BRCA1 breast cancer: metastasis and mouse models

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Abstract

The BRCA1 gene is a tumour suppressor gene frequently mutated in heritable forms of breast cancers. BRCA1 deficient tumours show a basal-like phenotype. Attempts have been made to model the initial steps of this cancer in mice. The metastasis process of breast cancer, frequently to the bone environment, has also been modelled in mice in different ways. However, a good model representing both the initial steps of BRCA1-deficient breast cancer and the clinically important advanced stage of metastasis formation has not yet been developed. To develop and pre-clinically test new therapeutics this would be valuable.

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Introduction

The cure of breast-cancer is greatly desired, but in approximately 30-40% of the cases not achieved. Breast-cancer is still the second-leading cause of cancer mortality in woman, affecting one in nine woman in their lifetime (1). Mouse-models have taught a lot about many different diseases occurring in human. They have provided an enormous amount of knowledge on the aetiology of cancer. Germline inactivation of the prototype tumour suppressor genes Rb (2) and Tp53 (3) using gene targeting technology in mouse embryonic stem cells, appeared to complete the necessary tools to model the scope of mutations present in human tumours (4, 5). Targeted inactivation of Rb in mammary epithelium induces mammary adenocarcinomas and adenosquamous carcinomas in mice (6). Mammary-specific deletion of p53 leads to ERα-positive and – negative tumours (7). Combined Rb and p53 deletion using MMTV-Cre, develop lethal lymphomas within 2-6 months of age (6).

The formation of metastasis in the process of progression of breast cancer is a clinically important step. Without metastasis, breast cancer would hardly be lethal, since breasts are organs non-essential to life. 70% of the patients dying of breast cancer have metastases to the skeleton (8). The prevention and treatment of metastasis of breast cancer is therefore the most important step in the clinical treatment of breast cancer. To study all aspects of tumour pathology, mouse models are needed that mimic not only tumour phenotype, but also the initiating steps of human tumour development. Studying the early stages is difficult because many genetic alterations have occurred prior to the clinical presentation of the disease (9). It is important that mouse models reflect the events that are common in human breast cancer pathology to develop new therapeutics. Owing to the complex nature of the metastatic process, models that mimic both de novo tumour development and spontaneous metastasis formation are scarce, but nevertheless emerging (10). What do mouse-models have to offer to investigate the metastasis process of heritable breast cancer?

Mouse models for metastasis

Many models have been developed to research the development of breast cancer and metastasis. All models have their specific advantages and disadvantages. Which models have been used to date will be discussed here. Mice normally hardly develop spontaneous mammary cancer and therefore mice have to be manipulated to create a cancerous mouse. Transplantation models have been widely used to create cancer in mice. Syngeneic models refer to mouse or rat cancer cell lines or tissues transplanted into inbred animals of the same genetic background as the derived cell line or tissue. The host is similar to the transplanted tissue, particularly important to consider the close interaction between tumour and its microenvironment (11, 12). Tumorigenesis is not simply the result of proliferative activation of a cell. Rather, it is a complex interaction between the neoplastic tissue and the stromal environment of a specific organism (11, 12). Mammary epithelial cell growth, differentiation, lactation and progression to cancer involve bidirectional interactions between the epithelial population and its surrounding stroma (13). Heterotopic transplantation refers to the transplantation of tumours to a different anatomic location or tissue from which the tumour was derived. Historically, tumours were characterised by growth after subcutaneous transplantation. In this model, spontaneous metastasis are uncommon. With orthotopic transplantation the cancer cells are transplanted to the same anatomic location and tissue the cells are derived from, like the mammary fat pad for breast cancer. This method more closely resembles human cancers. This supports the idea that host-tumour interactions are important for tumour development (11). In 1988 it was already

established that injection of a mouse mammary carcinoma cell line into the mouse mammary fat pad was significantly more tumorigenic compared to injection of the same cells subcutaneous. The mammary gland thus provides an advantageous environment for mammary carcinoma cells (11, 12, 14). However, these models lack the genetic complexity of human tumours (9, 11).

