate an anti-invasive mechanism that delivers clinical benefit is the key area that will ultimately determine the success of novel anti-invasive treatments

Markers of Antiinvasive Activity

Inhibition of tumor invasion may result in inhibition of tumor growth, there being good evidence that inhibition of a number of targets not directly linked to proliferation or apoptosis results in tumor growth inhibition. Equally, there are targets for which inhibition does not result in a consistently measurable effect on the primary tumor, although the incidence and progression of secondary spread is inhibited. The latter response provides a serious challenge to clinical drug development, given the reliance on radiologic response criteria (such as RECIST) in early development. An ideal pharmacodynamic biomarker of drug activity is linked to an outcome that can be measured and is linked to patient benefit. Although several markers of proliferation and cell death are readily available (such as Ki67, nuclear

counts, and caspase activation), there are few markers that have been validated as reporters of an invasive phenotype.

Given the huge investment required for clinical trials, some early indication that an anti-invasive agent is inhibiting its target at the tumor site is highly desirable. Demonstrating that target inhibition is correlated with the inhibition of a process causally linked to invasion and metastasis provides further confidence in achieving a successful PoP/PoC, reducing the risk carried into Phase III trials. Ideally, potential markers of tumor invasion are amenable to validation in pre-clinical models as well as being feasible for clinical measurement. Biomarker development may require significant research investment to establish clinical feasibility in respect of tissue sampling, variability, and stability [67].

Cellular Biomarkers of Invasion

Cell migration and invasion have been shown to be dependent on the phosphorylation and activation of proteins associated with cytoskeletal regulation, including paxillin [68], FAK [69], and ROCK [70]. Modulation of paxillin and FAK phosphorylation by the src kinase inhibitor AZD0530 (saracatinib) was demonstrated in tumor xenografts by both immunohistochemical and tissue lysate ELISA methods. Similar measurements in breast cancer biopsy tissue demonstrated the feasibility of applying such methods to Phase I trials to provide an early PoM that could be carried into PoP studies [71]. FAK and paxillin are downstream effectors of other regulators of invasion, such as c-met [72, 73], suggesting that they may be universally applicable to novel anti-invasive agents and epithelial tumor types. The expression or activation state of a number of proteolytic enzymes closely associated with tumor invasion, and measurement of specific breakdown products as reporters of enzyme activity, also provide many opportunities for biomarker selection [74, 75]. Of note, the presence of soluble urokinase receptor (uPAR) has been extensively studied in breast cancer and shown to correlate with tumor progression; soluble uPAR arising as a result of increased tumor cell proteolysis [76].

Enzymes, such as MMPs, urokinase, and heparanase; cytoskeletal proteins such as paxillin and cortactin; some integrins such as $\alpha v\beta 3$, $\alpha v\beta 6$, and $\alpha 3\beta 1$; and cytosolic kinases such as FAK and src are all associated with invadopodia [77]. In the absence of invadopodia, cells in vitro are unable to perform some of the key steps of invasion. Removal of a single protein, Tks5, a key component of the invadosome, inhibits invasion in vitro and also blocks the degradation of ECM proteins [78]. Furthermore, decreased Tks5 expression inhibited tumor growth in vivo [79]. Hence the "invadosome" may be a highly selective marker of tumor invasion in vivo.

Circulating Tumor Cells

Circulating tumor cells (CTCs) are intuitively a consequence of an active invasion process. Tumor cells may also enter the circulation as a result of passive shedding, so the presence of CTCs may not be the most reliable marker of an active process [80]. However, there is good evidence that the presence of CTCs correlates with disease progression and poor survival in response to treatment [81]. Although a reduction in CTCs alone may not be a reliable measure of inhibition of tumor invasion, combining a measure of CTC burden with other (aforementioned) markers could provide an early decision point for further clinical studies.

Markers Linked to Normal Tissue Changes

In addition to molecular markers that are directly linked to underlying mechanisms supporting the invasive phenotype, there are physiological processes that mimic tumor invasion in cell behavior and ECM turnover. Inflammatory cells, such as leukocytes and monocytes, have much in common with the wandering tumor cell, using cytoskeletal changes and proteolytic cascades to enter and exit tissues. Peripheral blood is a readily accessible compartment, whereas some of the markers referred to earlier require an invasive technique, such as fine-needle or core biopsy. Measurement of inflammatory cell function may be a suitable surrogate for some tumor invasion targets.

Similarly, processes analogous to tumor cell invasion may be seen in the process of normal bone turnover. The presence of bone degradation peptides in the circulation as a result of normal bone turnover is frequently elevated in cancers in which bone metastasis is a common feature such as prostate cancer [82]. Such markers were successfully applied to the development of saracatinib (unpublished data), in which an initial indication of both pharmacological activity and linkage to a disease-related process were obtained from Phase I volunteer studies.

DISEASE SETTINGS FOR DEVELOPMENT OF ANTIINVASIVE DRUGS

As discussed earlier, a major consideration for clinical development of novel anti-invasive agents is the time scale and size of Phase II/III trials. However, there are a number of clinical settings in which progression of occult metastases leads to an early recurrence. In these settings, an anti-invasive agent might be expected to delay TTP or improve disease-free survival (DFS).

Ideally, the drug target would be known to be related to patient survival in a particular cancer, through molecular pathology measures such as immunohistochemistry or expression profiling. Relatively short TTP/DFS would be compatible with the ideal of an early readout of activity, and the proposed biomarkers of invasion would be accessible and measurable. By implication, the clinical trials thus defined provide the basis for selection of drug targets based, at least in part, on feasibility of development and registration rather than the strength of the therapeutic hypothesis alone.

Candidate Clinical Endpoints in Trials of Antiinvasives

Occult liver metastasis is a key determinant of patient survival following primary surgery for colorectal cancer [83]. Although the molecular pathways underlying colorectal tumor progression have received much attention since the initial description of the sequential molecular events contributing to the adenoma–carcinoma sequence, the determinants of liver metastasis are less well defined. Recurrence rates following successful liver resection are comparatively high and occur within a relatively short time frame [84]. Furthermore, the detection of liver metastases by imaging and biomarkers is already established as part of clinical management for these patients. Thus, patients who have complete pathological clearance following liver resection are a patient population for whom anti-invasive agents might be expected to increase DFS.

The management of superficial and invasive bladder cancers carries significant morbidity, mortality, and costs [85]. One of the key goals is the prevention of progression to muscle-invasive disease, thereby reducing the need for major surgery and the incidence of cancer-related mortality. Recurrence following successful resection is a common event and often occurs within a relatively short time frame. Bladder cancers provide an almost unique setting for PoM and PoP studies, as watchful waiting with repeat biopsy is part of routine clinical management.

Hormonal treatments are well established in the management of prostate cancer, although the emergence of castrate-refractory tumor growth and metastasis to bone are major contributing factors to treatment failure and poor patient outcomes [86]. The current natural history of prostate cancer presents two distinct minimal residual disease opportunities for the development of anti-invasive therapy: first, in patients with established metastatic disease who have achieved a good response (defined by suppression of prostate-specific antigen [PSA] in the blood) to castration therapy, and second, patients with no clinically detectable metastatic disease who have evidence of disease progression based on rising PSA alone. In the former setting, we might expect an anti-invasive drug to prolong progression free survival; in the latter, it may delay time to distant metastasis. Molecular targets of the invasive phenotype are also implicated in the progression of bone metastases, and some targets are also key to osteoclast function. Therefore, anti-invasive agents may have the potential to have an impact on both the primary disease and progression of prostate cancer bone metastases.

