J Mammary Gland Biol Neoplasia
DOI 10.1007/s10911-007-9050-8

Modeling Metastatic Breast Cancer in Mice

Jos Jonkers · Patrick W. B. Derksen

Abstract Metastatic disease is the major cause of death in breast cancer patients. Patients presenting with metastases cannot be cured, and as a consequence, treatment is palliative and focuses on prolonging survival and maintaining quality of life. Numerous mouse models have been generated in which human breast cancer development and metastasis have been studied, ranging from spontaneous and carcinogen-induced models to transplantation models and genetically engineered mouse models. Here, we summarize past progress and highlight present developments in modeling breast cancer invasion and metastasis in genetically modified mice, and the impact it may have on the development of innovative anticancer therapies.

Keywords Mouse model · Breast cancer · Metastasis · Mammary gland · Transgenic

Introduction

Breast cancer is the most common malignancy among females in the Western world, resulting in approximately half a million deaths annually mainly due to metastatic disease [1]. While primary breast tumors can be treated with increasing success, many tumors are not eradicated by local treatment and systemic adjuvant therapy, and will relapse, often leading to the incurable phase of cancer, metastatic disease. Although frequently regarded as such, tumors are not a homogeneous population of immortalized cells. Recent data have shown that a primary tumor consists of several populations of malignant cells, each of which may respond differently to conventional treatment [2, 3]. Specifically, certain tumor cells that present 'stem cell like' characteristics were shown to be causal to radiotherapy resistance [4–6]. In analogy to this, approximately 40% of breast cancer patients show resistance to standard chemotherapy, resulting in tumors that will relapse and subsequently metastasize.

Metastatic disease is a complex phenomenon, in which cells have to detach from their microenvironment, counteract the resulting pro-apoptotic signals, invade surrounding stroma, enter the vasculature and colonize distant sites. These processes depend on the activation and inhibition of multiple signal transduction pathways and their targets, which are currently ill defined. Research on breast cancer metastasis has greatly profited from recent advances in modeling metastatic disease in mice, using both transplantation techniques and genetic modification [7, 8]. Due to the complex nature of the metastatic process, models that mimic the entire disease are scarce, but nevertheless emerging. This review will focus on past and present mouse models in which breast cancer invasion and metastasis are studied. We will discuss the lessons learned from these models and the usefulness of the resulting data in the identification of targets for drug development.

Human Breast Cancer

Breast cancer is a genetic disease and, consequently, its incidence increases with age. Among the numerous risk factors (e.g. late menopause, nulliparity, long term post menopausal hormonal replacement therapy, obesity and

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alcohol) the most predictive risk factor is a family history of breast cancer [9]. Approximately 5–10% of all breast cancers result from certain forms of hereditary cancer predisposition, such as Li-Fraumeni syndrome [10, 11], Cowden's disease [12], and BRCA mutation carriers [13]. All breast cancers, however, have acquired somatic genetic abnormalities, resulting in mutated or overexpressed cancer genes (e.g. TP53, MYC, BCL2, CCND1) [14]. Large-scale sequencing of breast cancer genomes is currently being performed by several groups [15, 16], but the exact number and type of mutations that are required for breast cancer initiation and metastasis in humans is still unknown.

Breast Cancer Progression and Metastasis

Breast cancer is a clinically heterogeneous disease. Aggressive breast cancers not only show multiple morphological phenotypes, the rate and onset at which metastasis occurs also varies considerably. For example, aggressive disease with distant metastases within 3 years, as well as manifestation of distant metastases more than 10 years after initial diagnosis are not uncommon [17]. Sites of metastasis may differ per subtype, but mainly comprise lung, pleura, liver and bone [18] (Fig. 1). Human pathology has defined a plethora of histological subtypes, based mainly on morphological criteria, of which ductal and lobular carcinomas represent the majority (Table 1). Although informative, the ductal and lobular classification is a poor prognostic indicator of metastatic risk [19] and can be misleading, as it implies cellular origin. To better define the basis of breast cancer heterogeneity, gene expression profiling has recently been used to identify molecular subtypes of breast cancer with distinct clinical features [20, 21]. Similarly, supervised classification of gene expression profiling data has yielded prognostic gene expression signatures that predict risk of breast cancer metastasis [22, 23]. Yet other groups have identified gene expression signatures that predict the site of metastasis [24, 25]. Tumor or metastasis-specific expression profiles can thus help to point to distinct signaling pathways that contribute to metastasis. In this way, "tailored" intervention strategies may be developed to improve the currently relatively poor clinical management in both the adjuvant and metastatic setting [9–14]. Conventional prognostic markers that predict or correlate with breast cancer metastasis are not very reliable and are mainly based on phenotypic criteria, such as tumor size, histological grade, lymph node status and (lymph)angioinvasion [26–29]. More recently, clinicians have also employed the aberrant expression of proteins (uPA/PAI1, steroid receptors, ERBB2) to predict disease outcome [28–32], or as targets for intervention strategies [33, 34].