Xenograft models are human cancer cell lines or tissues, transplanted into immune-compromised animals. This way, the tumours are a mix of human cancer cells and host stromal cells. Invasion into neighbouring tissue and ectopic survival are crucial events for cancer progression and are a prerequisite to forming metastasis (15). Invasion and metastasis are the cause of malignancy and responsible for treatment failure (12, 15). However, some pathways do not allow the interaction between cancer and stromal cells due to species specificity (9, 11). Implantation models do not recapitulate all of the interactions and micro-environmental components that may play important roles in tumour development. Mechanical disruption of the tissue may force escape from the primary site which may make seeding to distant sites easier (11).

Metastasis can also be experimentally induced by injection of tumour cells directly into the systemic circulation. Depending on the site of injection and characteristics of the tumour cells, metastasis will or will not develop. The site of injection influences the organ target through the first capillary bed the cells encounter following injection. Therefore, the target organ does not always represent the sites of metastasis in the clinic. This model also bypasses important events like escape from the primary tumour, invasion into adjacent tissue and extravasation into the circulatory system (11), see figure 1. Historically, immune-competent mice were used, but to reduce rejection and increase the incidence of metastasis, later models have used athymic mice. Culture conditions always select for populations of cells that are not necessarily representative for the parental population (9).

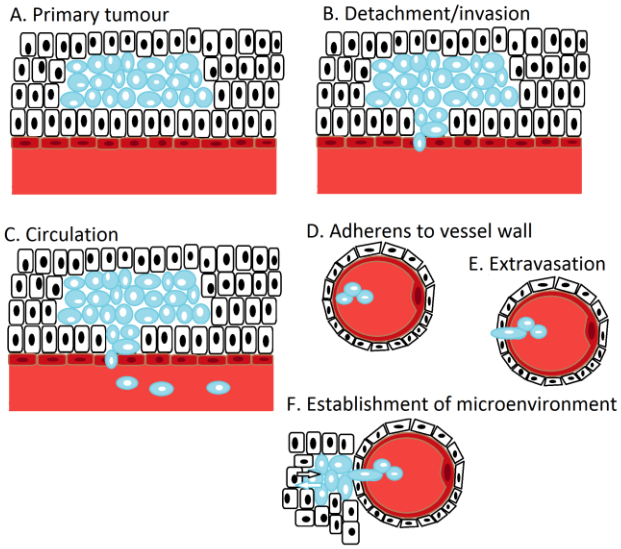

Figure 1 Main steps for cancer cells to establish a metastasis. Cancer cells have to detach from the primary tumour and invade the surrounding tissue to reach the circulation. From the circulation the cancer cells have to adhere to a vessel wall before extravasation to a new tissue can occur. After arrival in the new environment, the cancer cells establish a microenvironment to grow into a metastasis.

Also, the murine host must be immune-compromised to prevent rejection (9). The role of the immune system can therefore not be examined in xenograft models. Impaired angiogenesis has been shown to occur in nude mice which have impaired T and B cell function to prevent rejection. SCID mice, even though having deficits in both T and B cell number and function, show immune-mediated elimination of metastatic cells because of a high resting NK cell activity. Significant differences for angiogenesis have been reported between transplanted and autochthonous tumours. This may reduce the predictive power of xenografts for the clinic (11). Differences in the role and function of pathways for the development and progression of cancer between mouse and human cannot be ruled out. However, using genetically engineered mice as a model there is no selection of cancer cells due to cell culture or transplantation. Also the immune system is normally functioning and pathways do

not have to cross species boundaries. Naturally occurring or chemically induced tumours more truly replicate the normal initiation and progression observed in humans. Unfortunately the majority of the spontaneously arising tumours in mouse models do not metastasize or do so with a very long latency (11).

Genetically engineered animal models permit the investigation of the influence of genetic heterogeneity. In familial breast cancer, only 50-60% of carriers of BRCA1 mutations develop breast cancer, presumably due to the influence of polymorphic genes. Genomic polymorphisms cannot be evaluated using cell culture-based systems (11), because all cells in a cell culture are genetically identical. Models for breast cancer using genetically engineered mice have not always shown to be metastasising similar to the human situation or do not metastasise proficiently enough to provide the numbers necessary to investigate either the emergence of metastasis or do drug essays. However, a few models have been developed which metastasise similar to human breast cancers of which some will discussed hereafter.