PROSPECTS, CHALLENGES, AND REWARDS

A number of agents currently in clinical trials target key molecules and pathways implicated in tumor invasion and metastasis. However, none are to be developed specifically as anti-invasive/metastatic drugs; this aspect of activity will likely be addressed in post-registration trials. As evidenced by the foregoing chapters in this volume, there is an ever-increasing understanding of the signaling pathways and host–tumor interactions that lead to invasive and metastatic disease. Many potential drug targets have not been the focus of drug discovery research or are currently intractable. Other targets have found alternative paths to registration.

Although there remains a need for in vivo models of invasion and metastasis that are more compatible with drug discovery, there are now many models that better represent the biology and provide a more informative pre-clinical test of the anti-invasive hypothesis. Model development, in parallel with advances in live-phase imaging and reporters of target inhibition, should provide robust evidence of drug candidate activity.

Clinical development remains the most significant challenge and obstacle to the delivery of anti-invasive/anti-metastatic agents that have the potential to turn metastatic disease into a non–life-threatening chronic disease. Although few anti-invasive agents may be stand-alone treatments, when they are used in conjunction with other targeted therapeutics, confinement of disease and prevention of further metastasis is a worthy goal. Paradigm-shifting drugs in oncology are rare, but the headlines they incite often obscure the quiet revolution by which the majority of common cancers are increasingly managed as chronic diseases. Sadly, for many advanced cancers, the magnitude of benefit from drug intervention remains disappointingly small, so novel therapeutic strategies aimed at maintaining such cancers in a quiescent state are potentially of high impact, even in the absence of disease regression or, indeed, growth arrest. Cancer is defined in part by its ability to invade, so reversal of this process may result in disease containment, even in the disseminated state. Furthermore, many of the devastating symptoms of advanced cancer, such as bone pain and nerve damage, are a direct consequence of the tumor invading other

tissues, so prevention of these events would have dramatic benefits in terms of symptom control and overall quality of life.

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53 The Role of Radiotherapy in the Treatment of Metastatic Disease

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In the past twenty years, significant advancements in cancer treatment have been achieved. New surgical techniques such as robotic surgery [1], three-dimensional magnification, and intraoperative imaging have resulted in less invasive and more effective surgical procedures for the resection of both primary tumors and metastatic disease. Promising novel cytotoxic agents, including cancer-specific molecular targeting agents [2], have been discovered and continue to be investigated. Moreover, radiation therapy has undergone rapid advancement in its targeting capabilities with the introduction of technologies such as 3D-conformal radiotherapy, intensity-modulated radiotherapy, image-guided radiotherapy, and stereotactic body radiosurgery.

Metastatic disease presents a challenging therapeutic situation. Often, patients have widespread systemic disease without abundant treatment options. However, radiotherapy, first applied in cancer treatment more than one hundred years ago, has played an important role in patients with metastatic disease. Its primary use has been to efficiently provide palliation of symptoms such as pain, obstruction, bleeding, and intracranial pressure. Small doses of radiation have been given to provide effective relief of symptoms in patients who often have a high burden of disease and to minimize the impact on normal tissues. Recent improvements in both cytotoxic agents and targeting methods in radiotherapy have extended the survival time in patients with metastatic disease. Typical palliative doses of radiation such as 30 Gy in ten fractions, although effective at relieving symptoms, have been unable to biologically control disease on a long-term basis. Newer, more aggressive techniques and dose fractionation schemes are often warranted in certain patients to provide long-term local control and to possibly increase disease specific survival.

This chapter covers both traditional and novel techniques in radiation therapy used to treat metastatic disease. Our concentration is on treatment beyond palliation and focuses mainly on patients with a low burden of disease and with a life expectancy of greater than three months. We review radiotherapy's current status and potential future use, both as a single modality and in combination with newer targeted cytotoxic agents.

RECENT ADVANCES IN CLINICAL RADIOTHERAPY IN METASTATIC DISEASE

In the past decade, clinical radiotherapy has undergone numerous advancements that have ensured better targeting and delivery. A more precise delivery of radiation is now available using novel imaging techniques along with conformal dose calculation methods. In addition, increased understanding of the biologic basis of radiation therapy has allowed radiation to be combined with biologic agents and biologically targeted radionuclides. Our discussion of newer techniques in radiation therapy for the treatment of metastatic disease has been classified into subcategories: stereotactic radiosurgery in metastatic disease, conventially fractionated radiation therapy, targeted radionuclide therapy, and immunoguided radiotherapy. In addition, the definitions of each treatment, its rationale for use, and its experimental and clinical study thus far are summarized.

Stereotactic Radiosurgery in Metastatic Disease

Stereotactic radiosurgery (SRS) was first developed in the 1950s by Lars Leskell as a mechanism for delivering large single doses of radiation to the brain to treat intracranial neoplasms. Using novel targeting techniques along with various devices that allowed the focusing of numerous beamlets aimed at a well-defined target, Leskell's frame and fiducial markers defined a coordinate system that allowed treatment setup accuracy within 2 mm. This system makes high-dose

radiation therapy to the brain feasible. Typically, stereotactic radiosurgery is performed with the use of a Gamma Knife, a CyberKnife, or a traditional linear accelerator (Linac) and combines precision radiotherapy with novel immobilization techniques to deliver high-dose-per-fraction treatment. Using a single or a few fractions, this method delivers high doses in an "ablative" treatment designed to disrupt cell division and to eradicate disease. The combination of hypofractionation with high doses not only increases local control but also allows shorter treatment times. Because of the potential normal tissue side effects, pretreatment quality assurance is required.

Stereotactic Radiosurgery for Intracranial Metastatic Disease

Radiosurgery is used primarily in intracranial disease for brain metastases. Dose parameters in radiosurgery were first established in the Phase I RTOG 90-05 protocol [3]. This study attempted to determine the maximum tolerated single fraction dose for both primary and metastatic brain tumors previously treated with radiation therapy. The study included 100 patients who had recurrence of brain metastases after previous conventionally fractionated radiotherapy (average 30 Gy). They were then retreated with radiosurgery either using Gamma Knife (24%) or Linac (76%). For tumor sizes of 31 to 40 mm, 21 to 30 mm, and 20 mm or less, the maximum tolerated radiosurgical doses were 15 Gy, 18 Gy, and 24 Gy, respectively [3]. Based on these results, RTOG 95-08 analyzed 333 patients with one to three brain metastases under 4 cm. The patients all had a Karnofsky performance status ≥70 and were randomized to receive either whole-brain radiation therapy alone (37.5 Gy in 15 fractions) or whole-brain radiation therapy and stereotactic radiosurgery using the doses recommended by the 90-05 study [4]. In the group that received radiosurgery, the median survival was better for patients with solitary lesions (6.5 months versus 4.9 months). Post hoc subgroup analysis also suggested a benefit with SRS in patients who have up to three metastases, and performance status at six months was shown to be significantly better in all the patients treated with SRS. There was no significant difference in major toxicity between the two groups.

Local control of brain metastases using radiosurgery has been high in several institutional series. Swinson et al. [5] reported 84.3 percent local control in an analysis of 1569 metastatic brain tumor lesions in 619 patients treated with radiosurgery. They found that whole-brain radiotherapy (WBRT) prior to radiosurgery improved locoregional control in select patients.

