Topic Introduction

Methods to Study Metastasis in Genetically Modified Mice

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Metastasis is often modeled by xenotransplantation of cell lines in immunodeficient mice. A wealth of information about tumor cell behavior in the new environment is obtained from these efforts. Yet by design, this approach is “tumor-centric,” as it focuses on cell-autonomous determinants of human tumor dissemination in mouse tissues, in effect using the animal body as a sophisticated “Petri dish” providing nutrients and support for tumor growth. Transgenic or gene knockout mouse models of cancer allow the study of tumor spread as a systemic disease and offer a complementary approach for studying the natural history of cancer. This introduction is aimed at describing the overall methodological approach to studying metastasis in genetically modified mice, with a particular focus on using animals with regulated expression of potent human oncogenes in the breast.

METASTASIS THEORIES

A classical theory generated in the 1970s predicts that metastases arise from genetically determined subpopulations in primary tumors (Fidler 1973). Indeed, the ability of gene-expression profiles of human primary carcinomas to predict metastasis risk (van ‘t Veer et al. 2002; Ramaswamy et al. 2003; Wang et al. 2005) suggests that the metastatic ability of tumors is acquired by a linear progression to a distinct genetic program in cells of the primary tumor (Kang et al. 2003; Minn et al. 2005). On the other hand, analysis of single disseminated cancer cells led to a hypothesis that metastatic disease evolves independently from the primary tumor (Klein 2009; Stoecklein and Klein 2010). The parallel theory of metastasis is based on observations that the genomic alterations of disseminated tumor cells in patients often do not resemble those of the primary tumors (Schmidt-Kittler et al. 2003). In short, some cancers appear to evolve at the primary site and metastatic variants are then selected at the periphery, and others appear to disseminate early, so that metastatic evolution proceeds at the ectopic site. Genetically engineered mouse models of cancer serve as an important tool in the search for a unifying theory of metastasis, and provide experimental ways to take into account both of these theories (Husemann and Klein 2009).

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GENETICALLY MODIFIED MOUSE MODELS OF METASTATIC DISEASE

Despite significant advances in our understanding of cancer, metastasis remains the main cause of cancer-related mortality. Once established, metastatic disease is difficult to eliminate, as it often becomes inaccessible to surgical treatment and insensitive to chemotherapy. Different metastatic cancers can have vastly different natural histories that likely represent pathogenetically unrelated diseases. Mouse models of metastatic cancer are only beginning to capture this diversity, and new and improved models to study metastasis would greatly benefit the metastasis research field. Tissue-specific oncogene expression (and/or tumor-suppressor loss) has been used to induce metastatic cancer in many tissues, including liver (Shachaf et al. 2004; Lewis et al. 2005), breast (reviewed in Fantozzi and Christofori 2006; Vernon et al. 2007; Bos et al. 2010), colon (reviewed in Heijstek et al. 2005), lung (Meuwissen et al. 2003; Rapp et al. 2009; Winslow et al. 2011), skin (Landsberg et al. 2010; Damsky et al. 2011; Schiffner et al. 2012), pancreas (Tevethia et al. 1997; Hingorani et al. 2005; Bardeesy et al. 2006; Rhim et al. 2012), ovary (reviewed in Sale and Orsulic 2006), and prostate (Gingrich et al. 1996; Han et al. 2005; Moore et al. 2008). In transgenic cancers, metastatic sites are often identical in different models, perhaps reflecting tropism in the tissue of origin of the primary tumor. For example, mammary tumors produce metastatic colonies almost exclusively in the lung (reviewed in Vernon et al. 2007), and pancreas and colon tumors metastasize to the liver (Qian et al. 2001; Shachaf et al. 2004; Heijstek et al. 2005; Morton et al. 2008). Other tumors, such as lung or prostate, have a less predictable pattern of dissemination. Some cancers in humans also display a recurrent order of the systemic spread, but advanced metastatic disease in humans rarely affects only a single organ.