Modelling metastasis of breast cancer

Human breast cancers are molecularly and histologically heterogeneous, so it is difficult to compare patients. Breast cancer cells predominantly express the chemokine receptors CXCR4 and CCR7 and are known to metastasise to organs expressing the corresponding ligands CXCL12 and CCL21 (16, 17). Advanced disease breast cancers show a preferential affinity for the bone microenvironment (9, 16, 17). The ideal model for breast cancer would reproduce the genetic and phenotypic changes that occur with human cancers. This ideal model does not (yet) exist (9). However, a number of models have come very close to mimicking the clinical manifestation of a number of breast cancers. For example, Derksen et. al. have created a model which metastasises very similar to human breast cancer.

Derksen et. al found that mammary-specific loss of E-cadherin alone results in mammary epithelial cell death by apoptosis. Combined loss of E-cadherin and p53 confers anchorage-independent survival of mammary cells, resulting in a non-functional mammary gland owing to aberrant lobulo-alveolar development and a severely disrupted ductal architecture. These abnormalities are probably caused by loss of cell polarity and acquisition of anoikis (programmed cell death associated with loss of cellular contact) resistance. This results in loss of mammary gland organization and enhanced proliferative capacity of mammary epithelial cells, leading to non-functional mammary glands (10). Mammary specific somatic loss of E-cadherin and p53 results in a significant shift from expansive to invasive mammary tumours which shows strong phenotypic similarities to human pleomorphic invasive lobular carcinoma (PILC) (10). Mouse ILC (mILC) cells could be detected in organs such as skin, lungs, liver, gastrointestinal tract, pancreas, spleen and throughout the peritoneal cavity in 74% of the female mice with mammary specific somatic loss of E-cadherin and p53. Also, several mice developed bone metastasis. These characteristics indicate that the mouse model recapitulates the histopathology and tumour biology of human ILC (10). Nevertheless, human ILCs are mostly ER-positive, whereas in mILC ER-positive cells are only found sporadically. This may reflect a general shortcoming of the mouse as a model for ER-positive human breast cancer, since most mammary tumours induced in mice are ER and PR negative (9, 18, 19). Therefore, murine models do not precisely recapitulate steroid receptor signalling and are therefore not suited for preclinical testing of inhibitors of steroid receptor signalling and for drug resistance studies (9). Also, the mILC depends on loss of p53. In human ILC, only 4-25% has inactivated p53. However functional loss of p53

through alternative mechanisms is often not ruled out (10). This mouse model represents an excellent preclinical model to test novel intervention strategies for invasive and metastatic breast cancer (10).

BRCA – a basal-like breast cancer

What is basal-like breast cancer? In the human mammary gland two distinct types of epithelial cells are found: basal/myoepithelial cells and luminal epithelial cells (20, 21). Luminal cells line the ductal lumen and secrete milk upon terminal differentiation into lobulo-alveolar cells. Basal/myoepithelial cells reside between these luminal cells and the basement membrane to assure ductal contractility (22). Immunohistochemically these cell types can easily be distinguished by staining with antibodies to keratin 5/6 (basal) or keratins 8/18 (luminal) (21, 23). Although morphology is often associated with the pattern of molecular aberrations in breast cancers, tumours of the same histological type show remarkable differences in clinical behaviour (24). Basal-like tumours comprise a heterogeneous group that accounts for up to 15% of all breast cancers, affect younger patients, are more prevalent in African-American women, and often present as interval cancers.