The use of conventional WBRT in combination with radiosurgery for brain metastases remains controversial even after publication of these studies. Eichler and

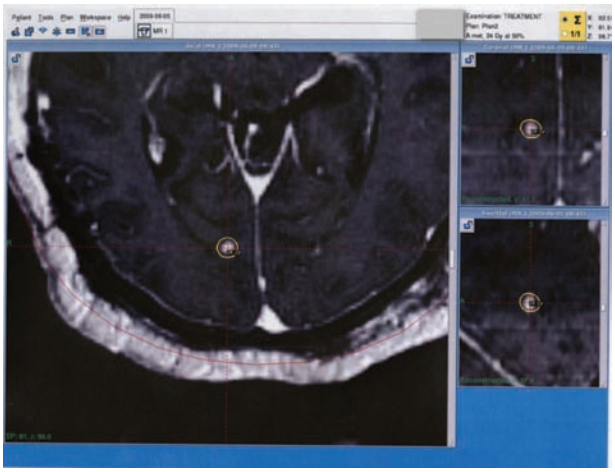

Figure 53.1. Example of GammaKnife radiosurgery for brain metastases. The lesion in the right cerebellum is being treated to 24 Gy in one fraction, following the dose parameters established in the RTOG 90-05 study.

Loeffler [6] summarized the typical treatment options based on patient performance, systemic disease burden, and number of brain metastases in their extensive literature review (Figure 53.1). Further studies are needed to determine the survival benefits and quality of life offered by using SRS for patients with brain metastases.

Stereotactic Radiosurgery for Extracranial Metastatic Disease

Based on the success of radiosurgery to treat intracranial lesions, new methods were developed to achieve similar treatment accuracy in other parts of the body. Patient immobilization, combined with methods for controlling tumor and organ motion, permits high doses of radiation to be administered extracranially for improved tumor control. This new method of radiation delivery has been termed *stereotactic body radiation therapy* (SBRT) [1]. Technologies including image guidance immediately prior to treatment are used to facilitate this large fraction dose treatment. The key to achieving high accuracy for SBRT requires four factors: (1) reliable and reproducible patient immobilization, (2) proper accounting of tumor and organ motion control, (3) planning and treatment correlation, and (4) daily targeting with pretreatment quality assurance. Patient immobilization and registration during SBRT treatment has most commonly been achieved with a variety of available body frame systems that mimic those used in intracranial SRS. SBRT is now being used to treat tumors of the lung, liver, pancreas, kidney, prostate, and spine. Other uses of SBRT in metastatic disease have included spinal, lung, and liver metastases.

Experience in treatment of spinal metastases with single-fraction high-dose radiation using stereotactic

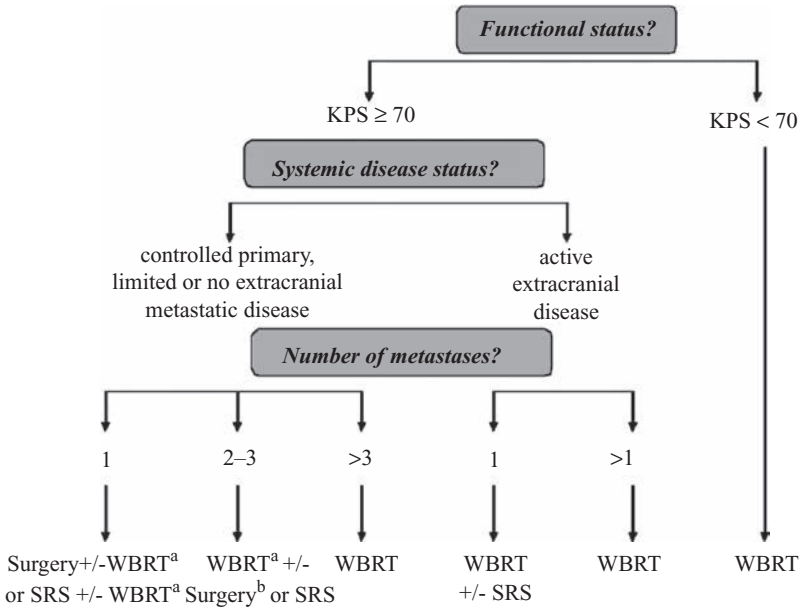

Figure 53.2. Algorithm for the initial treatment of brain metastases. (a) Omission of up-front WBRT is an alternative in patients who are followed closely for progression after surgery or SRS. (b) Consider for patients with one dominant lesion causing mass effect, or pathologic diagnosis required. KPS, Karnofsky performance status; SRS, stereotactic radiosurgery; WBRT, whole-brain radiation therapy. Adapted from *The Oncologist* 2007; 12:884–898.

methods continues to develop. A large study from Henry Ford Hospital involving 230 patients with metastatic lesions in the spine showed significant pain relief and local control with single fraction treatment of 10 to 16 Gy [8, 9]. Chang et al. [10] reported on the use of stereotactic methods to treat seventy-four spinal metastatic lesions first using 30 Gy in five fractions and then 27 Gy in three fractions, and reported 84 percent actuarial one-year tumor progression-free incidence with minimal toxicity. Single-institution data from the University of Pittsburgh showed that 86 percent of patients received significant pain improvement and 88 percent achieved tumor control in 500 spinal lesions treated using the Cyberknife to deliver a single fraction of 12.5 to 20 Gy [11]. As opposed to the other mentioned studies, 68 percent of the lesions had previously been treated with radiation. High-dose-per-fraction treatment for spinal metastases will continue to be popular as more results are reported and ongoing trials mature. Newer dose fractionation schemes are being proposed as primary treatment for patients with spinal metastases based on the encouraging results.

Limited metastatic disease within the liver has presented a challenging dilemma in cancer therapy. Surgical resection in the liver has been shown to produce reasonable clinical outcomes [12]. These results create an encouraging forum for exploration of noninvasive high-dose radiation treatment of this disease. Early studies by the pioneers of SBRT showed minimal side effects in treatment of one or two liver metastases with doses ranging from 20 to 40 Gy in two to four fractions. Other single-institution successes prompted initiation of a Phase I/II study in the United States recently reported by Kavanaugh et al. [13, 14]. Patients with one to three liver metastases smaller than 6 cm tolerated doses up to 60 Gy in three fractions. Interim analysis on thirty-six patients showed actuarial local control at eighteen months to be 93 percent, with only one episode of grade 3 toxicity. These data, although preliminary in nature, have provided encouraging evidence that combining novel targeting methods with an increasing dose can provide long-term control for metastatic disease in the liver. Ongoing trials will continue to provide data in patients treated in this noninvasive manner.

SBRT has been established as a treatment for primary lung lesions in patients with medically inoperable early-stage lung cancer [15–19]. This technique has now been applied to metastatic lung lesions, but very few Phase II data exist specifically for lung metastases treated with SBRT. In several single-institution studies, local control in patients was quite high using various different dose fractionation schemes, typically ranging from 48 to 60 Gy given in three to five fractions. Local control in these studies was promising, and ranged from 67 percent to 92 percent [17, 20–35]. Patients with metastatic lung lesions may benefit extensively in the future from this technology because they tend to have better pulmonary function than patients who have

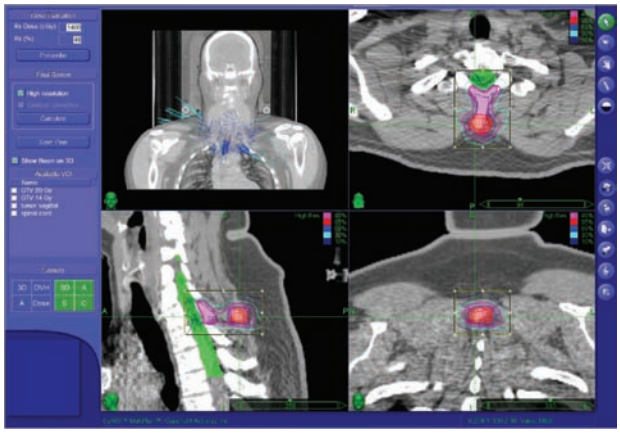

Figure 53.3. Example of using radiosurgery with the CyberKnife to treat a solitary spinous process metastasis in a patient with metastatic melanoma. Two volumes were designed to allow the gross tumor volume to be treated to 20 Gy in one fraction while the contiguous bone marrow cavity was treated to 14 Gy in one fraction.