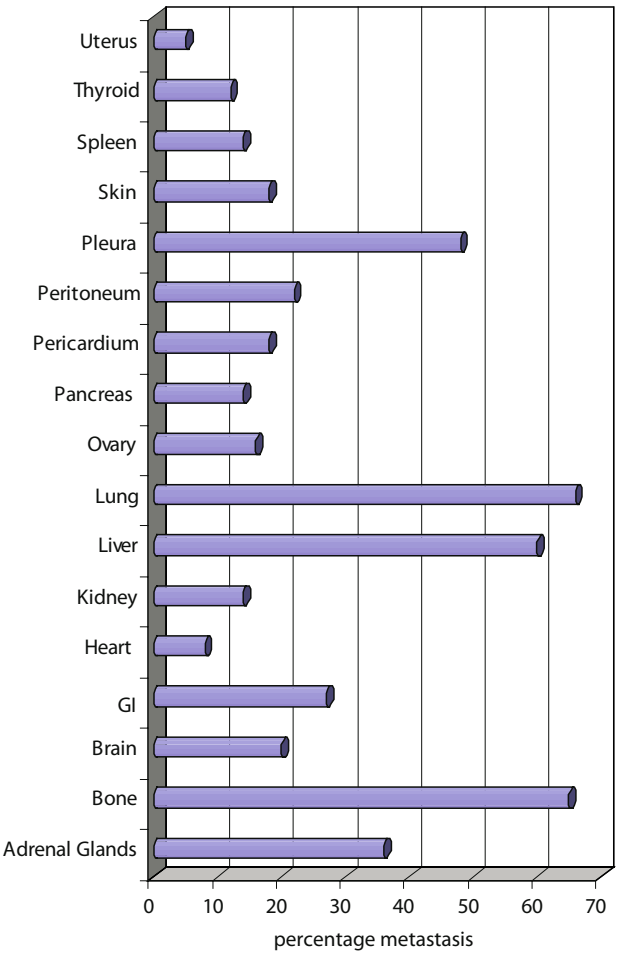

Figure 1 Dissemination sites of breast cancer metastasis at autopsy. Percentages obtained from 2,050 cases are shown. Adapted from [18].

Breast Cancer Diagnosis and Treatment

Systemic screening by means of mammography may result in early diagnosis, depending on tumor subtype. These clinical examinations have resulted in a 25–30% decrease in mortality from breast cancer, mainly in postmenopausal women [35]. Among women with breast cancer who present tumors less then 2 cm in diameter, or up to 5 cm without signs of axillary node metastasis, the 20 year recurrence-free survival is approximately 77 and 64%, respectively [27, 36]. Given the fact that predictions regarding recurrence and metastasis are difficult to make, systemic adjuvant therapy following local treatment is now widely applied to help eradicate tumor cells that might have already spread systemically [9, 37]. Currently, depending on the guidelines used, 50 to over 80% of breast cancer patients receive adjuvant therapy, even though only 25–40% of them will relapse and ultimately die of metastatic disease [38]. Hence, many women are 'over-treated' and will suffer unnecessarily from the harmful side effects of standard chemotherapy. Adjuvant chemotherapy regimens

Table 1 Histopathological subtypes of invasive breast carcinoma.

Breast cancer subtype	Frequency (%)	10–year survival (%)
Invasive ductal carcinoma (IDC)	50–80	35–50
Invasive lobular carcinoma (ILC)	5–15	35–50
Mixed	4–5	35–50
Tubular/invasive cribriform	1–6	90–100
Mucinous	<5	80–100
Medullary	1–7	50–90
Invasive papillary	<3	Unknown
Metaplastic	<5	Unknown

Adapted from [38].

often consist of repetitive administrations of combinations of drugs [39]. Frequently used combinations are CMF (cyclophosphamide, methotrexate, fluorouracil), FAC (fluorouracil, doxorubicin, cyclophosphamide), FEC (fluorouracil, epirubicin, cyclophosphamide) and AC (doxorubicin, cyclophosphamide), sometimes followed by a taxane [9]. These therapies are followed by endocrine therapy in case of hormone receptor-positive disease, and combined with the anticancer drug trastuzumab in case of *ERBB2* gene amplification [40]. Other targeted therapies against VEGF and EGFR are now in advanced stages of clinical testing. In addition to these therapies, scores of other small molecule inhibitors and biologicals that antagonize the activity of specific enzymes or protein–protein interactions are currently under development [33].