Historically, metastatic disease in a new cancer model has been detected by conventional gross and microscopic histopathological inspection at the time of tumor harvest. In the absence of clear “clinical” symptoms (usually signs of organ failure), the lung, liver, spleen, and kidneys of the tumor-bearing mice are commonly evaluated for evidence of visceral metastases, followed by the search for brain metastases and skeletal metastases in major bones. The more recent whole-body imaging methods such as magnetic resonance imaging (MRI), micro-computed tomography (micro-CT), positron emission tomography (PET), and bioluminescence imaging (Acuff et al. 2006; Deroose et al. 2007; Fushiki et al. 2009; Kirsch et al. 2010; Krupnick et al. 2012) permit a “live” metastasis scan of an entire body of a mouse with cancer; see Introduction: **Noninvasive Imaging of Tumor Burden and Molecular Pathways in Mouse Models of Cancer** (Wang et al. 2014).

In genetically modified mouse models, the metastatic phenotype is often linked to the genotype of the primary tumor. Loss of the tumor-suppressor genes *Trp53*, *Pten*, or *Rb* have all repeatedly been shown to promote neoplastic disease to the systemic stage, regardless of the tumor type studied (Maroulakou et al. 1997; Fedorov et al. 2003; Lin et al. 2004; Calbo et al. 2005; Lewis et al. 2005; Ridgeway et al. 2006; Chen et al. 2007; Berman et al. 2008; Morton et al. 2008; Dankort et al. 2009; Guigon et al. 2009; Puzio-Kuter et al. 2009; Schade et al. 2009; Antico Arciuch et al. 2011). Although the pleiotropic effects of inactivation of these genes, which include the induction of genomic instability (Yin et al. 1992; Fukasawa et al. 1996; Puc et al. 2005; Coschi et al. 2010; Manning et al. 2010; van Harn et al. 2010), altered cell-cycle progression, and the reprogramming of chromatin (for review, see Beckerman and Prives 2010; Fiorentino et al. 2013), make it challenging to link a specific effect of tumor-suppressor deficiency to a given step in a metastatic cascade, this does not diminish the value of tumor-suppressor-deficient backgrounds as a reliable way to reveal metastatic abilities in models that are not metastatic. It is particularly relevant in light of frequent tumor-suppressor loss in human cancers.

USING GENETICALLY MODIFIED MICE TO STUDY METASTASIS

Identification of Genes that Regulate Metastasis

Genetically modified mice have been long used to identify or validate specific factors that regulate metastasis. One of the most widely studied models of metastatic progression is an insulinoma model

(Hanahan 1985). Expression of the SV40 viral oncogene under the control of the rat insulin promoter leads to gradual transformation and tumorigenesis in the pancreatic islets. Recurrent changes associated with the advanced disease in this model (e.g., up-regulation of the insulin-like growth factor receptor, IGF-1R) have been found to be important for metastasis (Lopez and Hanahan 2002). Somatic delivery of candidate progression genes in this model has led to identification of metastasis-inducing properties of the antiapoptotic molecule BclX-L (Du et al. 2007) and of the receptor for hyaluronan-mediated motility isoform B (Du et al. 2011). Additionally, many metastatic factors have been validated using *Rip-Tag* mice, including neural cell adhesion molecule (Perl et al. 1999; Crnic et al. 2004), E-cadherin (Perl et al. 1998; Du et al. 2007), and vascular endothelial growth factor-C (Mandriota et al. 2001). One of these factors, β 1-integrin (Kren et al. 2007), regulates metastatic progression in other cancer models—for example, in the *MMTV-PyMT* (Guy et al. 1992) and in the *MMTV-Neu* (Muller et al. 1988) models of metastatic breast cancer (reviewed in Lahlou and Muller 2011).

