

PART I. BASIC RESEARCH

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 co-workers 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

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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, micro-RNAs 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 senescence 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.

MODELS AND TOOLS FOR METASTASIS STUDIES

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 preferen-

tially 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 pro-

files 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

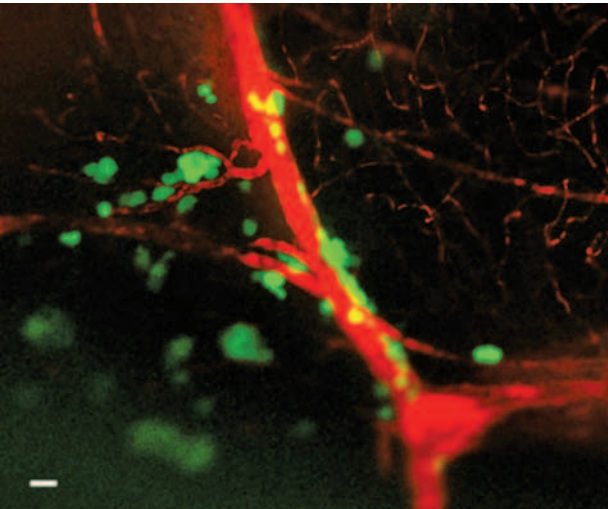


Figure 1.1. Perivascular growth of metastatic cells in the brain of a nude mouse, twenty-one days after left-heart injection of GFP-expressing MDA-MB-435 cells. The mouse was injected with rhodamine-albumin 1 hour before killing and exposing the brain for imaging with a laser scanning confocal microscope. 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.

cell types of recipient mice, provide powerful systems for imaging tumor–stroma interactions with multi-photon 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

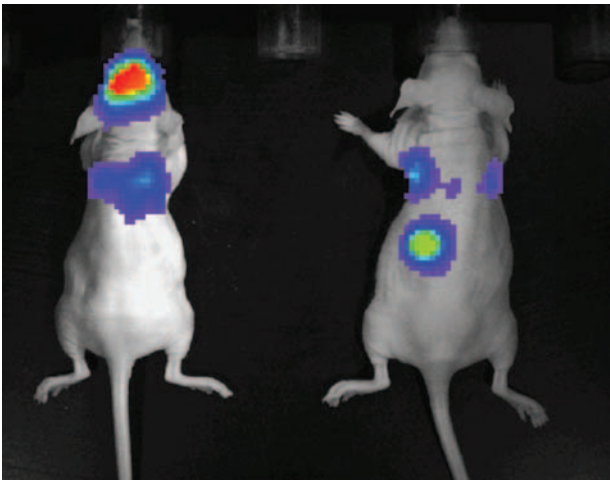


Figure 1.2. Bioluminescent detection of metastases in lungs, adrenal, and brain of nude mice previously injected with luciferase-expressing MDA-MB-435 human cancer cells. The cells were injected into the mammary fatpad, and the tumors removed when 1 cm in diameter. Six weeks later, before obvious signs 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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