Mouse Models of Human Breast Cancer

Mouse models have made an important contribution to our understanding of breast cancer progression and metastasis. Novel, 'humanized' mouse models are at present being developed and will be pivotal to pinpoint the cardinal events that govern intrinsic cues of tumor cells and microenvironmental interactions that propel metastatic disease. Also, they will be important tools to test and validate novel treatment strategies.

Mice are Not Little Humans

The laboratory mouse has been used extensively in cancer research for a number of reasons. First, the mouse is a mammalian organism that shares many anatomic, physiological and genetic similarities with humans. Second, the mouse germ line can be easily manipulated whereby genes may be overexpressed or inactivated, even in a time or tissue-specific manner. These characteristics render mice very attractive as a study object for human cancer pathogenesis. But how alike are mice and men? Does a mouse mammary tumor truthfully reflect human breast cancer?

Mice are about 3,000 times smaller, live on average 40 times shorter and undergo approximately 100,000 fewer cell divisions in their lifetime than humans. Yet, they are as susceptible to developing cancer as humans [41]. Despite the fact that mice should experience far less genetic damage during their relatively short life span, both mice and men show a cumulative cancer incidence of approximately 30%. Thus, humans must have evolved intrinsic anti-tumor mechanisms which have allowed them to decrease cancer susceptibility [42]. Mice mainly tend to develop tumors of mesenchymal origin, whereas human age-related cancers are mostly derived from epithelial progenitor cells [43]. Apart from the obvious environmental factors, such as alcohol, diet and tobacco, there are clear biological differences between mice an men that are likely to contribute to differences in cancer risk and spectrum. One major difference comprises telomere erosion during malignant transformation of human cells. In contrast to human cells, mouse somatic cells express biologically active telomerase, an enzyme that is required for maintenance of chromosome ends [44]. Accordingly, mouse telomeres are 40 to 60 times longer than human telomeres and, consequently, mouse cells are more easily immortalized than human cells, which must undergo a second genetic hit to overcome telomere crisis [45]. Despite these discrepancies, mouse models have demonstrated their utility in cancer research and have made a substantial contribution in understanding human tumor etiology.

The Mouse Mammary Gland

In order for the stem cells in the neonatal mammary bud to develop into an adult mammary gland, progenitor cells need to propel and maintain a delicate and fine-tuned balance of multiple signaling pathways. These pathways, which include among others Wnt, Notch, EGF, and TGF signaling, are triggered to enable cells to invade stroma, proliferate, acquire anoikis resistance, undergo apoptosis, differentiate and induce angiogenesis [46, 47]. Also, stromal elements present in the mammary gland, such as adipocytes, fibroblasts, endothelial and immune cells, will intricately interact with mammary epithelium in order to successfully form a functional mammary gland [48]. These processes not just are used for mammary gland development; during pregnancy, the mammary gland undergoes similar morphological and environmental changes. An enormous response is displayed upon release of female reproductive hormones, including the induction of mammary gland formation, differentiation of cells into milk-secreting units and subsequent massive involution after parturition. It is this remarkable flexibility of mammary

epithelial cells that breast cancer cells hijack during their progression and metastasis.

Transplantation Models of Breast Cancer

Transplantation of cells derived from breast cancer has been extensively used to study several aspects of breast cancer pathology *in situ*. A major factor to be considered, however, is the site of tumor cell injection. For example, several aspects of the metastatic process (tumor–stroma interactions, detachment and local invasion, extravasation) are omitted when injecting tumor cells into a vein (IV). Injections into the left ventricle of the heart (intracardiac; IC) which excludes the direct trapping of tumor cells in the lungs, facilitate a broader dissemination spectrum and are thus successfully being using to study bone metastasis [49]. Orthotopic transplantations, and to a lesser extent ectopic transplantations, mimic additional aspects of metastasis, because cells have to form a primary tumor as well as actively trigger local invasion and extravasation, which are key aspects of metastasis.

Allograft Models

Transplantation of tumor cells into syngeneic recipient mice with identical genetic backgrounds (allografting) prevents graft versus host reactions, which is a problem when studying human cancer cells in mice (xenografting). Most allograft models do not show extensive metastatic behavior, especially to bone. Nevertheless, specific selection in vivo has resulted in cancer cell lines (e.g. 4T1.2 cells derived from a spontaneous BALB/C mammary tumor) that show enhanced metastatic potential to lungs, liver, bone and brain when injected intracardiacally or at orthotopic sites [50, 51]. Gene expression profiling of several 4T1.2 sublines with different metastatic potential has led to the identification of Twist as a key factor in breast cancer metastasis [52]. Twist may contribute to metastasis by promoting an epithelial to mesenchymal transition (EMT) characterized by loss of E-cadherin-mediated cell–cell adhesion, activation of mesenchymal markers, and induction of cell motility.

