Conditional Mouse Models of Metastatic Breast Cancer

Jos Jonkers and Karin E. De Visser

Background

Breast cancer is the most common malignancy among women worldwide, affecting 12% of the Western female population and resulting in ~11,000 new cases and more than 3300 deaths annually in the Netherlands. The occurrence of distant metastases accounts for over 90% of breast cancer deaths and is currently the most common cause of death for women aged between 35 and 50 years. Patients with breast cancer are at risk of developing manifest metastases for their entire lifetime. Prevention of metastasis formation entails surgical removal of the primary breast tumor, sometimes combined with local radiotherapy, and systemic (neo)adjuvant hormonal therapy or chemotherapy. Despite these local and systemic therapies, approximately 30% of breast cancer patients will relapse and ultimately die of metastatic breast cancer. Once overt metastatic disease is diagnosed, patients are treated with chemotherapy in a palliative setting. Unresponsiveness of distant metastases to conventional forms of chemotherapy is the central unsolved problem in cancer therapy. There is an urgent need for adjuvant therapies that prevent systemic spread of cancer cells and eradicate disseminated cancer cells and established metastases.

Discussion

Breast Cancer Metastasis Formation

Metastasis formation starts with loss of contacts between cancer cells in the primary tumor, after which cells migrate away from the primary tumor, enter the blood stream or lymphatic system, extravasate from the circulation in distant organs and colonize distant organs. Disseminated breast cancer cells harbor a tremendous survival strategy, as overt metastasis formation frequently occurs more than 5 years after removal of the primary breast tumor. Patients without any clinical signs of metastases frequently have circulating tumor cells (CTCs) in peripheral blood and/or disseminated tumor cells (DTCs) in bone marrow, and their presence is associated with poor prognosis (2, 3). The mechanisms underlying long-term survival of CTCs and DTCs and signals that activate these cells from their dormant state are largely unknown.

In order to obtain insights into the metastatic cascade, much effort has been put into genetic and molecular analysis of paired samples from human primary breast tumors, CTCs, DTCs and manifest metastases (4–6). These analyses have led to two different models of metastatic progression, e.g. the ‘linear progression’ model in which a primary tumor fully develops before cancer cells disseminate and metastasis formation takes place, and the ‘parallel progression’ model in which cancer cells disseminate early before the primary tumor has acquired the
Animal Models of Metastasis

full malignant phenotype; however, neither model is supported by irrefutable evidence. Global gene expression profiling revealed that human primary breast cancers are strikingly similar to distant metastases of the same patient. On the other hand, most studies using genetic analyses have reported a high degree of discordance between matched pairs of primary tumors and metastases of the same patient (6, 8–10). Two recent studies have utilized massive parallel sequencing technologies to analyze the genomic features of paired primary breast tumors and metastases and revealed differential mutation frequencies and structural variation patterns in metastases compared with the matched primary tumors (11–13). Heterogeneity between primary breast cancers and their metastases has also been observed at the single protein level. For example, Erbb2 gain has been found in disseminated cancer cells from Erbb2 negative primary tumors. Together, these data suggest that significant evolution can occur during disease progression and may underlie the poor responsiveness of metastases to therapy, discouraging simple extrapolations from local to systemic disease.

Despite the discordance between matched primary tumors and metastases, primary breast tumors do harbor molecular traits that can be utilized to predict whether the tumor is likely to metastasize. Substantial efforts have been put into the identification of prognostic markers that characterize breast cancer patients at risk for metastasis development. DNA microarray technologies have been very useful to find gene expression profiles that distinguish primary breast tumors that will develop metastases from those that will remain localized (15, 16). At the Netherlands Cancer Institute, Van ’t Veer and colleagues have established a 70-gene profile from primary breast tumors that predicted the likelihood of distant metastases in young breast cancer patients with lymph-node-negative tumors (15). These profiles are useful to discriminate between breast cancer patients with a low risk profile, who do not have to undergo unnecessary treatment with toxic chemotherapy, and patients with a poor prognosis profile, who should be treated with more aggressive therapy. However, the ability to identify patients with a high metastatic risk profile does not guarantee prevention of metastases, as current treatment regimens fail to fully eradicate disseminated cancer cells and micrometastases.