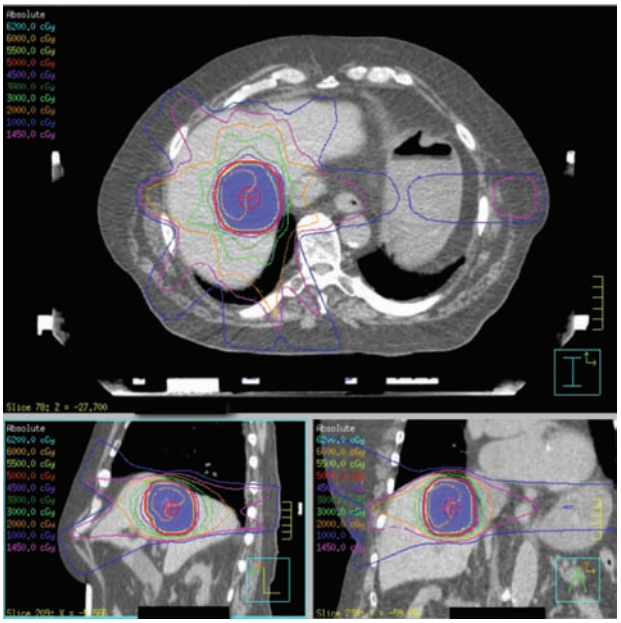

Figure 53.4. Example of using SBRT to treat a solitary liver metastasis in a patient with metastatic bladder cancer. This lesion was treated with 50 Gy in five fractions.

inoperable primary lung disease. Phase I dose escalation trials similar to those performed in primary lung cancer have been performed and are ongoing.

In summary, as we increase our ability to treat systemic disease with newer targeted therapies, it has become progressively more important to effectively treat oligometastases within the lung, liver, and spine to improve the patient's quality of life and survival benefit. New technologies such as SBRT show promise as a noninvasive method for achieving control of metastatic disease within the lung, liver, and spine.

Conventionally Fractionated Radiation in the New Era of Metastatic Disease

Thus far, we have concentrated our discussion on high-dose-per-fraction treatment of specific sites of metastatic disease. However, certain locations and levels of disease burden do not allow such treatment because of the potential for normal tissue toxicities or for ineffective local control. Traditional fraction sizes of 1.8 to 2 Gy can still provide effective palliation of symptoms. Newer techniques, including modulation of radiation dose based on disease burden, image guidance of dose delivery, and targeted drug therapy combinations, will continue to be generated in an attempt to increase local control of metastatic disease while reducing normal tissue toxicity.

Intensity-modulated radiation therapy (IMRT) has increased in favor as a method to modulate doses based on disease burden and normal tissue dose constraints. The use of IMRT in the brain for brain metastases allows modulation of dose with increasing fraction size and causes fewer effects in normal tissue. For tumors larger than those typically recommended for radiosurgery, IMRT techniques have increased local control for large tumors and have spared critical structures within the brain from toxicity [26–29]. IMRT allows for differing fractionation schemes, including simultaneous integrated boosts to areas of large disease burden. These procedures increase the probability of local control and are currently being expanded outside the brain to treat lung and other metastases [30, 31].

Newer imaging capabilities in radiation have also been developed and implemented to improve precision in radiation therapy delivery. The creation of onboard imaging (OBI) on traditional Linacs and newer machine designs that combine real-time imaging with treatment delivery has allowed better treatment of metastatic disease with conventionally fractionated radiotherapy [32, 33]. The introduction of image-guided radiotherapy (IGRT) and IMRT into clinical practice has allowed many dose escalation trials using conventionally fractionated radiotherapy. However, it may be difficult to integrate these technologies fully into the treatment of metastatic disease because of the increased total treatment time and cost. Nevertheless, increased advancement in treatment delivery will continue to help reduce the side effects of therapy and to potentially improve the quality of life in these patients.

Targeted Radionuclide Therapy

By nature, metastatic disease is systemic. Cytotoxic therapies are being developed that target systemic disease with fewer side effects than past agents. However, tumors are often unresponsive to these drugs. In addition, some locations within the body are less accessible to them, preventing adequate penetration and cytotoxic drug concentrations. Targeted radionuclide therapy is currently being explored to use the effects of radiation systemically.

The efficacy of targeted radionuclide therapy depends on the type of radionuclide, as well as on the antigen–antibody interaction. Radionuclides need to have an emission profile that is appropriate for both therapy and imaging. The antibody–antigen interaction must have an optimal level of binding affinity to allow selective tumor targeting. In current cancer therapy, radionuclides are used in conjugation with monoclonal antibodies to target selective tumor antigens, and alone to target non–antibody-associated targeted nuclides.

Non–antibody-associated, bone-seeking radionuclides have been employed in the treatment of metastatic bone disease. Examples studied thus far include phosphorus-32 (^{32}P), strontium-89 (^{89}Sr), samarium-153 (^{153}Sm), and rhenium-186 (^{186}Re). These radionuclides all have the necessary characteristics of preferential

uptake in bone and the ability to be cleared by the body. Ideal radiopharmaceuticals have a half-life of activity close to the physical half-life in tumors and mainly precipitate in the cortical bone, which decreases the effect on the bone marrow. This characteristic prevents severe bone marrow suppression.

The first radiopharmaceutical used to treat metastatic bone disease was ^{32}P. Its initial use showed effectiveness in relieving pain in prostate cancer patients with widespread bone disease. Unfortunately, ^{32}P incorporated itself into various cellular constituents, causing severe bone marrow toxicity. In the 1980s, its use gradually declined. ^{89}Sr chloride (Metastron) and ^{153}Sm ethylene diamine tetramethylene phosphonate (EDTMP) lexidronam (Quadramet) were introduced in the 1990s as pharmaceuticals in palliative treatments for painful osseous metastases. Because these agents are calcium analogs, they localize well to the bone. Any undeposited agents are cleared through the urinary tract. ^{153}Sm has a significantly shorter half life than ^{89}Sr, allowing faster recovery from pancytopenia and more frequent use. Both ^{153}Sm and ^{89}Sr have been reported to have a 60 percent to 80 percent palliation effect lasting more than six months [34, 35]. These agents are useful in certain conditions of widespread bone disease that require pain palliation. Extensive research is still ongoing for ideal radiopharmaceuticals, including ^{187}Re or ^{189}Re, tin (^{117m}Sn), ^{33}P, ytterbium-175 (^{175}Yb), lutetium-177 (^{177}Lu), and radium-233 (^{233}Ra). More tests combining radionuclides with chemotherapy or bisphophonates should be encouraged.

