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Modeling metastasis in the mouse

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Abstract

Metastasis is a complex clinical and biological problem presently under intense study, and several model systems are in use to experimentally recapitulate and dissect the various steps of the metastatic process. Genetically engineered mouse models provide faithful renditions of events in tumor progression, angiogenesis, and local invasion that set the stage for metastasis, whereas engrafting of human or mouse tumor tissues into mouse hosts has been successfully exploited to investigate metastatic dissemination and colonization of distant organs. Real-time, high-resolution microscopy in live animals, and comprehensive genetic and molecular profiling are effective tools to interrogate diverse metastatic cancer cell phenotypes as well as the metastatic tumor microenvironment in different organs. By integrating the information obtained with these complementary approaches the field is currently obtaining an unprecedented level of understanding of the biology, molecular basis, and therapeutic vulnerabilities of metastasis.

Introduction

Metastasis is a challenging clinical problem and the cause of most deaths from cancer. As a biological process, metastasis is quite complex as it reflects the many barriers that cancer cells that leave a primary tumor must overcome to generate aggressive secondary lesions [1]. Depending on the cancer type, metastasis may be achieved by just a rare minority of tumor-initiating cells that reach, survive and eventually overtake a distant tissue microenvironment over a long period of time, as in certain types of breast cancer [2,3], or it may represent a relatively common occurrence among primary tumor cell populations that are primed to perform many of the necessary steps and prone to forming rapidly growing metastatic colonies as, for example, in lung adenocarcinoma [4,5].

Certain fundamental properties of metastatic cells, including migration and invasiveness, have been the subject of many studies using a variety of *in vitro* model systems. Technological advances such as fluorescent or bioluminescent reporter molecules and sophisticated microscopy have allowed sensitive and accurate analysis of these processes and their molecular underpinnings at the single-cell or cell-cluster levels [6,7]. These experimental systems

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additionally provide comparatively inexpensive methods of choice to screen RNAi, cDNA or chemical libraries for mediators or inhibitors of these cancer cell functions [8–10]. *In vitro* model systems have contributed to define the role of candidate metastasis genes in particular steps of the metastatic cascade [11,12]. However, these models can provide surrogate systems for the analysis of only a limited set of events in the metastatic cascade, which *in vivo* involves multiple steps within specific tissue contexts.

To better understand tumor development and progression *in vivo*, two general strategies have been pursued in mice: genetically engineered models of cancer, and transplantable tumor model systems. These strategies provide complementary approaches to the dissection of specific metastatic steps. Cancer models driven by the introduction of oncogenic mutations in a tissue-specific manner can faithfully recapitulate important aspects of tumor initiation, local progression, and response to therapy [13,14]. In these models, cancer develops with high penetrance in a stepwise manner, enabling the study of tumor initiation and early steps of metastatic dissemination (Figure 1). However, metastasis in these models is often restricted to lymph nodes and the lungs, or is missing altogether (Table 1). Syngeneic and xenograft models in which mouse or human cancer cells are introduced into immunocompatible or immunocompromised mice provide at present methods of choice to experimentally address metastatic dissemination to, and colonization of relevant organs. Syngeneic models enable the study of the complete microenvironmental interface in the mouse but are limited to study mouse cancer cell metastasis. Conversely, in spite of the caveat of an incomplete immune system, xenograft models provide a superior alternative to the study of metastasis of human cancer cells *in vivo*.

Here we present an overview of the advantages and disadvantages of the principal mouse model systems currently used for the study of metastasis (Table 2), and how the combined use of these systems complemented with *in vitro* models is yielding an increasingly robust understanding of the multiple modes and steps of metastasis.

Modeling early metastatic steps

Cancer cells in a primary tumor have already acquired a number of aggressive functions that will remain important throughout the rest of their metastatic progression. These functions generally include motility, invasiveness, resistance to hypoxia and reactive oxygen species, survival after detachment, and evasion of immune surveillance. The accumulation of these properties in human cancer cells is thought to occur in the context of the primary tumor, and genetically engineered tumors in mice provide powerful models to delineate the acquisition of these functions (Figure 1).