Xenograft Models

Metastasis can be also studied by transplantation of human tumor cells in mice, a technique that is restricted to immune-compromised or immune deficient animals because of the inevitable host versus graft reactions [53, 54]. As a consequence, the contribution of an intact immune system—which plays a key role in the metastatic process—cannot be studied in these xenograft models. Also, stromal components that can foster carcinoma-associated fibroblasts have been shown to activate and maintain SDF1-mediated signaling and subsequent induction of angiogenesis [55, 56]. Despite these limitations, several studies have successfully utilized a human cell line, MDA-MB-231, in the search for factors that regulate metastasis. This cell line is derived from pleural effusion fluids of a breast cancer patient, and displays colonization of multiple target organs, such as liver, lung, brain, adrenal glands and bone upon orthotopic and subcutaneous transplantation [57, 58]. Massagué and colleagues have successfully used in vivo selection of MDA-MB-231 cells in nude mice to identify and validate genes that control metastasis to bone and lung [24, 25]. Bone metastatic subpopulations of MDA-MB-231 cells were selected by several rounds of intracardiac injections and isolation of metastatic nodules in the bones of the injected mice [25]. Lung metastatic variant sublines of MDA-MB-231 were selected by repeated tail vein injections and isolation of lung lesions [24]. Subsequent gene expression profiling of parental MDA-MB-231 cells and metastatic variants yielded sets of genes that promote metastasis to bone or lung. Bone metastasis of MDA-MB-231 cells appears to be mediated by a limited number of genes, including the angiogenesis factors *FGF5* and *CTGF*, the activator of osteoclast differentiation *IL11*, the bone matrix-degrading metalloproteinase *MMP1* and the bone-homing chemokine receptor *CXCR4* [25]. CXCR4, which is highly expressed in human breast cancer cells, is an important determinant for organ-specific metastasis because CXCR4-positive tumor cells are actively recruited by SDF1-expressing metastatic target organs [59]. Gene expression profiling of lung metastatic MDA-MB-231 variants yielded a set of 54 genes including—besides *CXCL1*, *MMP1* and *MMP2*—the EGF family member *EREG*, the cell adhesion molecule *SPARC*, the cell adhesion receptor *VCAM1*, the interleukin receptor *IL13RA2*, the inhibitor of cell differentiation *ID1* and the cyclooxygenase *COX2* [24]. Functional validation of these genes showed that some (e.g. *ID1*) serve dual functions in both the primary tumor and in the lung microenvironment, whereas others (e.g. *IL13RA2*, *SPARC* and *VCAM1*) contribute only to lung metastasis formation.

Xenografting in Humanized Mice

Obviously, caution should be taken when transplanting human tumor cells into mice, as human cells are not fully adapted to the mouse microenvironment. Indeed, attempts to recapitulate human breast epithelial morphogenesis by introducing human MECs into cleared mammary fat pads of mice have been unsuccessful. To circumvent this limitation, Kuperwasser and coworkers have developed an orthotopic xenograft model in which both the stromal and epithelial components of the reconstituted mammary gland

are of human origin [60]. To this end, mouse mammary fat pads were "humanized" by introducing human breast fibroblasts into cleared mouse glands, and at a later time point engrafting human breast epithelial and stromal cells into the humanized fat pads. This system allows for full developmental and functional outgrowth of human breast ducts and lobules and permits orthotopic grafting of primary human breast cancer tissue and cells. Another strategy to produce humanized mouse xenograft models of breast cancer metastasis involves injection of human breast cancer cells into immunodeficient NOD/SCID mice carrying human bone grafts [61]. Of 13 breast cancer cell lines tested, only SUM1315 cells formed lung and bone metastases. Importantly, bone metastasis was to the human implant and not to the mouse skeleton, suggesting species-specific homing characteristics.

Conventional Transgenic Mouse Models of Breast Cancer

Historically, mammary gland-specific overexpression of oncogenes has been the primary means to study breast cancer in transgenic mice. The initial oncogenes to be discovered were genes found overexpressed through genomic amplification in human breast cancers, or genes identified as targets of mouse mammary tumor virus (MMTV) insertional mutagenesis experiments [62, 63]. Consequently, the MMTV long terminal repeat (MMTV-LTR), which contains the retroviral promoter and enhancer elements, has been widely employed to drive mammary gland-specific overexpression of a gene of interest in transgenic mice [64]. Also, other promoters, mainly derived from genes encoding mammary gland-specific (lactogenic) proteins, have been used to create a multiplicity of transgenic mouse models of breast cancer. An overview of promoters used for mammary gland-specific transgenesis is shown in Table 2. A negative aspect of many of these models is that tumors have to be induced by hormonal stimuli, triggering not only transgene expression but also developmental cues, which may affect tumor etiology. Conversely, female reproductive stimuli are also present systemically in the absence of pregnancy or lactation and can therefore induce undesired expression of transgenes, a known phenomenon when using, for example, whey acidic protein (WAP) promoter elements. Also, lymphatic dissemination and subsequent metastasis, especially to bone, are rare in conventional transgenic mouse models, which is most likely due to the rapid onset and progression of the primary neoplasm [8] (Table 3). In an ideal world, the promoters used in mouse models should not only be mammary-specific, but also hormone-independent. This requirement induces an intrinsic paradox that has prevented the development of *the* ultimate model using the aforementioned tools.

