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Cancer

Disease models of breast cancer

Piyush B. Gupta¹, Charlotte Kuperwasser^{2,*}

¹Department of Biology, Massachusetts Institute of Technology and Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA

²Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111-1800, USA

The development of novel breast cancer therapies depends on appropriate experimental models of disease pathogenesis. In recent years, there has been a proliferation of sophisticated models for breast cancer research. We describe here novel models that have particular relevance for drug development, and discuss the advantages and disadvantages of each model with respect to the various aspects of breast cancer pathogenesis.

Introduction

It is estimated that nearly 216,000 women will be diagnosed with breast cancer and over 40,000 will die of the disease in 2004 [1], (http://www.cancer.gov/cancer_information/cancer_type/breast, http://www.cancer.org/docroot/STT/content/STT_1x_Breast_Cancer_Facts_Figures_2003-2004.asp). According to the American Cancer Society, there has been a steady increase in cases diagnosed each year since 1980, due primarily to increased mammography screening. Enhanced diagnostic methods that allow for early tumor detection, combined with improved treatments, have resulted in a slight decrease (2.3%) in breast cancer mortality during the past decade.

Risk factors associated with the development of breast cancer include family history, age at menarche, age of menopause, nulliparity and parity after the age of 30, and postmenopausal hormone therapy. While accounting for only 5%–10% of all breast cancer cases, inherited mutations in the tumor suppressor genes BRCA1 or BRCA2 can increase the risk of disease development to as much as 85%. The complexity of the factors

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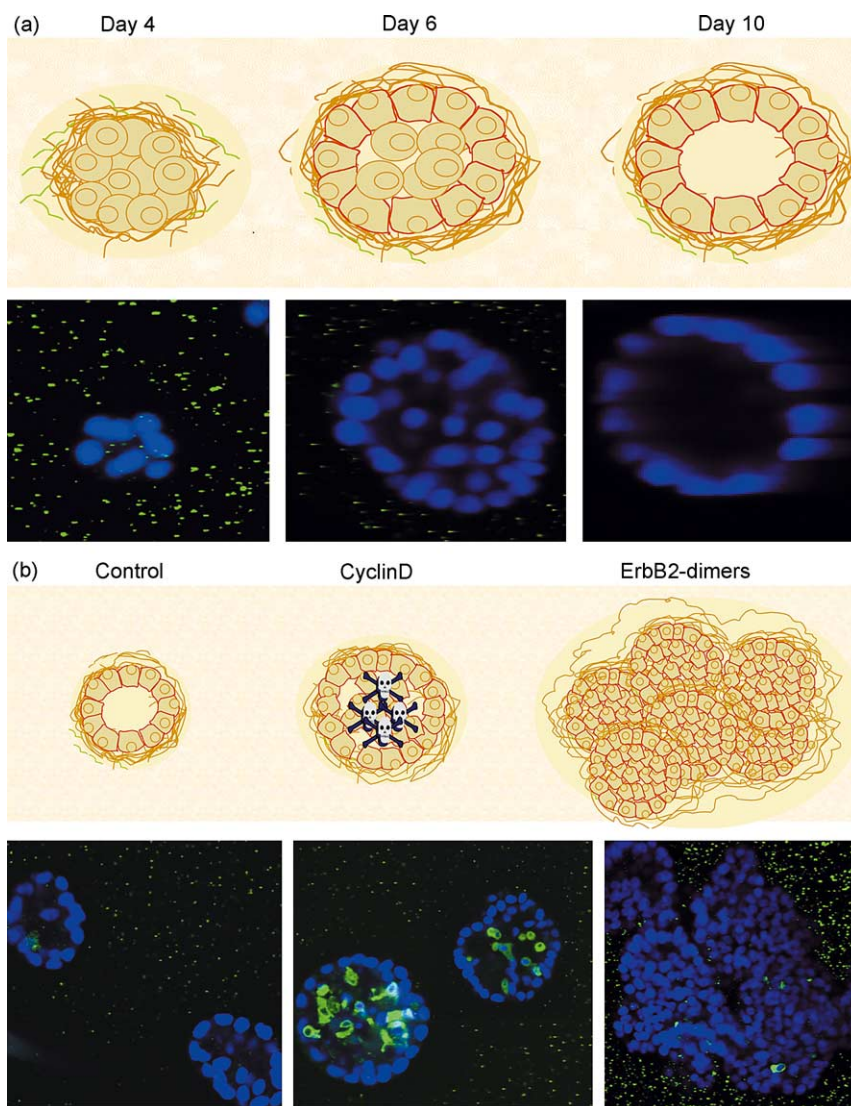
A decade has past since the isolation of the BRCA1 gene. Although diagnostics for breast cancer susceptibility have improved, the highly sought therapeutic target for this prevalent cancer has yet to be identified. Recent approaches have focused on developing assays that better reflect the native biology of this complex tissue. Gupta and Kuperwasser discuss the use of three dimensional mammary epithelial cell culture models, and advanced xenotransplantation assays, to dissect the pathways involved in mammary biology. These avenues hold great promise for preclinical validation studies of next generation therapies.