It has been proposed that secondary tumor formation involves rare cell variants that have accumulated a complete set of genetic mutations in the primary tumor that enables these cells to grow in a distant organ [15]. However, this hypothesis has been challenged based on the detection of widespread gene expression patterns in primary tumors that strongly predict metastatic competence [16], and the analysis of growth dynamics of human breast primary tumors and metastases [17]. Genetically marked transplantable tumors in mouse mammary carcinoma models were used to demonstrate that cancer cells can disseminate at the premalignant stage [18,19]. Moreover, *ex vivo* genetic manipulation of tumor-derived cells prior to implantation in cleared mouse mammary fat pads was used to identify genes that influence this process [19]. Other transplantation methods using inducible genetically engineered oncogenes have shown that non-transformed mammary cells introduced in the mouse circulation can extravasate in the lungs, and give rise to tumor foci in the lung parenchyma upon oncogene induction [20]. However, the question of whether early disseminated tumor cells (DTCs) are responsible for the outgrowth of secondary tumors

remains a matter of debate. In one approach, early-stage mouse tumors were transplanted for variable time periods, and metastatic outgrowth measured upon resection after a fixed number of weeks. The results suggested that early DTCs are inefficient at initiating secondary tumors [19].

Xenografting of limiting dilutions of cancer cells are standard for evaluating tumor-initiating capacity [21], and has been instrumental in linking epithelial-mesenchymal trans-differentiation (EMT) to tumor-initiating "cancer stem" cell phenotype in breast cancer [22]. A recently introduced interleukin-2 receptor-deficient NOD/SCID mouse strain has been used to demonstrate that one single human melanoma cell can develop into a lethal, metastatic tumor [5]. Tumor engrafting models are extensively used to delineate the role of genes of interest in early metastatic steps at orthotopic sites. Both, xeno- and allo-transplantation have been successfully used to delineate the role of genes and miRNAs in the invasive capacity of tumor cells [12,23–25], and to dissect the distinct contributions of particular genes in the context of mammary tumors versus the context of lung metastasis [11,26].

The importance of the microenvironment in tumor progression is widely recognized, and genetically engineered mouse models have provided key insights into the relevance of the tumor microenvironment in metastasis [27]. Dissenting the contribution of different components of the tumor stroma represent a significant experimental challenge. Nonetheless, progress has been made with the use of bone marrow transplantation from genetically engineered donor mice. Such bone marrow transplants into tumor bearing animals can shed light into the role of a candidate metastasis gene expressed in either the cancer cells or a particular cell type in the tumor microenvironment. Recent examples of this approach have revealed the specific contribution of macrophage-derived cathepsins in pancreatic cancer and breast cancer cell invasion [28]. Similar genetic manipulations in a double transgenic PyMT/RAG1−/− breast cancer model have implicated CD4 T effector cells in tumor cell invasion and metastasis [29]. This methodology is highly promising to define the role of the innate and adaptive immune system in tumor cell dissemination.

Metastatic dissemination and colonization: transplantable tumor models

Establishment of secondary tumors imposes different demands on disseminated cancer cells depending on the target organ. Xenograft models provide an effective system to investigate secondary organ colonization of human cells, and remain the model of choice for pre-clinical studies of human tumor-derived cells (Table 3). Intracardiac inoculation of cancer cells into the arterial circulation of mice allows the systemic distribution of these cells to all organs for the analysis of metastatic functions including organ-specific extravasation, survival in the newly invaded parenchyma, retention of tumor-reinitiating capacity, and overt colonization [30]. In contrast, tail-vein inoculation forces cancer cells to lodge in lung capillaries, which allows an assessment of lung extravasation and colonization functions [31]. Carotid artery inoculation likewise targets cancer cells to the brain [32].