^{131}I, a radioisotope of iodine, has been used to treat functioning metastases of thyroid cancer since 1946 [36]. Iodine, the prototype of systemic radionuclide therapy, has many favorable characteristics, such as selective localization and rapid clearance of nonabsorbed radioisotope. It also has the advantage of being used for both therapeutic and diagnostic purposes. Thyroid cancer may metastasize to the lung or the bones; if it travels to the bones, patients are diagnosed with a poor prognosis. The role of iodine radioisotope therapy in metastatic bone diseases from thyroid cancer remains controversial. However, recent reports have shown the ten-year overall survival in patients with bone metastasis treated with thyroidectomy followed by radioiodine to be 15 percent [37]. Patients with isolated lung metastases who are treated with radioiodine have a better ten-year survival, 60 percent [38]. In addition, patients who are younger and patients who have well-differentiated tumors have a better overall response to this therapy. The success of radioiodine in metastatic thyroid cancer reinforces the need to develop better targeted radionuclide therapies for other cancer types.

Experience with targeted radionuclide therapy shows that targeted cytotoxic agents can be effective in the treatment of metastatic disease. Although not curative, they can provide long-term disease control or palliation with little systemic toxicity in select cases. A focus in current research is to combine the biologic effects of radiation of cancer cells with a specific systemic targeting method.

Immunoguided Radiotherapy

Although chemical and metabolic targeting agents have a limitation to their specificity, immunoguided radiotherapy can target tumor cells preferentially by exploiting the antigen–antibody reaction [39]. This type of immunoguided radiotherapy has been termed *radioimmunotherapy* (RIT) [40]. The development of the hybridoma technique, which ensures enough antibody production to perform clinical investigation, has created a new widespread interest in RIT. Research in several other specific areas in RIT shows promise. Specifically, the ability to mass-produce both chimeric and humanized antibodies makes it possible to overcome exogenous antibody immunogenicity. Advances in genetic engineering have led to the reduction of the antibody molecule size, making systemic administration of these antibodies feasible. Better agents have been developed with more optimal half-life and energy. Finally, ongoing research in tumor biology has led to the discovery of more specific tumor-associated antigens (TAAs).

So far, more than 100 TAAs have been identified, although only a limited number of TAAs are being investigated for use in targeted therapies. With additional identification of specific types of TAAs, this treatment will have more potential for effective systemic treatment of metastatic cancer diseases. Generally, antigen expression is heterogeneous in a single tumor. Not all tumor cells will express the same amount of antigen; therefore, the effects of antibodies on tumors can be limited based on this heterogeneity. The advantage of RIT may lie in its ability to overcome this dilemma. Non–antigen-expressing cells have been shown to be targeted by effects of RIT on nearby antigen-expressing cells through a phenomenon called the "bystander effect." Radionuclides that have been used in immunoguided radiotherapies include ^{125}I, ^{131}I, yttrium-90 (^{90}Y), ^{186}Re, copper-67 (^{67}Cu), bismuth-212 and -213 (^{212}Bi and ^{213}Bi), actinium-225 (^{225}Ac), and astatine-211(^{211}At). This investigational therapy in metastatic disease is discussed briefly here.

At present, radiolabeled antibody anticancer treatments have been more effective in hematological malignancies than in solid tumors. This finding reflects the relative radiosensitivity of hematological malignancies compared with solid tumors. Factors that limit the efficacy of radiolabeled antibody therapy to the solid tumor include variable vascular supply,

elevated interstitial pressure, and heterogeneous uptake of monoclonal antibodies (mAbs) by solid tumor cells [41, 42]. In tumor sampling and clinical modeling, mAb uptake and tumor size show an inverse log relationship. This correlation suggests that reduction in tumor size may increase the effectiveness of mAb therapy in solid tumors.

Vogel et al. [43] showed that RIT had better efficacy in microscopic metastatic disease versus macroscopic disease in a live animal model. This study led to clinical trials that tested the effect of ^{131}I-hMN14 anti-CEA antibody in the adjuvant setting after surgical resection of a limited number of liver metastasis in primary colon cancer [44]. All thirty patients had small-volume disease (<3.0 cm) and were chemorefractory to 5-FU. One dose of ^{131}I-hMN14 anti-CEA antibody was given based on dosimetric calculations. The overall response rate was 58 percent, and the mean duration of response was nine months. Five patients with disease stabilization were treated again without significant toxicity. Despite the limited data to date, this study is encouraging for the further use of RIT agents in combination with other therapies in the treatment of solid metastatic lesions. Other studies are looking at RIT in combination with chemotherapy and external beam radiation therapy [45, 46]. Early stage clinical trials are under way to evaluate RIT for application in other solid tumors, such as breast [47], prostate [48], brain [49–51], and others [52, 53].

The recent FDA approval of two drugs, Y-90-ibritumomab (Zevalin) and ^{131} I-tositumomab (Bexxar), has led to the use of RIT agents in the treatment of patients with hematological malignancies, specifically chemotherapy-refractory non-Hodgkin lymphoma. Zevalin is a monoclonal murine antibody to the CD20 Ag that is attached to a pure beta emitter, Y-90. It is administered over a two-week period commencing with an initial infusion for biodistribution imaging and ending with a second infusion one to two weeks later. Bexxar targets the same CD20 Ag and is a beta and gamma emitter. It is approved for use in low-grade or transformed non-Hodgkin lymphoma. Both drugs have shown encouraging response rates in the treatment of patients with non-Hodgkin lymphoma [54–57]. Similar agents are being developed and tested based on these results. Other biological targeted radiation agents that are under study include radiolabeled liposomes [58] and radiolabeled nanoparticles [59].

Combining Radiation Treatment with Other Anticancer Modalities in Treatment of Metastatic Disease

Significant advancements have been made in recent decades in the primary modalities for the treatment of patients with metastatic disease. A multidisciplinary approach to combine these modalities is required. Determining how to combine different modalities can be challenging not only to define the treatment of choice for each clinical situation but also to identify ways to combine these newer technologies. It is imperative that these therapies be combined in a clinical trial setting to discover their potential synergistic effects. The clinical setting is increasingly important in the evaluation of high-dose, hypofractionated radiation treatment in which the potential normal tissue effects are greater. The biologic effects of these therapies in combination with systemic cytotoxic agents are unknown.

One form of combined treatment, radiotherapy with molecular targeted therapy, has received much attention recently. A number of tumor specific targets, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) have provided opportunities for the development of targeted agents such as cetuximab (Erbitux) and bevacizumab (Avastin). These agents have become popular in use with other cytotoxic agents in the treatment of metastatic disease. Other potential targets that are being actively investigated include specific tumor marker, angiogenesis inhibitor, and hypoxia-inducible factor (HIF) inhibitor [60, 61]. The combination of innovative high-precision radiotherapy with biologically targeted therapy may also have promise in the treatment of metastatic disease.

Another area of interest in multimodality therapy is the combination of targeted radiation therapy with immunotherapy. The synergistic effects of these two therapies have been well documented, but unproven. Immnunotherapy has a better response in combination with radiation. In addition, radiation increases the immune response, providing a theoretical enhancement of potential immunomodulating agents. This provides hope that radiation in combination with immunotherapy can potentiate antitumor immune response through cross-priming, antigen presentation, and enhancement of cytotoxic immune cells.