Gene expression of basal-like breast cancer

Basal-like breast cancers defined by immunohistochemical markers have been proposed to: <1> lack ER, PR and HER2 expression, <2> express one or more high-molecular-weight/basal cytokeratins (CK5/6, CK14 and CK17), <3> lack expression of ER and HER2 in conjunction with expression of CK5/6 and/or epidermal growth factor receptor (EGFR), and <4> lack expression of ER, PR, and HER2 in conjunction with expression of CK5/6 and/or EGFR (24, 25). The majority of basal-like breast cancers lack or show low levels of ER and PR, lack HER2 protein overexpression and HER2 gene amplification, and express genes and proteins usually found in 'basal'/myoepithelial cells of the normal breast, including CK5/6, 14 and 17, P-cadherin, caveolins 1 and 2, nestin, αB crystalline, CD109 and EGFR. p53 immunohistochemical expression or TP53 gene mutations is observed in up to 85% of cases . Also, alterations of the pRB and p16 G1/S cell-cycle checkpoint are remarkably prevalent in these cancers (24). The majority of triple negative cancers are of basal-like phenotype and the majority of tumours expressing 'basal' markers are triple negative, but not all basal-like cancers determined by gene expression profiling lack ER, PR and HER2 and not all triple negative cancers show a basal-like phenotype by expression array analysis (24). Modelling basal breast cancer by genetically engineering mice led to the findings that deletion of the retinoblastoma (*RB1*) gene in common progenitor cells gives rise to either a luminal-B subtype or basal-like mammary tumours with an EMT phenotype. However, *RB1* loss leads to basal-type tumours only when there is an accompanying *TP53* mutation. Since both EMT and *TP53* mutations are associated with a cancer stem cell (CSC) phenotype, this suggests that the type of mutations, rather than the cell-type origin, has a greater effect on CSC phenotype (26).

Heritable breast cancer

Germline mutations in BRCA1 are associated with a substantially higher risk of developing basal-like breast cancer (24, 27). Basal-like cancers and tumours arising in BRCA1 germ-line mutation carriers show a peculiar pattern of cell-cycle protein expression; they express significantly lower levels of p27 and higher levels of Skp2, cyclin E and caspase-3 when compared to sporadic breast carcinomas and BRCA2 mutation tumours. Also, they both rarely harbour CCND1 gene amplification (24). BRCA1 was first isolated as the gene responsible for increased susceptibility to familial breast and ovarian cancer. 50% of the familial breast

cancers show germline mutations of BRCA1 (1, 28). Germline mutations of BRCA2 are responsible for about one third of the cases of hereditary breast cancer. BRCA1 and BRCA2 interact with RAD51, which is essential to homologous recombination and DNA double strand-break repair. RAD51 co-immunoprecipitates with p53 (1, 28).

It has been a mystery that mutations of BRCA1, most commonly mutated in familial breast and ovarian cancers, do not appear in sporadic forms of breast cancer and are rare in ovarian cancer (29). The BRCA1 gene promoter is methylated in >60% of medullary and metaplastic breast cancers with a basal-like phenotype (24). The significance of epigenetically mediated loss of gene function in cancer has recently been highlighted by the recognition that this process precedes and appears to be essential for several genetic events that drive tumour progression. This link can be an indirect one, and is associated with promoter hypermethylation of key genes in early tumour evolution (29). Sporadic invasive ductal carcinomas with basal-like phenotype express ID4, a negative regulator of BRCA1, at significantly higher levels than grade-matched controls. This mechanism may account for the low levels of BRCA1 expression in sporadic basal-like carcinomas of ductal morphology (24).

BRCA1 and cancer stem cells

BRCA1 mutation in committed progenitor cells can lead to stabilisation of SLUG/SNAI2 protein. SLUG, a transcription factor that can induce epigenetic changes, is a developmentally regulated gene and is downstream of the Wnt and Notch pathways. SLUG has been shown to increase the expression of genes and cell-surface markers associated with CSCs. This reinitiation of the stem-cell programme integrates driver mutations with the CSC hypothesis. This theory postulates that CSCs are cells that can help tumours progress, contribute to tumour heterogeneity, withstand the effects of therapy and reinitiate tumours subsequent to treatment (26, 30). Breast cancers with high levels of CSCs, by gene expression, are associated with poor outcome. Expression of CD44+/CD24-, ALDH1+, $CD49f^{high}$/$DLL1^{high}$/$DNER^{high}$, retention of PKH26 dye, and low proteasome activity have all been associated with stem cells activity in breast cancer. Cancer cells can acquire a CD44+/CD24- phenotype through epithelial-to-mesenchymal transition (EMT) and ALDH1-positivity through HER2 overexpression. A combination of genetic events leading to EMT and HER2 overexpression could generate an aggressive clone with a CD44+/CD24-/ALDH1+ CSC phenotype (26). Analysis of 13 breast cancer cell lines showed that all cell lines with a CD44+/CD24- subpopulation were basal breast cancer cells that had undergone EMT (31, 32).