Table 2 Promoters used for mammary gland-specific expression.

Promoter element	Abbreviation	Expression	Activation	Strengths	Weaknesses	Reference
Mouse mammary tumor virus long terminal repeat	MMTV	Mammary epithelium, several other tissues	Steroid hormones	Strong promoter	Requires pregnancy Expressed in other tissues	[118]
Whey acidic protein	WAP	Mammary epithelium	Lactogenic hormones	Mammary gland-specific	Requires pregnancy	[119, 120]
Bovine β-lactoglobulin	BLG	Mammary epithelium, salivary gland	During mammary development and lactation	Mammary gland-specific	Requires pregnancy	[121]
Rat prostate steroid-binding protein	C3(1)	Prostate and mammary epithelium	Estrogen	Does not require pregnancy	Expressed in other tissues	[122]
Metallothoinin	MT	Many tissues, including mammary gland	Zinc ions	Does not require pregnancy Expression inducible with Zn^{2+}	Expressed in many other tissues	[123]
Cytokeratin 14	K14	Skin, mammary epithelium salivary gland, thymus		Does not require pregnancy	Expressed in other tissues	[124]
H19	H19	(Embryonic) mammary gland, lung, liver	Genetic imprinting, de-methylation		Expressed in other tissues	[125]

Conventional Transgenics: Polyoma Virus Middle T Antigen

The Polyoma middle T-antigen (PyMT) has the ability to convert established cell lines to an oncogenic state. Middle T antigen 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 [65]. Mammary gland-specific overexpression of PyMT using the MMTV promoter results in multifocal adenocarcinomas with a short median latency and the formation of metastasis to lungs and lymph nodes [66]. This mouse model has since been used extensively because it shares many characteristics with human breast tumors. First, tumors develop with high penetrance and show gradual loss of estrogen and progesterone receptors. Second, the multistage progression from hyperplasia to a full-blown

Table 3 Mouse models of breast cancer metastasis.

Promoter element	Transgene			Tumor Latency (days)	Site of metastasis	Reference
	1	2	3			
C3(1)	SV40 LT			180	Lung	[126]
H19	Igf2			>280	Lung, liver, spleen	[125]
MT	Met (Y1248H and M1268T)[a]			300	Lung, LN, kidney, heart	[88]
MMTV	Cox-2			210	LN	[127]
MMTV	Wnt-1			240	Lung, LN	[118]
MMTV	Wnt1	Fgf-3		120	Lung, LN	[128]
MMTV	Ron			200	Lung, liver	[92]
MMTV	ΔN β-catenin	$Trp53^{\Delta/+}$		300	Lung	[128]
MMTV	$Chk2^{D347A}$			290	Lung, Spleen	[129]
MMTV	ErbB2			200	Lung	[77]
MMTV	ErbB2	TGFβ		200	More lung metastases	[80]
MMTV	ErbB2	TGFβ (SR2F)		240	Less lung metastases	[82]
MMTV	ErbB2	S1004A		330	More lung metastases	[130]
MMTV	ErbB2 activated			120	Lung	[76]
MMTV	ErbB2 (YB and YD)			~150	Lung	[78]
MMTV	ErbB2 (YB)	TGFβR1 (AAD)		270	Lung	[131]
	ErbB2 (YB)	TGFβR2 (Δcyt)			Less extravascular	
	ErbB2 (YD)	TGFβR1 (AAD)			More extravascular	
MMTV	ErbB2 (YD)	$ItgB4^{1355T}$		150	Inhibition of metastasis	[132]
MMTV	PyMT			84–175	Lung, LN	[66]
MMTV and MMTV-rtTA	PyMT	TetO-$TGFβ1^{S223/225}$		55	More lung metastases	[81]
MMTV	PyMT	VEGF		40	Lung	[72]
MMTV	PyMT	$Irs1^{\Delta/\Delta}$		80	More lung metastases	[70]
MMTV	PyMT	$uPA^{\Delta/\Delta}$		50	More lung metastases	[69]
MMTV	PyMT	$CD44^{\Delta/\Delta}$		105	More lung metastases	[68]
MMTV	PyMT	$Plg^{\Delta/\Delta}$		50	Less lung metastases	[71]
MMTV	PyMT	$MEKK1^{\Delta/\Delta}$		90	Lung, delayed	[133]
MMTV	PyMT	$TGFβR2^{MG\Delta}$		60	More lung metastases	[83]
WAP	HGF			50	Lung	[89]
WAP	RAS			180	Lung	[134]
WAP	SV40 LT			300	Lung, LN	[135]
WAP	Notch-4			200	Lung	[136]
MMTV-rtTA	ErbB2 activated				Lung (reversible)	[107]
MMTV and WAP-rtTA[b]	Cre	$Trp53^{F5-6}$		280/330	Lung, liver	[102]
K14	Cre	$Trp53^{F2-10}$	$Cdh1^{F4-15}$	200	Lung, LN, liver, GI tract, peritoneum, pancreas	[103]