A gene that consistently regulates metastatic behavior of cancer cells across a variety of transgenic cancer models is *TGF- β 1* (Yang et al. 2002, 2008; Siegel et al. 2003; Tu et al. 2003; Muraoka-Cook et al. 2004; Borczuk et al. 2011). TGF- β 1 enhances the proliferation of cells of mesenchymal origin and inhibits the proliferation of many types of epithelial cells. TGF- β 1 acts by binding to the type II TGF- β receptor (T β RII), which is constitutively active as a serine/threonine kinase (Heldin et al. 1997; Massague and Chen 2000). TGF- β signaling appears to play a role in the development of disseminated disease by acting both cell autonomously and as a systemic factor (Forrester et al. 2005; Fang et al. 2011). In this regard, either gain or loss of TGF- β signaling may promote metastasis, depending on the cell type or cellular context (reviewed in Brier and Moses 2009). It is speculated that the metastasis-related effects of TGF- β 1 are mediated by the tumor microenvironment, such as endothelial cells, immune cells, and fibroblasts. For example, acute repression of TGF- β signaling in *Kras*-induced lung tumors promotes lymph node metastasis associated with an immunosuppressed tumor microenvironment (Borczuk et al. 2011). Similarly, acute ablation of TGF- β signaling in *MMTV-PyMT* mammary tumor cells leads to a fivefold increase in lung metastasis (Brier et al. 2008), possibly via increase in the number of infiltrating Th17 (Novitskiy et al. 2011) and/or *Gr-1+CD11b+* myeloid cells (Yang et al. 2008). However, transgenic mice expressing a soluble TGF- β antagonist (T β RII:Fc fusion protein) are resistant to the development of metastases at multiple organ sites in an *MMTV-Neu* breast cancer model (Yang et al. 2002). Cross-validation of a metastasis factor in cancers induced in different genetic contexts or in distinct tissues is an excellent strategy to broaden the relevance of a metastasis study.

The metastatic process consists of a number of steps: local invasion and intravasation, transport through the circulation, extravasation, formation of a micrometastasis, and, finally, development of overt metastatic tumors (Faltas 2012). Some of these steps, especially at the beginning and at the end of the cascade, appear to be more challenging for tumor cells to accomplish (Chambers et al. 2000). Intuitively, a number of distinct changes must occur within tumor cells to enable them to complete the metastatic process. In support of this view, a number of genes have been shown to modify the metastatic behavior of primary cancers. Many of these genes have been validated in vivo by demonstrating that in complex genetically altered mice known to develop metastatic cancer, the loss of expression of a metastatic modifier decreases penetrance of a metastatic phenotype (recent examples include urokinase [Almholt et al. 2005], caveolin 1 [Williams et al. 2004, 2005], flotillin-2 [Berger et al. 2012], tetraspanin CD151 [Copeland et al. 2012], *Elf5* [Chakrabarti et al. 2012], macrophage inhibitory cytokine-1 [Husaini et al. 2012], breast cancer metastasis suppressor 1 [Cook et al. 2012], *KiSS1* [Cho et al. 2012], and *Nm22* [Marino et al. 2012]). On the other hand, there is evidence suggesting that both dissemination and colonization at the ectopic site can be determined by the same genes that drive growth of the primary tumor (Husemann et al. 2008; Podsypanina et al. 2008a; Rhim et al. 2012).

Determination of the Therapeutic Value of Metastasis-Related Genes Discovered in Mouse Models

One possible prediction from the multistep model for the establishment of metastatic disease is that the additional mutations acquired during the metastatic process would supercede the cells' continued

requirement for the initial oncogenic driver mutation that was necessary at the primary site of disease. However, this appears to not be the case, as several examples show an exquisite sensitivity in both the primary lesion and distant metastatic lesions to the continued activity of the initiating oncogenic mutation. Although the published record of this dependency in experimental models is still short (Moody et al. 2002; Gunther et al. 2003; Shachaf et al. 2004; Dankort et al. 2009), it faithfully recapitulates clinical situations where effective targeted therapies are used to treat patients with metastatic cancer (Stahel et al. 2003; Hotta et al. 2004; Schneeweiss et al. 2004; Flaherty et al. 2010). These observations argue that metastasis-promoting mutations are not, in many instances, capable of protecting the metastatic lesions from therapeutics that inhibit the primary oncogenic driver. However, this observation does not exclude the importance of testing whether inhibition of metastatic modifier genes might effectively block tumor relapse at metastatic sites and could therefore represent potential therapeutic targets against advanced cancers.