Recent research has attempted to discover the potential mechanisms related to the effect of radiation on the immune system. In animal studies, ionizing radiation increases the immune response by inducing a “danger signal” to which the immune system responds. The combination of dendritic cell response, T cells, and release of tumor antigens can result in a therapeutic immune response [62–67]. Radiation combined with enhancement of the immune system may help to overcome immune tolerance to weak immunogenic tumor-associated antigens [68–70]. The radiation-induced upregulation of MHC class I, death receptors (Fas/CD95), costimulatory molecules B7-1, intercellular adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen (LFA)-3 (together

called TRICOM) results in an immune-mediated killing of tumor cells [71–74]. Studies are being done to evaluate whether higher doses of radiation may enhance these processes.

APPLICATION OF RADIATION THERAPY IN METASTATIC DISEASE

Thus far, we have reviewed the use of advancements in radiotherapy for the treatment of metastatic disease. The application of these therapies depends on the clinical symptoms, disease burden, type of histology, other comorbidities, and quality of life. In addition, other prognostic factors that may affect treatment modality and its outcome include the size of metastases, number of metastases, performance status, age, and previous treatment. All these factors can affect life expectancy and thus should be considered appropriately in the choice of recommended treatment. The treatment choice should be tailored to best improve the patient's survival without jeopardizing quality of life. In many cases, supportive care alone is the best choice for patients with an aggressive, and a high burden of, disease. If radiation is offered as a therapeutic option, the patient's sensitivity to this procedure and to the aggressive nature of the specific cancer must be considered [75, 76]. Some of the decision parameters used to help guide treatment recommendations in patients with metastatic disease are discussed here.

Clinical disease that is aggressive in nature but responds quickly to radiation therapy includes lung and gastrointestinal cancer. These histologies respond rapidly to radiation therapy, thus enabling local control of disease that is possible in specific clinical situations. Unfortunately, these histologies are also aggressive in nature and spread quickly to other sites, often limiting the potential benefit of therapy. In cases in which the tumor itself responds well to radiotherapy but systemic disease carries a poor prognosis, newer treatment modalities that allow completion of therapy within a shorter time span will increase the potential therapeutic ratio. In patients with a life expectancy greater than three months, hypofractionated radiotherapy treatments can be recommended both as palliative and potentially life-extending. Conventionally fractionated radiation therapy for palliation remains quite reasonable and effective for patients who are diagnosed with a life expectancy less than three months and who manifest significant symptoms.

Undifferentiated carcinomas are histology types that not only respond poorly to radiotherapy but also are aggressive in nature. Patients with widespread disease in this category have short life expectancies. Without potential systemic options, use of radiotherapy in these patients is typically limited to palliative intent.

Primary breast cancer and prostate cancer represent the two most commonly diagnosed cancers in the United States today. When these cancers metastasize, they can remain sensitive to modern systemic therapies for several years. Radiation treatment for these patients provides potential benefit. For example, median survival in patients with bone metastasis from breast cancer can be twenty-four to thirty-two months [77, 78]. Median survival in patients with bone metastasis from prostate cancer is even longer, at thirty-six months. Novel techniques in radiation therapy in these patients offer the most potential benefit. It is also important to look at inclusion of these patients in studies describing potential therapies in metastatic disease. Studies with a high proportion of inclusion of these patients may have artificially elevated survival benefits and should be scrutinized carefully based on historical controls.

Some cancer histologies, such as malignant melanoma and renal cell carcinoma, have been shown to be radioresistant. However, metastatic renal cell carcinoma, compared with other cancer metastases, may be affected by the use of high-dose, hypofractionated radiotherapy to overcome relative radioresistance. Extended survival benefits have been achieved. Many of the first studies in stereotactic body radiation therapy discussed earlier were performed in patients with metastatic renal cell carcinoma.

In conclusion, a number of complex variables must be considered in the treatment of metastatic cancer. Ideal treatment combinations will offer benefits not only for the patient's survival but also for the patient's quality of life. New technologies in radiotherapy have reduced the normal tissue side effects and have alleviated the disease burden in many patients. When used in combination with other therapies, these new advances in radiation therapy may lead to increased survival in specific patients. Specific metastatic disease types should be studied and these therapies adjusted accordingly to individualize treatment based on as many clinical factors as possible. A multidisciplinary approach, available in primary cancer treatment, is needed in metastatic disease to best combine the synergistic effects of these newer therapies.

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54 Prospects for Clinical Trials of Metastasis Inhibitors

George W. Sledge Jr.

TARGETS FOR METASTASIS SUPPRESSION

The process of metastasis has been carefully delineated in recent decades and includes intravasation, survival in the circulation, and arrest in a distant organ; extravasation, survival, and growth after extravasation; and persistence of growth. Although an enormous amount of research has examined the early steps of the metastatic process, it seems unlikely that the earliest steps are amenable to therapeutic intervention. In general, distant metastasis has occurred in the majority of human cancers prior to detection of the primary tumor. In some tumors (e.g., pancreatic cancer) overt metastatic disease is common at diagnosis, whereas in other cancers (e.g., breast and colorectal cancers) micrometastatic disease is present at the time of diagnosis. In either situation, circulating tumor cells have lodged in a distant site long before a patient meets a physician.

Where in the metastatic process will the therapeutic line be drawn? Certainly this will occur somewhere following intravasation and spread through the circulation, and quite possibly following organ arrest (depending on whether tumor dormancy occurs at this point or at a subsequent point, as discussed later). Regardless, metastasis suppression strategies posited solely on interruption of the earliest steps of the metastatic process are unlikely to prove successful in most cases.

There are possible exceptions to the above rule that might render treatment of the early portions of the metastatic process valuable. The process of secondary sites of metastasis, in which a secondary wave of metastatic cells is derived from an initial metastatic site rather than the primary tumor, may play an important role in the eventual demise of the patient.[1,2] Such secondary sites of metastasis may be particularly important with regard to brain metastases in diseases such as breast cancer. In this disease, brain metastasis as an initial site of distant metastasis is rare, although later central nervous system disease is common, suggesting that metastasis to an intervening organ may serve as a nidus for subsequent spread. In theory, agents interfering with intravasation, tumor circulation, and organ arrest might prevent the development of a secondary metastasis, although testing such an agent in the clinic might prove difficult.

TREATMENT OF MICROMETASTATIC DISEASE: THE PATH TO ADJUVANT THERAPY

In most human epithelial malignancies, overt metastatic disease is incurable. Although exceptions exist (e.g., surgically resectable colorectal metastases to the liver,[3] metastatectomy for soft tissue sarcoma pulmonary metastasis,[4] and rare metastatic breast cancers[5]), volume of metastatic disease currently determines an upper boundary for potentially curative metastasis inhibition. As argued earlier, a lower boundary also likely exists in the clinic, so successful metastasis inhibition may follow a "Goldilocks principle" (i.e., "not too hot, not too cold, just right") from both a biologic and a volumetric standpoint.

In this sense, metastasis inhibitor therapy will resemble, and may indeed be identical to, current adjuvant therapy for micrometastatic disease. Adjuvant therapy, as practiced across a wide variety of clinical scenarios, has the following characteristics: it is systemic therapy (i.e., it is administered to the whole body, although the actual target may be organ-specific), its target is micrometastatic disease, and its goal is the eradication of micrometastatic disease and/or the delay or prevention of overt metastatic disease. Metastasis inhibition, when developed, will likely have similar characteristics. It is therefore reasonable to ask how agents enter the adjuvant arena.