[a] Only one animal was tested for each mutation.
[b] Tet–responsiveness was not functional in this promoter.

malignancy is represented in MMTV-PyMT mice. Finally, metastatic potential appears to be independent of hormonal fluctuations with a reproducible progression rate [67]. Several labs have employed the MMTV-PyMT model to define and substantiate a role for genes that have been implicated in tumor progression and metastasis, such as CD44 [68], uPA (69), Irs1[70] and Plg [71] (Table 3). Also, overexpression of the blood vessel angiogenic factor VEGF-A in MMTV-PyMT mice resulted in accelerated formation of lung metastasis, not only by promoting tumor angiogenesis but also by sustaining tumor proliferation and survival [72].

Conventional Transgenics: Receptor Tyrosine Kinases

The 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. Hence it is being used as a strong prognostic indicator, predictor of metastasis and a target for treatment [73]. *ERBB2* is amplified in approximately 15 to 30% of all breast cancers [30], especially tumors from patients with lymph node metastases [74]. ErbB2 is an EGF family-type RTK, which normally regulates mammary growth and differentiation. Tissue culture experiments have shown that overexpression leads to transformation and invasion in the absence of ligand [75]. Transgenic mice that have been engineered to express wild type and mutant forms of ErbB2 under the control of MMTV promoter, show formation of multifocal adenocarcinomas that metastasize to lung [76–78].

Like the MMTV-PyMT model, the MMTV-ErbB2 mouse has formed the basis for many experiments to investigate cooperating events in breast cancer. A potent inducer of invasiveness is TGFβ, a secreted cytokine that exerts its activity by binding to distinct serine/threonine kinase receptors. Interaction of TGFβ with its receptor can have a dual outcome; it can suppress initial tumorigenesis, but conversely, can also stimulate invasion and metastasis, which is accompanied by an epithelial to mesenchymal transition (EMT) and proangiogenic and immune suppressive effects on the tumor microenvironment [79]. Furthermore, it has been shown to play a role as an important mediator of bone metastasis by increasing the expression of tissue-specific cytokines [25]. Expression of TGFβ in MMTV-ErbB2 mice caused more circulating tumor cells and lung metastases than ErbB2 mice alone. Furthermore, MMTV-ErbB2;MMTV-TGFβ1 tumors contained higher levels of active Smad2, Rac1, Pkb/Akt, MAPK and p38 [80]. Interestingly, primary tumor burden was unaltered regardless of TGFβ1 signaling. Inducible expression of TGFβ in the MMTV-PyMT mouse model of metastatic breast cancer also corroborated these findings [81]. In vivo inhibition by expressing either TGFβ antagonist SR2F, TGFβ receptor type I (TGFβR1), or dominant negative TGFβRII, reduced the number of lung metastases, thus substantiating the pro-metastatic functions of TGFβ1 signaling in MMTV-ErbB2 mice [82]. Somewhat intriguing is the finding that conditional ablation of TGFβRII in the MMTV-PyMT mouse resulted in an increased number of metastatic lung foci [83].

Hyperactivation of hepatocyte growth factor (HGF) and its receptor MET can cause transformation of cells, leading to the initiation or progression of malignancy. Under these conditions, MET can disturb the subtle balance between growth and apoptosis, and induce unrestricted growth and motility, accounting for cellular transformation, invasion and metastasis [84]. Although activating mutations or genomic amplification have not been reported in human breast cancer, Met amplification is frequently observed in BRCA1- and p53-deficient mouse tumors [85]. Moreover, overexpression of MET has been found to predict metastasis and survival in early-stage breast cancer [86]. Zinc-inducible overexpression of HGF in MT-HGF mice resulted in diverse tumorigenesis, including mammary gland tumors, without apparent metastasis [87]. Overexpression of a mutant form of the Met receptor, but not the wild type protein, resulted in mammary tumors which metastasized to lung, lymph node (LN), kidney and heart [88], suggesting ligand-independent functions of mutant Met. Recently, HGF was overexpressed in the mammary gland using a WAP promoter, resulting in mammary tumors with a median latency of 210 days, which metastasize to the lungs [89]. The RON receptor is a family member of the Met proto-oncogene, and recently, RON has been found to be overexpressed and constitutively activated in approximately 50% of primary breast cancer cases [90]. In addition, increased expression of the RON receptor strongly correlates with the more aggressive phenotype observed in node-negative breast tumors [91]. Transgenic overexpression of Ron in MMTV-Ron mice is sufficient to induce mammary tumors that metastasize to the liver and lungs with high penetrance. In contrast to the MET RTK, activating mutations in Ron did not confer a higher tumor incidence or metastasis rate [92].