governing breast cancer incidence and progression has led to the development of numerous experimental models to study the disease. In this review, we describe some of the more recent models that researchers are currently utilizing to understand breast cancer pathogenesis.

In vitro models of morphogenesis

The growth of breast epithelial cells as monolayers on tissue culture plastic does not accurately replicate the molecular and morphogenic (see Glossary) behaviors of this cell type in its normal tissue microenvironment. Indeed, neither the apical-basal polarity of single cells, nor the lobular and ductal structures formed by populations of epithelial cells *in vivo*, is replicated in such systems. To address these deficiencies, researchers have developed 3-dimensional (3D) systems in which breast epithelial cells are cultured, suspended within a reconstituted basement membrane (see Glossary) matrix (Matrigel) [2]. In these 3D culture systems, epithelial cells form well-organized spheroid structures, called *acini*. Acini, which are the smallest functional unit of the breast, have hollow lumens, polarized epithelium, and specialized basement membrane (BM)-anchored cell–cell contacts (Fig. 1).

*Corresponding author: (C. Kuperwasser) Charlotte.Kuperwasser@tufts.edu



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Figure 1. Schematic depiction and immunofluorescence of epithelial structures in 3D culture. (a) Upper cartoons depict cross sections through acini-like structures that are generated in basement membrane gels from control MCF-10A cells at four days (left), six days (middle) or 10 days (right) after seeding as single cells. Between day four and day six, the outer cells develop an axis of apicobasal polarity, and between day six and day 10, the centrally localized cells die to form the hollow structures [3]. Lower images show photomicrographs of confocal images of structures stained with DAPI to highlight nuclei. (Images from J. Debnath from the Brugge laboratory, Harvard Medical School.) (b) Upper cartoons depict cross sections through acini-like structures that are generated in basement membrane gels from control MCF-10A cells (left), cells over-expressing Cyclin D (middle) and cells expressing homodimers of ErbB2 as described by Muthuswamy *et al.* [4] and Debnath *et al.* [3]. The lower panels show confocal images stained with DAPI to identify nuclei (blue) and with an antibody to activated caspase3 (green) to stain apoptotic cells. Cells expressing cyclin D proliferate constitutively; however, they undergo cell death in the luminal space. ErbB2 cells also hyperproliferate but they escape apoptotic signals and fill the luminal space. In addition, the ErbB2-expressing structures are able to divide such that daughter cells are generated outside the acini and form new acini. (Images were obtained with permission from J. Debnath from the Brugge laboratory, Harvard Medical School.)

Thus, these 3D systems serve as useful models to study the molecular mechanisms governing normal epithelial cell morphogenesis, as well as the mechanisms behind the disruption of normal breast architecture during cancer pathogenesis [2,3].

In an application of this system, researchers compared the effects of various oncogenes that were introduced into immortalized epithelial cells before culturing the cells in 3D.

Remarkably, certain oncogene effects on epithelial cell morphogenesis were undetectable in standard monolayer cultures, and were unmasked only in the context of 3D cultures. For example, cyclin D1 over-expression or ErbB2 activation, both of which induce hyperproliferation in monolayer cultures, results in distinct morphogenic phenotypes discernable only in 3D [3,4]. ErbB2 activation resulted in the formation of aberrant multi-acinus (see Glossary)

Glossary

Acinus: a spherical hollow structure composed of epithelial cells.