Tumor Microenvironment and Breast Cancer Metastasis Formation

Establishment of metastases in distant organs involves reciprocal interactions between disseminated cancer cells and a foreign microenvironment. Microenvironments consist of extracellular matrix and normal host cells such as infiltrating innate and adaptive immune cells, fibroblasts, endothelial cells and adipocytes. Products of these so-called stromal cells include growth factors, cytokines, chemokines, reactive oxygen species and proteases. Tumor microenvironments are distinct from normal tissues. Differences include the presence of hypoxia that drives recruitment of certain immune cells, accumulation of chronically activated innate and adaptive immune cells, activation of angiogenesis, activation of carcinoma-associated-fibroblasts, low pH, low glucose concentrations, alterations in extracellular matrix proteins and liberation of previously bound growth factors by proteases. The composition of the microenvironment of a distant metastasis is largely dependent on the organ site in which metastasis outgrowth occurs.

Although it is generally accepted that the microenvironment of a distant organ influences metastasis outgrowth, not much is known about the exact nature of the microenvironment of
metastases and underlying mechanisms by which the microenvironment of a particular distant organ facilitates metastasis outgrowth. More data is available about the microenvironment of primary tumors, and -interestingly- certain traits of the microenvironment of primary tumors have been associated with increased dissemination and metastasis formation. Strong indications for a metastases-promoting role of the microenvironment come from a large number of studies in breast cancer patients reporting associations between the presence of abundant intratumoral macrophage infiltrations and unfavorable clinical prognosis (17–21). Also increased levels of various types of matrix metalloproteinases (enzymes that remodel the extracellular matrix) in primary breast tumors have been linked with poor prognosis (22, 23), and absence of expression of caveolin-1 in stroma of primary breast cancers has been linked with early tumor recurrence, metastasis formation and poor clinical outcome (24). Poor-prognosis gene signatures of primary breast tumors that predict increased risk for metastasis formation also often contain genes related to the immune system, angiogenesis or remodeling of the extracellular matrix (15, 16), underlining the influence of the tumor microenvironment on metastasis formation. Likewise, primary breast cancers with an activated wound healing gene-expression signature, representing genes involved in hemostasis, migration, inflammation, ECM remodeling and angiogenesis, were more likely to metastasize than tumors that did not express the wound healing signature (25, 26). Together, these data indicate that the ability of a primary breast cancer to metastasize is not only dependent on cancer-cell intrinsic processes, but is also clearly influenced by processes that are orchestrated by the tumor microenvironment.

Conventional GEMMs of Metastatic Breast Cancer

Several conventional GEMMs of breast cancer have been used to investigate cell-intrinsic and extrinsic mechanisms of metastasis formation. Although some of these transgenic models develop metastatic mammary tumors with short latency and high penetrance, they also have their limitations, as tumor formation is driven by organ-wide overexpression of extremely potent oncoproteins, which is not reflecting the human situation. Consequently, primary mammary tumor development in these models is extremely fast and aggressive and often occurs in several or all glands, thus limiting their utility as models for spontaneous breast cancer metastasis.

The MMTV-PyMT model. The Polyoma middle T-antigen (PyMT) oncoprotein has the ability to convert established cell lines to an oncogenic state. PyMT is a membrane bound polypeptide that can be regarded as a constitutively active analogue of a receptor that harbors docking sites for a number of effector proteins used by tyrosine kinase receptors to stimulate mitogenesis (27). MMTV-PyMT transgenic mice develop multifocal adenocarcinomas with a short median latency and frequent formation of metastasis in lungs and lymph nodes (28).

The MMTV-PyMT model has been used extensively to delineate the role of the innate and adaptive immune system in breast cancer metastasis formation. These studies have shown that genetic elimination of colony stimulating factor-1 in the MMTV-PyMT mouse model inhibited macrophage recruitment and macrophage-derived EGF production in mammary tissue resulting in delayed development of invasive mammary carcinomas and arrested pulmonary metastasis formation (21, 29, 30). A recent study showed that EGF production by macrophages and subsequent metastasis formation in MMTV-PyMT mice was dependent on IL-4 expressing...
CD4+ T cells (31), indicating that the adaptive immune system can stimulate metastasis formation through polarization of innate immune cells. Together, these data suggest that macrophages play a major role in facilitating late-stage metastatic progression of breast tumors.