Conditional Mouse Models of Breast Cancer

Human tumors are caused by accumulating genetic mutations in a distinct subset of cells that have a given susceptibility for the molecular consequences of such a mutation. In sporadic cancer, these mutations will affect a single cell that is embedded in a normal microenvironment. Conventional mouse models of human breast cancer have largely been based on the activation of a single dominant gene in a pan-organ setting. As a consequence, a large cellular compartment is targeted for oncogenesis, a situation not reflective of the human condition. In human cancer, mutations occur sporad-

ically in a stochastic manner and accumulate over time, leading to tumors with a distinct gene expression signature that determines metastatic behavior and clinical outcome [22]. Furthermore, most mouse mammary tumors arising in conventional transgenic models do not recapitulate the morphology of common human breast cancers [93]. Finally, tumor metastasis in conventional breast cancer models is largely restricted to lung, whereas most human tumors initially show lymphatic spread.

These limitations of conventional models have urged scientists to develop more advanced mouse models based on inducible systems that permit somatic and stochastic mutation of target genes in a wild type background [94]. Successful conditional gene (in)activation can be achieved by using site-specific recombination systems, such as the Cre/*loxP* from bacteriophage P1 or the FLP/*FRT* system from S. cerevisiae. To obtain a conditional mutation, coding regions of a gene are flanked by *loxP* or *FRT* elements, which are inserted in intronic sequences, resulting in functional alleles and phenotypically wild type animals. Mice carrying conditional mutations are then crossed to mice expressing the Cre or FLP recombinase enzyme in the mammary gland, and as a consequence, only those cells that have expressed the appropriate recombinase will undergo site-specific recombination resulting in deletion of the genomic sequences flanked by *loxP* or *FRT* elements [95] (See http://www.mshri.on.ca/nagy/floxed.html posted by the Nagy lab for a database containing all published floxed genes). Future models may involve 'double-layered' systems in which tissue-specific, reversible gene mutations can be induced in selected (Cre-expressing) cells at any given time through the application of inducible (e.g. tetracycline-responsive) elements incorporated in mammary-specific promoters [96, 97]. Compound mutant mice can thus be bred to investigate the contribution of multiple mutant alleles, in space and time, to breast tumorigenesis and metastasis, providing an unsurpassed degree of flexibility in mouse modeling.

Another interesting system, which may facilitate in vivo testing of candidate metastasis genes, is based on avian leukosis virus RCAS (replication-competent avian sarcoma-leukosis virus LTR splice acceptor)-mediated somatic gene transfer [98]. This system employs retroviral gene delivery whereby expression can be targeted to cells of mice transgenic for the avian subgroup A receptor gene (tva). This system has recently been employed successfully to introduce PyMT and ErbB2 in mammary epithelial cells of MMTV-tva transgenic mice [99]. Moreover, oncogene collaboration could be assessed by infection of MMTV-tva; MMTV-Wnt bitransgenic mice with RCAS virus carrying an activated ErbB2 oncogene. The RCAS-tva system may thus be a promising approach to develop flexible mouse mammary tumor models for rapid evaluation of candidate metastasis genes.

Conditional Mouse Models of Breast Cancer Metastasis

Conventional mouse models of breast cancer employ activation or inhibition of genes in a "pan-organ" setting, and, as a consequence, produce mammary tumors that only poorly resemble human breast cancer etiology and metastatic behavior [93]. Hence, new mouse models are needed that reproduce the salient features of human breast cancer development and metastasis. Whereas these conditional mouse models of breast cancer metastasis are obviously more complex, the first successful studies show promising results.

Inhibition of p53 function as a consequence of mutation or genomic loss is found in approximately 50% of human breast cancer cases [100, 101]. Conditional inactivation of p53 in the mammary gland using WAP promoter-driven Cre expression resulted in presumptive Erα-positive tumors that metastasized to the lungs in 36% of the female mice [102]. However, major discrepancies exist between this model and human p53-mutated breast cancers, tumor morphology and metastatic spread. Possible reasons for these differences may be that (1) conditional mutations are induced in the wrong mammary gland compartment when using a WAP promoter, or (2) conditional induction of an additional hit is required to obtain a 'humanized' mouse mammary tumor with the appropriate metastatic spectrum.