Lineage Tracing in Metastatic Models

The inefficiency of the metastatic process and lack of putative metastasis-specific markers make it challenging to trace the origin of metastatic cells. No clonal lineage-tracing experiments have been performed to date to trace the fate of individual metastasis-initiating cells in transgenic mice, although many studies have investigated the tumor-initiating potential of the specific cell subsets from transplantable tumors (for reviews, see Charafe-Jauffret et al. 2009; Asselin-Labat and Filby 2012; Hynes and Kelly 2012). The quantitative lineage-tracing strategies that have proven successful in resolving cell fates in normal epithelial tissues either using tissue-specific or stem-cell-specific transgenes (Barker et al. 2007; Clayton et al. 2007; Doupe et al. 2010; Lopez-Garcia et al. 2010; Snippert et al. 2010; de Visser et al. 2012; Mascré et al. 2012) or retroviral transduction (Mackenzie 1997; Ghazizadeh and Taichman 2001) are only starting to be used to follow primary tumor cells (reviewed in Alcolea and Jones 2013). Although marking tumor cells in their native environment offers certain advantages over tumor cell tracing in transplantation assays, the propensity of recombination-based approaches to activate double-strand break repair checkpoints (Zhu et al. 2012, 2013) may complicate the interpretation of data from cancer cells that have dysregulated these pathways.

The first cancer cell lineage-tracing reports focus on defining clonal architecture and clonal heterogeneity in two types of mouse tumors. To study clonal expansion in a colon adenoma model, Hans Clevers' group marked APC-deficient descendants of normal Lgr5⁺ colon stem cells (Barker et al. 2007) by means of retracing provided in a R26-confetti reporter line (Snippert et al. 2010). Under these conditions, single Lgr5⁺ adenoma cells appear to continuously contribute to adenoma growth at least up to 24 d after the concurrent activation of transformation and labeling and to give rise to multiple cell types in the adenoma (Schepers et al. 2012). If Lgr5⁺ cells and their long-lived progeny are retained upon progression to colon carcinoma, then a similar marking strategy could be used to evaluate the contribution of individual clones to colon cancer metastasis. In skin papillomas, which were labeled at clonal density using conditional keratin 14–restricted Cre expression (Vasioukhin et al. 1999), Cedric Blanpain's group showed that cells at different stages of differentiation contribute to tumor formation, mature for only a few divisions and primitive for multiple cell divisions, resulting in a large number of uniformly small clones at early time point, day 9 postlabeling. Interestingly, these small size clones were virtually absent in squamous cell carcinomas of the skin (SCC) 9 d after a similar labeling protocol, where instead heterogeneity in clone size was observed (Driessens et al. 2012). Whether the clone size heterogeneity reflects a range of tumor-propagating abilities in individual cells dividing at the same rate or, conversely, a range of cell-cycle times in cells with similar tumor-propagating activity remains to be determined. In either case, once traced tumor cells can be followed for longer time periods, it may become possible to determine the timing and the frequency with which the marked cells can give rise to metastatic SCC. There is also an emergent strategy aimed at combining cell-separation approaches with lentiviral tracing of individual tumor-cell subsets. Early evidence from Jeff Rosen's group suggests that it may be possible to study both

clonality and propagation kinetics of barcodes in the tumor outgrowth by combining antibody-based flow sort analysis with lentiviral-borne 60mer genetic barcodes (Chang and Rosen 2012).

Circulating Tumor Cells in Genetically Modified Models of Metastasis

An early tumor graft study in mice showed that circulating tumor cells (CTCs) can spread from solid tumors (Liotta et al. 1974). However, isolation of CTCs from mouse blood remains a challenge, perhaps because of an extremely low number of tumor cells in the circulation, or perhaps because of lack of sufficiently sensitive detection methods. Clinically approved CTC platforms developed for the concentration of rare tumor cells from human blood rely on human-specific antigen expression (Kling 2012). Many experimental platforms are also antigen-dependent (Shah et al. 2012; Yu et al. 2013). Although there are reports of isolation of mouse tumor cells by marker expression (e.g., anti-EpCAM antibodies were used to isolate mouse breast 4T1 tumor cells spiked into plasma-depleted blood [Shi et al. 2013], and FVB C3(1)-SV40 T-antigen mammary tumor cells were captured using a microfluidic-micromagnetic cell separation device [Kang et al. 2012]), variations between human and mouse antigens may compromise the analysis of mouse circulating cells. A label-free ISET (Isolation by Size of Epithelial Tumor cells) CTC technology is well suited for working with mouse models, as it is compatible with processing small sample volumes, is not restricted by tumor cell of origin, and allows the filtering of fixed as well as live cells, which is an advantage for study of metastasis-related factors in CTCs (Paterlini-Brechot 2011; Yu et al. 2012). As a putative marker of stem-cell capacity, at least in the mouse breast (Machado et al. 2013), cell size itself may be a promising parameter for the detection of the clinically relevant metastasis-initiating cells. The difference in size among tumor cells is sometimes a concern for this technology (Maheswaran and Haber 2010), but tumor cells in genetically modified models are perhaps best suited for size-based isolation, because their size is often uniform as a result of activation of an identical transformation program.