In vivo selection of organ-specific metastatic variants from human malignant samples and cell lines, coupled with analysis of mRNA and microRNA expression patters has allowed the identification of organ-specific metastasis genes and functions. By comparing the results of this type of analysis with clinical gene expression data sets, it is possible to identify metastasis-associated genes of clinical relevance. Several gene sets have been identified in this manner that are associated with organ-specific relapse in breast cancer patients [33,34]. This information in turn can be used to guide functional studies for the discovery of genes that mediate metastasis, including genes that prime cancer cells for extravasation across the tight endothelial walls in the lungs or the brain [11,34]. Variants of this approach have identified new mediators of circulating cancer cell interaction with vascular capillary walls [35], and

genes that support the tumor-reinitiating capacity of disseminated cancer cells in the lung parenchyma [36].

Another approach is based on interrogating clinical gene expression data sets for associations between specific pathways and particular disease outcomes. By combining this information with functional assays it was recently shown that a hyperactive Wnt pathway in lung adenocarcinoma tumors supports aggressive multi-organ metastasis to brain and bones [37], whereas a high level of Src activity in breast tumors endows disseminating cancer cells with an enhanced capacity to survival in the bone marrow microenvironment and may contribute to late-onset bone metastasis [2].

Transplantation studies have also illuminated other aspects of metastasis including the systemic effect of transplanted tumors. Allografted tumors can cause the mobilization of $VEGFR1^+$ bone marrow-derived cells to the lungs for the establishment of a “pre-metastatic niche” to host incoming cancer cells [38]. The use of xenografts has also allowed a demonstration that an indolent tumor can be stimulated to grow by bone marrow-derived cells mobilized by systemic signals from a separate tumor [39]. Inducible RNAi technology in a syngeneic model of lung metastasis has shown that recruitment of endothelial progenitor cells is essential for the angiogenic switch that facilitates macrometastatic growth [40].

A self-seeding mechanism by which circulating cancer cells reinfiltrate and populate the tumor of origin has been proposed to explain certain aspects of tumor growth and metastatic population dynamics [41]. A recent experimental demonstration of this phenomenon employed xenograft and allograft models of orthotopic tumor seeding by cancer cells entering the circulation from a separate tumor mass, from lung metastatic nodules, or from direct intra-arterial inoculation [42].

Although genetically engineered mouse models provide good systems for the pre-clinical evaluation of therapeutic agents [43,44], the response of human cancer cells to therapy *in vivo* requires the use of xenograft models. Of particular relevance is the xenografting of metastatic cell lines in orthotopic locations, followed by resection of the primary tumors and initiation of therapy. This set up approximates the situation observed in patients with advanced disease [45].

Visualizing metastasis

Tracking cancer cells in real time in whole animals has provided a tremendous advantage in the dynamic monitoring of metastatic development. Of particular relevance, multimodality imaging technology such as the triple fusion protein reporter with herpes simplex virus 1 thymidine kinase (HSV1-TK), fused to enhanced green fluorescent protein (eGFP), and firefly luciferase has enabled the use of nuclear imaging, bioluminiscence and fluorescence imaging in a single experiment. In addition, the same tissues can be further analyzed by histological detection of the fluorescence GFP in frozen sections, or immunohistochemical detection of the reporter [46]. Variants of these reporters with different emission wavelengths, duration of the signal, and split versions for complementation studies exist that are useful in different applications [47]. The development of more advanced reporter systems based on similar technology has also permitted the *in vivo* monitoring of gene pathway activity and inhibition [48].

Intravital microscopy is a key resource to visualize cancer cells performing various metastatic steps *in situ* (reviewed in [49]). Macrophage-assisted tumor cell migration involving an autocrine loop has relied on the use of this approach for the analysis of polyoma middle-T transgenic mammary tumors in mice [50]. In this assay, a fine needle containing a chemoattractant is introduced into the tumor, and migrating cells can be recovered for further

analysis. The use of this technology has also shed light on the interaction between migrating cancer cells and macrophages (reviewed in [51]).