**The MMTV-NeuT model.** The EGF-family type receptor tyrosine kinase (RTK) ErbB2 (aka Her2 or Neu) has a long track record of clinical interest because of its overexpression in many breast tumors. Her2 overexpression is used as a strong prognostic indicator, a predictor of metastasis and a target for treatment (32). ERBB2 is amplified in approximately 15 to 30% of all breast cancers (33), especially in tumors from patients with lymph node metastases (34). Transgenic mice that have been engineered to express wild type and mutant forms of ErbB2 under control of the mouse mammary tumor virus (MMTV) promoter, show formation of multifocal adenocarcinomas that metastasize to lung (28, 35, 36).

Also the MMTV-NeuT model has been used to study the role of the adaptive immune system in breast cancer metastasis formation (37). In contrast to the MMTV-PyMT model, spontaneous HER2 driven mammary tumorigenesis and metastasis formation are neither suppressed or altered by immunosurveillance mechanisms, nor promoted by the adaptive immune system, suggesting that the impact of the immune system on tumor metastasis may differ between breast cancer subtypes (37).

**Regulatable GEMMs of Metastatic Breast Cancer**

An important question is whether an oncogene or tumor suppressor that is crucial for initial tumor development is also required for tumor maintenance. Tumors that are “addicted” to a specific genetic lesion might be effectively treated by genetic or pharmacological neutralization of this lesion, e.g. by inhibition of oncoprotein activity or restoration of tumor suppressor activity (38). This question has been successfully addressed using GEMMs with doxycycline- or tamoxifen-inducible gene expression (39), which permits assessment of the requirement for sustained oncogene overexpression for maintenance of primary tumors and metastases. An elegant example is a conditional mouse mammary tumor model with doxycycline-inducible, mammary epithelium-specific expression of activated ErbB2 (40). Interestingly, addiction to activated ErbB2 is maintained during tumor progression and metastasis, since both primary mammary carcinomas and lung metastases rapidly and fully regress following transgene deinduction by doxycycline withdrawal. However, ErbB2-independent tumors recurred in these animals over time.

**Conditional Mouse Models for Invasive and Metastatic Lobular Breast Cancer**

Invasive lobular breast carcinoma is the second most common human breast malignancy, representing 10–15% of invasive breast carcinomas. Invasive lobular breast carcinomas are typically characterized by loss of E-cadherin expression, a critical cell-cell adhesion molecule involved in maintaining epithelial integrity and homeostasis (41, 42). Loss of E-cadherin is an indicator of poor prognosis and is associated with metastasis formation in multiple distant organs, in particular peritoneum, gastrointestinal tract, ovaries, lungs and bone marrow (43, 44). The estimated 10-year survival rate of patients with invasive lobular carcinomas is 35–50%. We have developed a conditional mouse mammary tumor model based on epithelium-specific
deletion of p53 and E-cadherin \((K14\text{cre};\text{Ecad}^{F/F};\text{p53}^{F/F})\) mice. These mice develop invasive carcinomas that resemble human invasive lobular carcinomas \((45)\). This model was generated by introduction of \(\text{LoxP}\) elements in the mouse genes for E-cadherin and p53 and combining this with cytokeratin 14-driven Cre recombinase \((K14\text{cre})\) expression. \(K14\text{cre};\text{Ecad}^{F/F};\text{p53}^{F/F}\) female mice develop breast tumors and/or skin tumors with a median latency of approximately 200 days (Fig. 1A). Like human invasive lobular carcinomas, mammary tumors arising in \(K14\text{cre};\text{Ecad}^{F/F};\text{p53}^{F/F}\) mice are characterized by cancer cells that abundantly infiltrate dense connective fibrous stroma as individual rows, so called Indian files (Fig. 1B). Approximately 50% of all tumor-bearing \(K14\text{cre};\text{Ecad}^{F/F};\text{p53}^{F/F}\) mice develop micrometastasis in distant organs including lung, lymph nodes, liver, gastro-intestinal and urogenital tract, and pancreas (Fig. 1C) \((45)\), thus mimicking the metastatic pattern of human invasive lobular carcinoma.
A critical barrier against tumor metastasis is anoikis: cell death induced by loss of cell-cell or cell-matrix interactions. This process is thought to ensure homeostasis of epithelial tissues, for example during post-lactational mammary gland involution. In order for tumor cells to survive in the blood stream or in lymph vessels, they have to somehow become resistant to detachment-induced apoptosis, a.k.a. anoikis. Intriguingly, invasive lobular carcinoma cells derived from tumors arising in \(K14\text{cre};Ecad^{FF};p53^{FF}\) mice show intrinsic resistance to anoikis, suggesting that these \(Ecad^{Δ/Δ};p53^{Δ/Δ}\) tumor cells have intrinsic capacity to survive in circulation (45).