In the absence of effective methods of CTC isolation from live animals, earlier reports focused on quantification of disseminated tumor cells isolated after cardiac puncture or digestion of peripheral organs. For example, Carlos Arteaga's group cultivated disseminated cells from *MMTV-PyMT* transgenic mice (Muraoka et al. 2003; Biswas et al. 2007) using established protocols (Wyckoff et al. 2000). Another group reported using lungs from mice with PyMT-induced mammary tumors for harvest and RNA profiling of disseminated tumor cells (Franci et al. 2013). In the *TRAMP* metastatic prostate cancer model, cells isolated from blood after cardiac puncture were used to produce experimental metastases in immunodeficient recipients (Carvalho et al. 2013). Isolation of circulating cells from the blood or peripheral tissues may also be used to study the early spread of metastasis-initiating cells in genetically modified models. To date, several examples document the early spread of cancer in transgenic mice. For example, Christoph Klein's group detected cytokeratin-positive cells in the bone marrow of young *MMTV-PyMT* and *MMTV-Neu* transgenic mice, but not in the wild-type controls (Husemann et al. 2008). Another group used dissociated lung preparations to show the presence of colony-forming mammary cells in the lungs of very young (26-d-old) *MMTV-PyMT* transgenic mice (Weng et al. 2012). Finally, a YFP reporter was used to detect circulating pancreatic cells in the blood of tumor-free mice, in which pancreas-specific expression of Cre was used to delete *Trp53*, activate expression of mutant *Kras*, and induce a fluorescent reporter (Rhim et al. 2012). Tumor-specific fluorescent reporters will facilitate the development of continuous monitoring strategies for CTC levels in transgenic mice required to investigate early or late tumor-cell dissemination.

In summary, the current strategies that allow the sampling of genetic events that may be required for metastatic properties include both forward and reverse genetic approaches. The former are aimed at molecular characterization of the putative somatic alterations acquired with progression to metastasis, for example, by identifying the genotype or the transcriptional profile of tumor cells isolated from colonized organs or from the systemic circulation (reviewed in Paterlini-Brechot and Benali 2007; Yu et al. 2011). The latter are aimed at evaluation of a putative metastatic role of candidate molecules or signaling programs in genetically defined models—for example, using crossbreeding with animals that carry candidate alterations or using lentiviral delivery of candidate cDNAs

or shRNAs to tumor cells, often concomitant with permanent marking of the transduced cells (reviewed in Nguyen et al. 2009; Bos et al. 2010).

Doxycycline-Regulated Oncogenes for Metastasis Studies

The main advantage afforded by regulated expression of potent oncogenes is the ability to control the timing of tumor development, and thus to allow better kinetic control of tumor dissemination and/or colonization. For an in depth description of the tetracycline-inducible model, see Introduction: **Tetracycline-Regulated Mouse Models of Cancer** (Yeh et al. 2014). Our laboratory uses the reverse tetracycline transactivator (rtTA) configuration for temporal control of oncogene expression during different steps of the metastatic cascade (Podsypanina et al. 2008a,b). To do so, we first dissociate the solid tissue (normal or a primary tumor) into a single-cell suspension; see Protocol: **Tissue Dissociation for Metastasis Studies** (Kabeer and Podsypanina 2014).