Basement membrane: a specialized extracellular matrix, comprised primarily of collagen IV and laminin, to which epithelial cells are anchored by integrin contacts.

Cre-lox: recombinant DNA technology system based on the properties of the Cre recombinase enzyme. This protein recognizes lox sequences and mediates the excision of DNA between lox sites and recombination of DNA that contain a specific lox sequence.

Dysmorphic: relating to the disruption of the normal process of 3D structure formation.

Fat pad: the stromal compartment of the mouse mammary gland, composed primarily of adipose tissue.

Morphogenic: relating to the process by which cells develop 3D structures in a well-defined manner.

Orthotopic: relating to the tissue in which the cells of interest originate (e.g. the mammary gland for breast cells).

structures, with filled lumens consisting of epithelial cells. By contrast, cyclin D1 over-expression, which also resulted in increased acinus size, did so while retaining normal hollow lumens. These morphogenic differences are intriguing given the poor prognosis of ErbB2-positive breast cancers, and, importantly, would have been undetectable in the context of monolayer cultures (Fig. 1).

Normal epithelial cells are anchored to an underlying BM by transmembrane proteins called integrins, which provide contextual survival and/or motility signals to the overlying epithelium. During malignant transformation, the normal constellation of integrin proteins is altered, resulting in variant binding specificities for different extracellular matrix components. These alterations result in the disruption of normal epithelial architecture, with the subsequent potential for invasive growth. As such, breast cancer cells grown in 3D fail to generate normal acinus structures, but rather form irregular, disorganized clusters. In an attempt to reverse the dysmorphic (see Glossary) properties of breast cancer cells, researchers treated cancer cells with β 1-integrin blocking antibodies before culturing them in 3D (β 1-integrin is over-expressed by breast cancer cells). Antibody treatment caused the dysmorphic cancer cells to reorganize into normal-looking acini, displaying apical-basal polarity, BM, and normalized cell-cell contacts [5]. Inhibition of other signaling pathways, including the MAPK and PI3K pathways, was also able to induce reversion to a normal morphology. These findings are important, because they reveal potential targets for therapies directed against later-stage invasive carcinomas.

As the examples above illustrate, 3D models are useful for characterizing the effects that genes have on cancer cell behaviors, beyond the proliferation and apoptosis assays typical of studies relying exclusively on monolayer cultures. Moreover, in the context of cancer drug development, use of these models might facilitate the production of novel therapies geared towards reversing the dysmorphic properties of

advanced-stage carcinoma cells. The superiority of 3D models over standard cultures in the drug development process remains to be empirically established. Nevertheless, these 3D models have already revealed valuable insights into the contributions of molecular factors towards breast epithelial cell behaviors that, in some cases, could not have been identified through conventional monolayer culture strategies.

In vivo models

While *in vitro* models of breast cancer pathogenesis are cost-effective, expedient, and amenable to high-throughput screening, the full extent of most physiological processes is only observed in the context of a living animal. Therefore, studies of complex interactions, including those involved in immune surveillance, angiogenesis, and stromal-epithelial signaling, often require *in vivo* models to be meaningful.

In vivo models of breast cancer fall broadly into two categories: mouse models and xenograft models. We describe some of the recent developments for both types of models below.

Mouse models

A myriad of transgenic mouse models of cancer have been developed, with increasing sophistication over the years. Mouse models that use widespread oncogene-expression or tumor suppressor-inactivation rarely survive long enough to form mammary tumors, because the mice often succumb to tumors arising in other tissues. To circumvent this problem, researchers refined their approach and targeted gene expression or loss specifically to the mammary epithelium, in many cases by placing the regulation of transgene expression under the control of the mouse mammary tumor virus (MMTV) promoter.