The path to adjuvant therapy is – or has been until now – fairly stereotypical. Novel agents are tested in small numbers of patients in Phase I trials that

TABLE 54.1. The path to adjuvant therapy

Trial	Number of patients
Phase I trial Toxicity, pharmacokinetics	12–40
Phase II trials in advanced cancer Activity signal (RR, PFS)	20–200
Phase III trials in advanced disease Clinical benefit signal (PFS, OS)	400–1200
Phase III trials in early disease Clinical benefit signal (DFS, OS)	2000–12,000

determine toxicity and pharmacokinetic parameters. Phase I trials may be either single-agent trials, or trials in which the novel agent is combined with existing therapies. These agents are then tested in Phase II trials in advanced (and often far advanced) cancers to establish an efficacy signal (typically an objective tumor response), prior to a proof-of-concept Phase III trial that demonstrates a clinical benefit signal (e.g., progression-free survival or overall survival) in an advanced disease setting. After this signal is obtained, the agent is then ready for examination in a Phase III proof-of-concept trial in the adjuvant (micrometastatic) disease setting, in which a clinical benefit signal of disease-free or overall survival is obtained. As shown in Table 54.1, the transition from Phase I trial to Phase III micrometastatic disease trial typically requires increasing numbers of patients and (owing to time required for accrual and follow-up) increasing times for study completion. It is not unusual for the total process to take a decade or longer.

This approach represents a potentially significant challenge to the development of metastasis inhibitor therapy. If a metastasis inhibitor has antitumor efficacy, as usually measured in the overt metastatic disease setting, typically involving the ability to shrink overt disease, then a metastasis inhibitor can be expected to follow the "standard" transition to the adjuvant disease setting described earlier. If, in contrast, a metastasis inhibitor is incapable of shrinking overt metastatic disease (as might be expected for many potential therapeutic approaches affecting micrometastases), then the "standard" path to the adjuvant setting is likely to prove stillborn.

The path to the adjuvant setting offers up other requirements for novel agents. Because patients with micrometastatic disease are typically healthy, and because in many adjuvant settings only a minority are doomed to recurrence, the balance of toxicity and benefit becomes an important determinant of an agent's acceptability. Toxicity may be acute (occurring during active therapy) or chronic (occurring or continuing following completion of therapy). Ethical administration of novel or investigational agents requires physicians to offer study participants some reasonable understanding of the risks faced by trial participants. Virtually no agent is without toxicity.

Similarly, and related to the above, duration of therapy affects one's ability to perform trials in the micrometastatic disease setting. Is the treatment to be administered for brief periods (weeks to months), for intermediate duration (months to years), or for a long duration (perhaps for the remainder of the patient's life)? Duration depends in part on the mechanism of action of the agent used, and in part on the toxicity of the agent. Because drugs frequently have multiple mechanisms of action, some of which are unknown or uncertain at the time of study initiation, assumptions regarding duration may prove flawed. In addition, because chronic toxicities often manifest themselves during the course of adjuvant trials, risk–benefit ratios may take years to establish.

Determining which population of patients is most likely to benefit is another important consideration in the development of trials testing metastasis inhibitors. Is the agent being tested a targeted therapy? All therapeutic agents are targeted in the simplistic sense of ultimately having a molecular target, but this represents a relatively low-level definition of targeted therapy.[6] Is the target well defined at the molecular level, and does its presence have definable biologic consequences? Can the target be measured reproducibly in the clinic (e.g., HER2 in breast cancer[7]) in a primary tumor sample? Does the presence of the target correlate with therapeutic benefit (e.g., the estrogen receptor in breast cancer[8])? True targeted therapeutics (those meeting the aforementioned criteria) have an inherent advantage in clinical trials in that smaller patient populations may be required to establish clinical benefit.

Finally, clinical investigators necessarily initiate trials with some reasonable estimation of predicted clinical benefit of the novel agent. This estimation of benefit (with regard to endpoints such as disease-free or overall survival) is an important determinant of the number of patients required to be enrolled in an adjuvant trial. Indeed, estimating the number of patients required to establish benefit may be the most important decision facing a clinical research team. An underestimate may result in a falsely negative trial result, and an overestimate is profligate of clinical and financial resources.

"FIRST-GENERATION" METASTASIS INHIBITORS: LESSONS FROM ADJUVANT THERAPEUTICS

If, as suggested previously, there is considerable parallelism between current adjuvant therapy and potential metastasis inhibitor therapies, what lessons can be learned from existing adjuvant therapies? To date, most successful adjuvant therapies have involved either antiproliferative or proapoptotic therapeutics (or

agents providing some mixture of antiproliferative and proapoptotic effects). Breast cancer represents the best-studied human epithelial malignancy with regard to testing agents in the micrometastatic disease setting.

From a genomic standpoint, breast cancer can be divided into several subtypes expressing differing biology and differing responsiveness. Adjuvant studies dating back over a decade suggest that estrogen–receptor-positive tumors may have a continuing risk of relapse, extending more than a decade following initial therapy. The Early Breast Cancer Trialists' Group meta-analysis of randomized clinical trials suggests that in the case of adjuvant tamoxifen, more than half of all recurrences and deaths occur following the completion of five years of adjuvant therapy.[9] A significant "carryover" effect occurs with adjuvant hormonal therapy, with much of the benefit occurring after completion of hormonal therapy.

In contrast, estrogen-insensitive tumors (typically treated with adjuvant combination chemotherapy) relapse at a relatively early time point, generally within the first five years following diagnosis.[9] In this population, adjuvant benefits (with regard to separation of disease-free survival curves) occur early on, in contrast to the pattern seen with hormonal therapies. It is currently unknown whether adjuvant HER2-targeted therapy, which has a relatively short clinical trial history, will follow the pattern of hormonal therapy or chemotherapy.

Clinical investigators have only recently begun to probe the genomic influences underlying clinical benefit in the adjuvant setting, although the existence of multigene predictors of prognosis and clinical benefit suggests that the influences are likely to be complex. In the case of estrogen–receptor-positive, lymph–node-negative breast cancers, genomic analyses suggest the existence of a subpopulation driven by either HER2 or by a proliferation gene cassette, and that these patients tend to be especially sensitive to proapoptotic agents.[10] In contrast, another subpopulation is characterized by significant sensitivity to estrogen-blocking agents, a weak proliferation cassette of genes, and HER2-negativity. This latter population predictably derives little benefit from chemotherapeutic interventions.[10]

IMPLICATIONS OF TUMOR DORMANCY FOR METASTASIS INHIBITOR THERAPY

Tumor dormancy has been the subject of several reviews in recent years.[11,12] In clinical human datasets, not all cancers are characterized by the existence of significant dormant populations fated to recur at a late time point. As the breast cancer experience described previously suggests, individual tumor types may harbor subtypes with rapid recurrences as well as subtypes that may lie dormant for prolonged periods.[13] Furthermore, in contrast to estrogen–receptor-positive breast cancer, with its continuing hazard for recurrence, cancers such as colorectal and ovarian cancer are (currently) associated with high initial hazard rates for recurrence and relatively few late recurrences.[14]

The mechanisms underlying tumor dormancy are explored elsewhere in this volume. The existence of tumor dormancy, however, raises important questions regarding the clinical development of metastasis inhibitors. First, as antiproliferative and proapoptotic adjuvant therapies improve in efficacy, dormant cells are likely to become the major cause of relapse and death for certain tumor types (e.g., breast cancer[14,15] and melanoma[15]). Second, as therapies administered for short periods of time may have little effect on dormant subpopulations, regardless of their mechanisms of action, novel metastasis inhibitor approaches (again depending on mechanism of action) may require prolonged exposure to the antimetastatic agent. Finally, targeting dormant cells will be important for many, but not all, human tumor types.[14]

Assuming the existence of dormant cancer cells in specific tumor types, how do we attack tumor dormancy? Investigators have suggested the existence of several types of tumor dormancy,[12] including (1) what has been called cellular dormancy (in which individual cells remain quiescent at distant metastatic sites); (2) angiogenic dormancy, in which the lack of new blood vessels prevents the progressive growth of tumors composed of actively dividing tumor cells; and (3) tumors that remain dormant because of immunosurveillance.