In vitro growth properties of normal breast tissue of BRCA1 mutation carriers show a reduction in the mammary stem cell (MaSC) subset of the various epithelial subpopulations and an increase in the luminal progenitor cell fraction compared to age-matched normal breast tissue. The primary cellular manifestation in BRCA1-associated and other basal cancers is the luminal progenitor cell rather than the MaSC. This epithelial cell hierarchy in human mammary tissue closely parallels that occurring in the mouse mammary gland. Despite some differences in the expression of cell surface markers between species, it is likely that the analogous subpopulations have highly conserved functions. For example, both the human MaSC enriched population and the mouse MaSC subset lack expression of the steroid hormone receptors. Furthermore, the luminal progenitor subsets in both human and mouse BRCA1-mutant mammary tissue show B27 factor independence in vitro. Notably, oestrogen receptor-α was expressed by a substantial fraction of human luminal progenitor cells. Oestrogen receptor-α may therefore directly mediate the partial efficacy provided by prophylactic oophorectomy in the prevention of

basal breast tumours in BRCA1 mutation carriers. This is also compatible with reports suggesting that tamoxifen chemoprophylaxis may be protective (27).

Mouse models for BRCA1 breast cancer

Xu et. al. created a mouse model for BRCA1 breast cancer by deleting exon 11 of the BRCA1 gene using the Cre *LoxP* system (1). The Cre transgene was under control of the WAP promoter (whey acidic protein) or mouse mammary tumour virus-long terminal repeat (MMTV-LTR). After 10-13 months, 3 out of 10 MMTV-CRE mice and 2 out of 13 Wap-CRE mice developed mammary tumours of diverse types. They found that Trp53 expression was altered in 2 of 3 tumours (1). The alterations of Trp53 expression in Brca1 conditional mammary gland tumours are reminiscent of human BRCA1 familial breast tumours, which frequently contain TP53 mutations (33). To test the additional value of loss of Trp53, they introduced a loss-of-function allele into *Brca1* conditional mutant mice. 8 of the 11 MMTV-Cre mice developed mammary gland tumours after 6-8 months (1). The model of Xu et. al. provides evidence about the functional relationship between BRCA1 and p53. However, the tumours found in these mice show diversity in their histological patterns and thus do not accurately resemble human BRCA1 breast cancers.

Lui et. al. therefore used a different strategy. They used a conditional model for p53 and BRCA1 where Cre was expressed under the skin tissue and mammary gland epithelium K14 promoter. Additional Brca1 conditional knockout over only p53 knockout resulted in a significantly reduced tumour formation latency; after 213 days half of the animals developed mammary tumours compared to 288 days when only p53 is conditionally knocked out. Both p53 and Brca1 contribute to tumorigenesis. Unsupervised clustering of gene expression patterns showed that $Brca^{null}/p53^{null}$ mouse mammary tumours are most similar to human BRCA1 tumours, whereas $p53^{null}$ mouse tumours are more similar to human sporadic basal-like breast cancers. This supports the $Brca^{null}/p53^{null}$ mouse model as a good model for human BRCA1 breast cancer (34). This models was successfully used to pre-clinically test the PARP inhibitor AZD2281 (35). However, due to the high incidence of development of skin tumours additional to the mammary tumours, this model is not useful for studying metastasis for human BRCA1 breast cancer.