Her2/Neu models

The Neu/Her2/ErbB2 oncogene is over-expressed in ~30% of breast cancers, and is correlated with invasive behavior and poor prognosis. Herceptin, which targets the Her2 receptor, is effective in the treatment of ~20% of erbB2-positive cancers. In an effort to develop an *in vivo* model of this important class of breast cancers, researchers expressed a mutated, activated *neu* allele under the control of the MMTV promoter, enabling mammary-specific expression [6]. This MMTV-*neu* model developed multifocal mammary tumors at approximately eight weeks of age and displayed progression to metastatic disease. A second MMTV-*neu* model, generated under identical conditions, yielded unifocal tumors, with a significantly longer latency, which also progress to metastasis.

Given the immense significance of metastasis with regard to the clinical prognosis of cancer patients, investigators have attempted to elucidate molecular factors modulating the metastatic phenotype. Accordingly, several second-generation

models have been developed in which MMTV-neu mice are crossed with other mammary-specific transgenic mice, to study potential modifiers of the metastatic phenotype. One such model is the MMTV-neu \times MMTV-TGF- β 1 mouse; these bigenic mice develop tumors with the same frequency and latency as MMTV-neu mice [7,8]. However, the tumors that arise in the TGF- β 1-crossed mice have reduced rates of apoptosis, release increased numbers of cancer cells into the circulation, and form lung metastasis. TGF- β is implicated in tumor invasion and progression, and these mice therefore serve as relevant models for studying the contributions of TGF- β to breast cancer metastasis.

p53-deficiency models and hormone-responsive models

The observation that 30%–60% of breast cancers lack functional p53 has spurred the generation of several mouse models of p53-deficiency mediated breast cancer. The first p53 knockout mice, developed on the C57Bl6/129Sv strain ~15 years ago, die largely of sarcomas and lymphomas. Because these mice lacked a mammary cancer phenotype, the knockout allele was transferred to a strain known to be more susceptible to spontaneous mammary tumors. As a result, when back-crossed onto the Balb/C strain, over 50% of p53^{+/-} heterozygotes contract mammary tumors within a year [9]. However, due to the constitutive nature of p53 loss, these mice develop tumors in various other tissues as well.

To construct a tissue-specific model of p53 deficiency-mediated breast cancer, researchers engrafted mammary gland-derived donor p53^{-/-} epithelium into surgically cleared mammary glands of recipient mice. The clearing procedure physically removes the portion of the breast tissue containing the mouse's epithelium, before full infiltration of the epithelial tree into the glandular tissue. This results in mammary fat pads (see Glossary) devoid of endogenous epithelium, and allows for the introduction of donor epithelium of a specified genotype [10]. In this system, mammary tumors develop in essentially all of the transplanted mice, with one-third of the tumors giving rise to metastases. Additionally, the great majority of p53^{-/-} premalignant epithelial cell lines derived from these mice are estrogen receptor (ER)-positive and responsive to ovarian hormones [11]. Moreover, the tumors that develop in these mice are largely ER-negative. These observations are significant, because while overt human breast cancers segregate into ER-positive and -negative classes, human premalignant breast lesions are invariably ER-positive. Thus, this mouse model represents an excellent opportunity to study the loss of hormone sensitivity that accompanies malignant conversion in ~30% of human breast cancers.

More recently, researchers have generated a p53-based model of ER-positive mammary cancers. p53 loss was targeted to the mammary epithelium by adopting the well-established Cre-lox (see Glossary) strategy. Specifically, MMTV-Cre mice

were crossed with transgenic mice that express a p53 allele flanked by two lox sequences (a "floxed" allele). Cre recombinase expression results in a recombination-mediated excision of the floxed-p53 allele from the genome of cells comprising the mammary epithelium. These breast epithelium-specific p53-deficient mice develop mammary tumors at a high frequency, some of which are also ER-positive [12]. Moreover, tumors arising in these mice display high rates of metastases to the lung and liver.