The latter has been explored by studying the consequences of somatic loss of E-cadherin in a noninvasive mouse mammary tumor model based on epithelium-specific inactivation of p53. In this setting, stochastic cytokeratin (K)14 cre-mediated inactivation of E-cadherin induces the formation of mouse invasive lobular carcinoma (mILC), reminiscent of the human situation [103]. In contrast to previous mouse models, metastases in mILC are mostly lymphatic and spread to lung, liver, gastric tract and peritoneum, similar to human pathology. Moreover, angiogenesis is strongly increased in mILC, a phenotype that may be facilitated by autocrine production of proangiogenic factors. In summary, the mILC mouse model not only mimics a human form of breast cancer, but also represents the first physiologically relevant model to study all aspects of breast cancer progression and metastasis. The 'humanized' phenotype in mILC may be to a significant extent credited to a combination of the tissue-specific promoter used and the nature of the secondary induced conditional mutation. Given the tumor incidence and timing, carcinomas are likely to originate from a mammary gland progenitor cell that expresses CK14 before or during puberty. The main disadvantage of using a CK14 promoter to drive cre expression in mammary epithelium is the fact that this promoter is also active in other epithelial tissues, including skin. Consequently, a substantial fraction of the females develop skin tumors, which arise either prior to or concurrent with the onset of mILC. To circumvent these problems, we have developed a

model, based on WAPcre-mediated deletion of E-cadherin and p53. Here, mammary tumors develop with a similar latency in a pregnancy-independent fashion and show a comparable metastatic spectrum and incidence (Derksen et al., manuscript in preparation).

Regulatable Mouse Models of Breast Cancer Metastasis

It is commonly accepted that tumor formation is a multistage process driven by stepwise acquisition of oncogenic capacities through (epi)genetic mutations in oncogenes and tumor suppressor genes [42]. 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 [104]. Using genetically engineered mouse models with doxycycline- or tamoxifen-inducible gene expression [95], this question has been successfully addressed for several oncogenes [105] and tumor suppressors, such as p53 [106]. These mouse models also permit assessment of the requirement of sustained oncogene overexpression for maintenance of tumor metastases. This issue has been elegantly tackled using a conditional mouse mammary tumor model with doxycycline-inducible, mammary epithelium-specific expression of activated ErbB2 (MMTV-rtTA; TetO-ErbB2) [107]. 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.

The Course Ahead

Clearly, mouse models that recapitulate the multiple stages of breast cancer development and progression have greatly contributed to our understanding of the molecular mechanisms governing tumor progression and metastasis. Whereas comparative histopathology is often used to judge a mouse model on its merits, there is a growing appreciation for the underlying molecular mechanisms as the key parameters regulating tumorigenesis and progression. The fact that tumor progression in some mouse models does not 'look' like a human malignancy, does not exclude the possibility that the affected genes and pathways are similar or overlapping. Today, more and more emphasis is being directed towards molecular profiling techniques to identify deregulated pathways that drive tumor progression and metastasis. Humanized mouse models are increasingly being used to extract distinct genetic signatures that correspond to mammary tumors with specific metastatic capacity. This will allow a thorough comparison of mouse versus human expression profiles to extract common denominators for a given tumor phenotype or (organ-specific) metastatic pattern. These efforts are expected to yield candidates which may lead to the discovery of new diagnostic markers as well as drug targets for intervention strategies to fight metastatic disease. The final frontier will nonetheless be the testing of new strategies for clinical applicability. Although xenografts have extensively been used as preclinical models, they are end-stage tumors that do not recapitulate the natural history of cancer in a histocompatible host and, consequently, have not excelled in predictive power. Furthermore, they lack particular interactions with the microenvironment, which contains key elements that influence tumor development and metastasis [108–110]. Novel models of in vivo metastasis are being developed at an expeditious pace and will yield mice that closely resemble human tumor development and progression, thus allowing accurate testing of newly designed agents.

Moreover, tumor development and progression can be monitored noninvasively for long periods using a growing array of imaging modalities, such as bioluminescence imaging (BLI) [111], fluorescence imaging [112], magnetic resonance imaging (MRI) [113], positron emission tomography (PET) [114], computer tomography (CT) and single photon emission computed tomography (SPECT) [115]. Finally, intravital imaging using multiphoton or two-photon microscopy permits real time observation of the dynamics of tumor growth, extravasation and cell migration, as well as the contribution of stromal fibroblasts, endothelial cells and immune cells [116, 117]. We believe the best is yet to come.

Acknowledgements We apologize to those whose work was not included because of space considerations. This work was supported by grants from the Dutch Cancer Society (NKI 2002-2635 and NKI 2006-3486 to JJ) and the Netherlands Organization for Scientific Research (NWO-Vidi 917.36.347 to JJ and NWO-Veni 916.56.135 to PWBD).

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