We also use the inducible system to study oncogene cooperation in metastatic progression. Specifically, we use bicistronic retroviruses expressing the tetracycline transactivator (tTA) and the candidate gene (MIT-gene) or the tTA alone (MIT-Rx) (Beverly and Capobianco 2003) to infect primary mammary cells prepared from mice harboring doxycycline-inducible transgene(s). This system allows for constitutive expression of the candidate gene from the provirus and tTA-dependent expression of the inducible transgenic oncogene(s). Following infection of primary mammary cells *ex vivo* (see Protocol: **Murine Stem Cell–Based Retrovirus Production for Marking Primary Mouse Mammary Cells for Metastasis Studies** [Beverly and Podsypanina 2014]), cells are injected into the tail vein of syngeneic or immunodeficient recipients, and metastatic colonization is measured 3–6 wk after cell transfer. Cell marking with lentiviral vectors can be enhanced by lineage restriction using flow cytometry–based sorting of specific cell subsets in hierarchically organized tissues, as we and others (Chang and Rosen 2012) do in the mammary gland (see Protocol: **Enrichment of Mammary Basal and Luminal Cells for Cell-of-Origin Metastasis Studies** [Kabeer et al. 2014]). Such lineage restriction is helpful in addressing the cell-of-origin debate in metastasis research.

To measure the efficiency of metastatic colonization in the lung, we use intravital bioluminescence imaging, whole-organ bioluminescence imaging, and standard histopathological techniques, such as hematoxylin/eosin or immunohistochemical staining. A more recent approach is to enumerate tumor cells circulating in the blood of tumor- or metastasis-bearing animals. In the absence of specific markers for mammary cells, models with genetic marking of tumor cells give the best experimental advantage, but the low volume of peripheral blood that can be obtained from live animals limits the application of this technique to postmortem studies. We are looking forward to developing techniques that will allow us to monitor the kinetics of tumor burden in mouse models during anticancer treatments, as is being done for human patients (Maheswaran et al. 2008; Pachmann et al. 2008; Stott et al. 2010; Dawson et al. 2013; Hofman et al. 2013). Meanwhile, isolation of tumor cells from a colonizing tissue (e.g., the lung or bone [Husemann et al. 2008; Weng et al. 2012] [see Protocol: **Tissue Dissociation for Metastasis Studies** (Kabeer and Podsypanina 2016)]) can be an acceptable surrogate for measurement of colonization efficiency as well as for subsequent functional analysis of tumor cells.

In principle, the accompanying protocols cover the entire methodological approach to modeling metastasis in mice, whether one aims to begin with benign, transformed, or already metastatic cells (Fig. 1). Although we use these procedures to study metastatic colonization (i.e., using experimental metastasis-based approaches), these procedures are universal and can be applied to studies of spontaneous metastasis and invasion. In contrast, the extent of metastatic burden, although being the end result of each study, is often measured by methods unique to the study system (i.e., bioluminescence imaging, small-animal magnetic resonance imaging, fluorescence imaging, and histopathological or molecular analysis) and must be selected based on individual applications. In addition to the associated protocols that describe techniques to study metastasis in breast cancer models (see Protocol: **Tissue Dissociation for Metastasis Studies** [Kabeer and Podsypanina 2014], Protocol: **Murine Stem Cell–Based Retrovirus Production for Marking Primary Mouse Mammary Cells for Metastasis**

FIGURE 1. A schematic overview of the major steps in modeling metastasis. Steps shaded in gray are described in Protocol: **Tissue Dissociation for Metastasis Studies** (Kabeer and Podsypanina 2014), the step shaded in green is described in Protocol: **Murine Stem Cell–Based Retrovirus Production for Marking Primary Mouse Mammary Cells for Metastasis Studies** (Beverly and Podsypanina 2014), and steps shaded in blue are described in Protocol: **Enrichment of Mammary Basal and Luminal Cells for Cell-of-Origin Metastasis Studies** (Kabeer et al. 2014)

Studies [Beverly and Podsypanina 2014], and Protocol: **Enrichment of Mammary Basal and Luminal Cells for Cell-of-Origin Metastasis Studies** [Kabeer et al. 2014]), two additional protocols, Protocol: **Isolating Epithelial and Epithelial-to-Mesenchymal Populations from Primary Tumors by Fluorescence-Activated Cell Sorting** (Aiello et al. 2014a) and Protocol: **Orthotopic Injection of Pancreatic Cancer Cells** (Aiello et al. 2014b), highlight procedures to study cell lineages in pancreatic cancer models and to orthotopically inject tumor cells directly into the pancreas.

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Methods to Study Metastasis in Genetically Modified Mice

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