BRCA1-deficiency models

The importance of BRCA1 mutations in predisposing women to breast and ovarian cancers is well established on the basis of numerous epidemiological studies [1]. However, the molecular basis for these predispositions remains a mystery. Indeed, beyond a general role in DNA repair and/or maintaining genomic integrity, little is known about the breast-specific functions of BRCA1. This fact has, in part, hindered the development of appropriate BRCA1-based mouse models of breast cancer. Because BRCA1-knockout mice die during embryogenesis, due to increased p53-dependent apoptosis, researchers have attempted to bypass the embryonic lethality by constructing mammary-specific BRCA1 deletion models. However, tumors in such mice only develop after a very long latency, and, moreover, invariably exhibit loss of the p53 protein [13]. Beyond providing a generalized mutator phenotype that promotes p53 loss, a specific contribution of BRCA1 loss to mouse mammary tumorigenesis remains questionable. This notion is supported by experiments demonstrating that ablation of BRCA1 function in mouse mammary epithelium lacking p53 does not appear to accelerate tumorigenesis, compared to p53 loss alone [14]. Furthermore, a recent mouse model in which BRCA1 function is ablated using a pan-epithelium specific promoter exhibits a spectrum of epithelial tumors, with the notable absence of a mammary tumor phenotype [15]. The generation of more refined BRCA1 mouse models of breast cancer will probably have to await further detailed elucidation of its function(s).

Human cell models

Mouse models of cancer have revolutionized research, and have facilitated the elucidation of genetic interactions central to the initiation and progression of this multistep disease. Nevertheless, in spite of the significant evolutionary conservation between the two species, there are important differences between the biology of mice and humans that are of relevance to studies of cancer. For example, fundamental differences in telomere regulation enable murine cells to bypass the requirement for telomerase upregulation, which is a rate-limiting step in human cancer formation. As another example, certain ligand-receptor interactions are incompatible between mice and humans. Additionally,

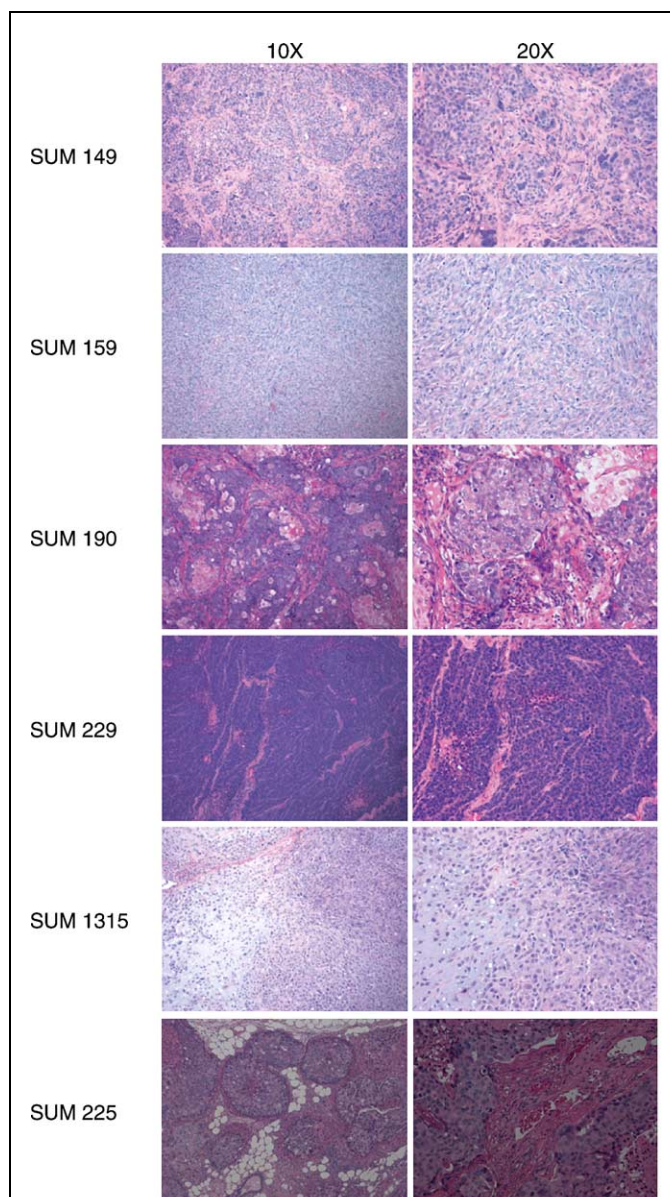
experiments have demonstrated important and significant differences in the ability to transform human cells, compared with cells of murine origin. For these reasons, it remains essential to develop models of cancer that employ human cells *in vivo*.