Long-term spinning disk confocal microscopy represents another application of advanced microscopy and improved tracing techniques to the examination of cellular movements and interactions in the tumor microenvironment. This imaging technique enables the dynamic visualization of stromal cells in defined tumor areas, and its combination with fluorescent reporter knock-in mice in which different immune cell types are marked, or injection of fluorescent antibodies or dextrans, enables the rapid collection of images for studying behavior of moving cells [52]. Rapid and prolonged visualization of tumor growth parameters such as angiogenesis, lymphangiogenesis, tissue viability, and response to therapy in larger tumor areas have also been achieved by the introduction of optical frequency domain imaging. This approach has the advantage of not requiring labeling or contrasting agents [53].

Multiphoton laser scanning microscopy has enabled live imaging of challenging metastatic sites like the brain. The use of cranial windows and fluorescently labeled tumor cells and dextrans has allowed a visualization of brain metastatic cells closely interacting with the microvasculature [54]. A longer term imaging study using genetic labeling of the vasculature has made possible to image several steps in the process of brain metastasis, demonstrating active extravasation of tumors cells into the brain, perivascular growth, and responses to therapy [55]. Advances in the visualization of protease activity such as the use of ACPP (activatable cell penetrating peptide) has also enabled the *in vivo* labeling of MMP protease activity in xenograft and genetically engineered tumors and small metastatic foci in the lungs [56]. The rapidly evolving field of intravital microscopy is likely to provide in the future new means to analyze metastatic behavior and performing detailed functional analysis of genetically engineered cells.

Conclusions and perspectives

A great deal of progress has been made in the study of the various metastatic steps in the past few years. However, many clinically relevant questions remain unanswered, owing partly to a lack of suitable animal models. The problem of metastatic dormancy and the role of the immune system in metastasis are prominent in this regard. Clinically, metastasis may take decades to become manifest in certain types of cancer [4], and there is a dearth of mouse models to study the biology of the latent metastatic state [2]. Both of these questions would benefit from the development of additional immunocompromised models (rev. in [57]). Progress in this direction is being made with human tissues implanted in the mouse to serve as recipients for human cancer cells [58,59]. Such systems may provide important new models for pre-clinical studies of anti-metastatic agents. Similarly, the ability to uncouple primary tumor growth from specific metastatic steps is a coveted feature of genetically engineered mouse models of cancer. The use of reversibly inducible oncogenic alterations [60–62], and stable [63] or reversible [64] RNAi targeting of genes of interest represents only some of the new tools that can be applied to the analysis of metastatic progression in genetically engineered mouse models. The development of new and improved experimental metastasis methods, and a better integration of the results with clinically data promises to support a sustained expansion of our ability to understand and fight metastasis.