A similar mouse model for BRCA2 associated breast cancer was created by Jonkers et al. Their mice carry conditional *Brca2* and *Trp53* alleles with the Cre *Lox*P system expressing the *cre* transgene under control of the K14 gene promoter. 72% of the female mice with complete loss of *Brca2* and *Trp53* function developed mammary tumours with a latency between 100 and 300 days. Both BRCA2 and Trp53 contribute to tumour development. The authors suggest their model as a valuable tool for testing chemoprevention and therapeutic intervention strategies, as well as for the identification of additional factors involved in tumour onset and progression and the genetic dissection of pathways involved in human hereditary breast cancer (28). However, because of the use of the K14 promoter, these mice also develop skin tumours with a high incidence.

The earlier mentioned model of Derksen et. al. inactivated E-cadherin and p53 using a conditional model with Cre under the expression of the WAP promoter (10). Earlier, the same group made a model inactivating the same genes, but expression Cre under the K14 promoter. This model does not represent as a good pre-clinical model due to the skin related problems because of the use of the cytokeratin promoter (K14) (10, 36). A good model to study metastasis in BRCA1 breast cancer, would probably be to conditionally inactivate BRCA1 and p53 expressing Cre under the WAP

promoter. Unfortunately the experiment done by Xu et. al. only conditionally inactivated Brca1 and Trp53 together using MMTV-Cre and not Wap-Cre. Could we have had this model in 1999 already? It is not clear from the publication why Xu et. al. choose to use the MMTV-Cre over Wap-Cre for combined inactivation of Brca1 and Trp53. Possibly due to technical limitations, hopefully overcome thirteen years later.

Discussion

The tendency to characterise the value of a model in terms of the similarities between the animal and the human cancer leads to the attempt to define the best model of a certain cancer. However, it may be unlikely that the human cancer in patients can be entirely modelled by one system alone (11). Paget's Seed and Soil hypothesis postulates that cells travel around the lymphatic and blood circulatory system until the cells find the right environment to grow in (9). Injection of cells into the circulatory system is a good model to test this hypothesis. Cancer cells will only metastasise to the right "soil" environment, like breast cancer cells with CXCR4 and CCR7 having a preference for tissue expressing CXCL12 and CCL21. However, this model for metastasis overcomes some boundaries which cancer cells normally have to overcome by themselves. Models in which many traits are overcome by the experimental procedure probably make the model more efficient, like shorter latency, but very likely also less reliable to human cancer in the clinic.

This also applies to the use of athymic mice for the injection models. Of course this will increase the frequency of metastasis in mice, but are these clinically relevant? Most breast cancer patients are in possession of a good working immune system which will clear many cancer cells circulating in the blood or lymphatic system before they get the chance to find the right "soil". The increase in metastasis found in athymic mice compared to "normal" mice can be attributed entirely to clinically non-relevant tumours, because they would normally have been cleared by the immune system. Adjusting any model to make it more efficient by overcoming natural boundaries is very likely to also make it less relevant to the clinic. Easier, faster and cheaper experiments will be harder to draw conclusions from and in the end turn out to be wrong more often. So much for easier, faster and cheaper experiments.

Genetically engineered mouse models for basal-like cancers show a good gene expression profile reminiscent of human basal-like cancers, compared to models for luminal types which lack the ER-signature. This makes genetically engineered mice good models to research basal-like breast cancers. The impairment in RB function is an important shared feature between human and mouse mammary carcinomas (25).

Gene expression analysis confirm that there is not a single murine model that perfectly represents a human breast cancer subtype. However, the murine models do show shared features with specific human subtypes and it is these commonalities that will lay the groundwork for many future studies (25). Although the perfect model does not yet exist, many improvements have been made and continue to be made. A mouse will never be human, but genetically engineered mouse models have already proven to be able to recapitulate many aspects of human cancer and have proven useful for pre-clinical drug essays. Both the initial steps in cancer development and the complicated steps required for metastasis formation have properly been modelled in mice. The next step to be made, is the accurately modelling of different specific breast cancers occurring in human, from initiation until the last phase in which human breast cancer often present itself in the clinic.

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