The existence of these types of dormancy suggests several potential modes of attack.[16,17] Quiescent individual cancers might be *maintained* in their dormant state with prolonged antiproliferative therapy (e.g., chronic hormonal therapy targeting the estrogen receptor) or via immunotherapy. Alternatively, single dormant cells might be *eliminated* with any of a number of potential antimetastatic therapies described elsewhere in this volume.

Angiogenic dormancy might be targeted with antiangiogenic therapies (e.g., anti-VEGF therapies) administered chronically to inhibit the "angiogenic switch" thought to occur in many progressing tumors.[18] An example of this approach is shown in Figure 54.1, in which the schema for the current Breast Cancer Intergroup trial E5103 is shown. This large adjuvant trial of close to 5000 patients randomizes women with early-stage breast cancer to receive either a backbone adjuvant chemotherapy regimen, chemotherapy plus the anti-VEGF agent bevacizumab administered during the course of chemotherapy, or chemotherapy plus bevacizumab administered both during and following chemotherapy for a total duration of one year. The questions surrounding metastasis inhibitor therapy are emphasized by this trial. Will anti-VEGF therapy potentiate the proapoptotic effect of chemotherapy (against both cancer cells and tumor endothelium),

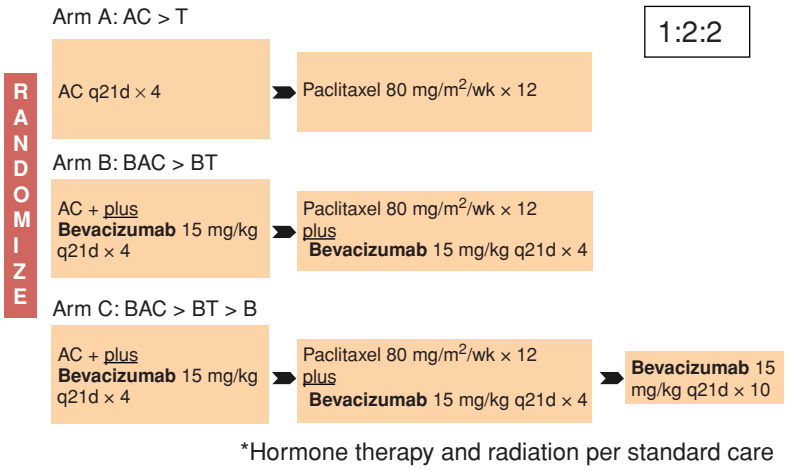

Figure 54.1. Prevention of metastasis with antiangiogenic (anti-VEGF) therapy in early-stage breast cancer: E5103.

providing efficacy with relatively short duration of therapy? Or will anti-VEGF therapy require prolonged administration (perhaps longer than allowed for in the current trial) to prevent the development of new blood vessels? Will anti-VEGF therapy have any effect on quiescent single cells? Will microscopic tumors of various sizes (and various degrees of tumor vessel development and maturation) respond similarly to anti-VEGF therapy? Can we target anti-VEGF therapy (using genomic or pharmacogenomic approaches) in any meaningful fashion, or will all patients require therapy? Will toxicities seen in the metastatic setting be similar to those seen in the adjuvant setting?

The targeting of dormant cells with prolonged antiproliferative therapy is currently being examined in the setting of micrometastatic disease in the form of trials examining prolonged durations of adjuvant hormonal therapy. JMA-17, the first of these trials, randomized patients receiving five years of adjuvant hormonal therapy with tamoxifen to receive either a placebo or five years of the aromatase inhibitor letrozole.[19] This trial demonstrated a significant reduction (though not elimination) of late metastasis. An ongoing example of this approach is shown in Figure 54.2, in which estrogen–receptor-positive breast cancer patients treated with five years of aromatase inhibitor therapy are randomized (in a double-blind fashion) to receive either a further five years of aromatase inhibitor therapy or a placebo. This and similar trials will help define a role for prolonged inhibition of micrometastatic (and presumably dormant) disease.

IMPLICATIONS OF NOVEL SURVEILLANCE TECHNOLOGIES

Current surveillance technologies are generally incapable of detecting micrometastatic disease, and are totally unable to distinguish dormant micrometastatic disease from actively growing micrometastases. This era of "silent" micrometastases may be coming to an end, as novel imaging and proteomic approaches push back the boundaries of detectable disease. For instance, the resolution of noninvasive brain imaging is doubling about every twelve months,[20] and current investigational MRI imaging is capable of resolution in the sub-millimeter range.[21] Similarly, improvements in proteomic technology have the potential to detect micrometastatic disease.

When, in the not-too-distant future, investigators are capable of detecting small deposits of micrometastatic tumor cells, what effects will this have with regard to either the development of metastasis inhibitor therapy or clinical outcomes? The ready assumption that the detection of smaller volumes of metastatic disease will improve outcome is unproven and not without major challenges for patients and their physicians.

That this might be the case is shown by surveillance trials in the early breast cancer setting. Two large randomized controlled trials allocated patients with early-stage breast cancer to either receive or not receive intensive surveillance with (then-extant) imaging and serum-based tumor markers.[22,23] Patients randomized to receive intensive surveillance fared no better than patients receiving regular physical examinations and screening mammography. Based on these data, the American Society of Clinical Oncology technology guidelines currently recommend against regular surveillance for metastatic disease other than the use of physical examination, screening mammography, and opportunistic imaging based on clinical signs and symptoms.[24]

Why might this be the case? First, patients receiving standard adjuvant therapy at the time of initial diagnosis may have already received the most active therapy available for micrometastatic disease. Cancer cells slipping through the initial net of adjuvant therapy are likely to be relatively resistant to the drugs

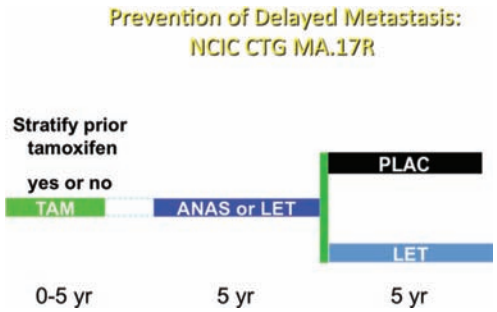

Figure 54.2. Prevention of late metastasis in ER-positive early stage breast cancer: MA17R. TAM = tamoxifen; ANAS = anastrazole; LET = letrozole; PLA = placebo.

to which they have been exposed, and indeed may be multidrug-resistant. Second, the mere detection of microscopic disease does not render those cancer cells treatable by any therapy. Finally, quiescent dormant cells, as the recent stem cell literature suggests, may require distinctly different therapies than actively dividing micrometastatic disease.[25,26]

This suggests that the challenge to investigators involved in developing novel surveillance technologies (whether protein-based or imaging) involves more than simple detection of micrometastatic disease. Novel technologies will need to assess whether micrometastatic deposits are quiescent or actively growing, and if quiescent, whether they have the potential to become actively growing. Ultimately, surveillance technologies should go beyond the determination of prognosis (i.e., whether the patient is fated to develop overt metastasis to demonstrate the sensitivity of micrometastases to specific therapeutic agents).

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