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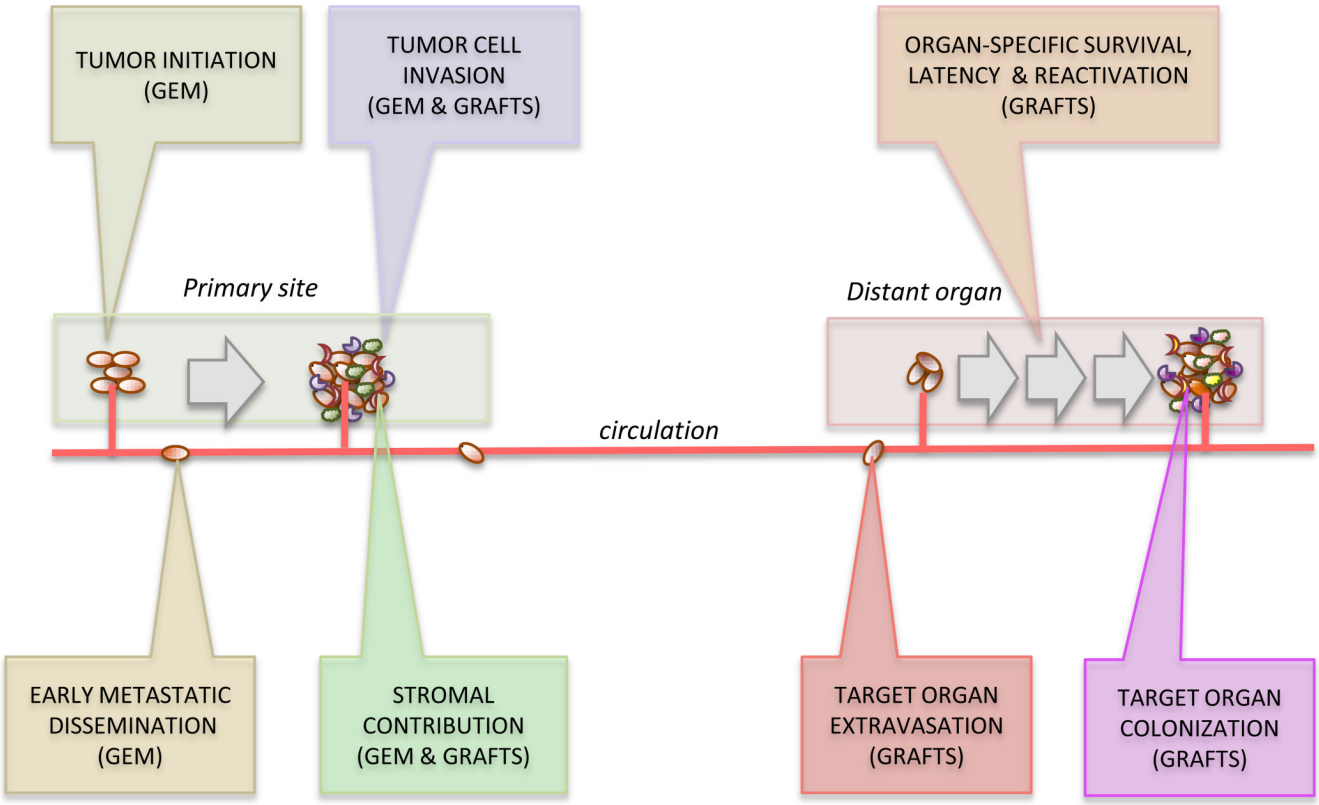

Figure 1.
Contribution of different mouse models to the various steps of metastatic dissemination. GEM: Genetically engineered mouse models, GRAFTS: xeno- or allograft transplantation.

Table 1

Metastatic patterns of dissemination

Tumor Type	Clinical Site Of Relapse	Metastatic pattern observed in GEMs	Metastatic sites in transplantable models
Breast	Bone, lungs, brain, liver, lymph nodes	Lungs, lymph nodes	Bone, lungs, brain, liver, lymph nodes
Prostate	Bone, lymph node	Lymph node, lung, rare bone	Bone, lymph node
Lung	Lung, brain, bone, lymph node	Lymph node	Lung, brain, bone, lymph node
Melanoma	Multiple organs, mostly lymph node, lungs, liver, brain and bone.	Lymph node and lungs	Lymph node, lungs, bone, brain.

Table 2

Advantages and disadvantages of genetically engineered and transplantable models for the study of metastasis

Tumor Model	Advantages	Disadvantages
GEM	- Immunocompetent host - Defined genetic background - Tumors arising in tissue of origin, usually from clinical relevant mutations	- Limited and/or atypical metastatic spread - Laborious uncoupling of initiation from progression - Burden markers metastasis - Requires validation in human
Xenografts	- Wide range of human samples - Range of orthologens metastatic sites - Short latency or long latency	- Lack of adaptive immune interactions - Some species-specific incompatibility
Allografts	- Immunocompetent host - Wide range of metastatic sites - Short latency	- Limited range of useful mouse cell lines - Requires validation in human samples

Table 3

Sources and utilization of clinical material.

Clinical source	Research use
Primary tumor biopsy	Gene validation, xenograft transplantation for stem/progenitor cell capacity, establishment of orthotopic tumors, gene discovery, therapeutic response.
Metastatic tumor, lymph node biopsies	Validation of gene expression by IF, establishment of xenograft tumors, gene discovery and functional studies.
CTCs (Circulating tumor cells)	Analysis of genetic changes, validation of marker expression, single cell xenograft.
DTCs (Disseminated tumor cells from bone marrow aspirates)	Similar as CTCs. In addition, essential contribution to metastatic dormancy and survival studies.