**Mammary Gland Reconstitution Models to Assess the Contribution of Cell-Intrinsic and -Extrinsic Factors to Mammary Tumorigenesis and Metastasis Formation**

We envisioned that the genetic complexity of the \(K14\text{cre};Ecad^{FF};p53^{FF}\) mouse model would hamper our ability to easily test the functional significance of cancer cell-intrinsic and -extrinsic processes via tedious intercrossing with various knock out mice. We have therefore modified the current model to increase our ability to manipulate stromal compartments independent of the epithelial compartment, and vice versa, to more accurately study complex host-tumor interactions. We have developed a flexible transplantation-based model for human breast cancer using primary mouse mammary epithelial cells (MMECs) with conditional alleles for p53 and E-cadherin (47). Primary MMECs were harvested from young \(K14\text{cre};Ecad^{FF};p53^{FF}\) mice (Fig. 2). After short-term culturing, these cells were transplanted into cleared mammary fat-pads of young female wild type recipient mice. Development of the mammary gland, as well as tumor formation and progression were subsequently assessed at several time points post-transplantation. Upon transplantation, primary mammary epithelial cells from \(K14\text{cre};Ecad^{FF};p53^{FF}\) donors first developed into phenotypically normal mammary glands, without contributing to the surrounding stroma. Between 6 and 20 months after transplantation, these mice developed invasive tumors with full penetrance (47). Tumor formation in the recipients mimics tumor spectrum, morphology and immunophenotype of the original conditional tumor model. Moreover, invasive tumors derived from transplanted MMECs maintain their metastatic capacity, as 50% of tumor-bearing recipient mice did develop micrometastases in distant organs. To test whether it is possible to genetically alter MMECs with a gene of interest before transplantation, we have lentivirally transduced MMECs from wild type FVB mice with GFP and transplanted these cells into recipient mice. The resulting ductal outgrowths contained GFP+ cells, indicating that it indeed is possible to genetically modify MMECs before transplantation (47).

Unlike the spontaneous \(K14\text{cre};Ecad^{FF};p53^{FF}\) tumor model, the MMEC transplantation model permits relatively easy genetic manipulation of cancer cell-intrinsic and -extrinsic processes involved in mammary tumorigenesis, as MMECs can be manipulated before transplantation (48, 49) and the stromal compartment can be manipulated by using recipient mice with altered stromal traits (Fig. 2).
Isolate MMECs from donor mouse in vitro culturing

Infect with viruses containing Conditional oncogenes or TSG

Orthotopic Transplantations

Immune proficient Wild-type mice

Mice with altered Stromal compartment

Mammary gland Outgrowth and subsequent Tumor watch

Study influence of genetic alteration on tumorigenesis

Study influence of stromal compartment on tumorigenesis

Fig. 2. Schematic overview of MMEC transplantation model. Primary MMECs are isolated from a donor mouse that expresses Cre recombinase under control of the cytokeratin14 promoter and conditional alleles for p53 and E-cadherin. These cells can subsequently be transduced with viruses carrying conditional oncogenes or TSGs to study the effect of genetic alterations on tumorigenesis. Alternatively, the primary MMECs can be transplanted into mice with normal or modified stroma to assess the contribution of any particular stromal component to tumor development and progression.

Acknowledgements

Work in the Jonkers and De Visser labs is supported by the Dutch Cancer Society (KWF) and the Netherlands Organization for Scientific Research (NWO).

References

Animal Models of Metastasis