Traditional xenografts

For decades, researchers have been using immunocompromised athymic nude mice as vehicles into which to implant established human breast cancer cell lines. These xenograft systems, while convenient, do not replicate many of the histopathological properties breast cancers display in human patients. Most cell line-derived xenograft tumors fail to display appreciable stromal infiltration, and consist largely of proliferating epithelial cells. By contrast, human breast cancers frequently exhibit a reactive stroma (desmoplasia), which is believed to provide important contributions to tumor growth.

In general, the use of cell line-derived xenograft tumors has limited the study of heterotypic interactions in cancer, because most established cancer cell lines form aggressive tumors efficiently without requiring significant stromal involvement. Owing to the difficulty of successfully culturing cells from tumors themselves, most cancer cell lines have been derived from pleural effusions taken from fluids of advanced-stage patients. An important recent advance was made with the successful derivation of breast cancer cell lines from human breast cancer samples, using culture techniques that facilitate the outgrowth of cancer cells from primary tumor masses [16]. (http://www.cancer.med.umich.edu/breast_cell/Production/index.html). When these cell lines are orthotopically (see Glossary) injected into mammary glands of NOD/SCID mice, the resulting tumors display a wide spectrum of histologies that are, in general, different from those of standard xenograft tumors. In particular, these cell lines tend to display increased stromal involvement and extracellular matrix deposition (Fig. 2). Some of these lines exhibit highly invasive anaplastic histologies, where tumor cells can be found within blood and lymphatic vessels. In addition, at least one of these lines (SUM225) forms tumors that are indistinguishable from actual breast cancer samples. This line exhibits histologies of ductal carcinoma *in situ*, including tumor cell growth within murine mammary ducts (Fig. 3). As these SUM225 tumors grow larger, they become invasive and provoke a significant desmoplastic stromal response.

In addition to histologies, some of the genetic alterations in these lines have been characterized and include those commonly found in breast cancers. The continued establishment of novel cell lines from primary tumors, rather than exclusively from pleural effusions, will enable significantly improved replication of the properties of human cancers in the mouse.

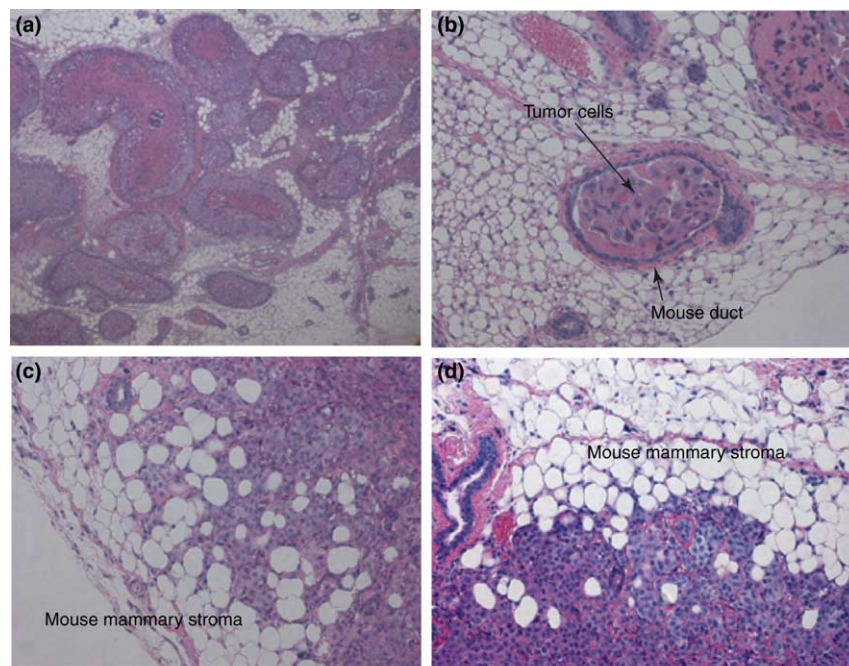


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Figure 2. Histologies of breast cancers from SUM cell line xenografts. Haematoxylin and eosin (H&E)-stained sections of human breast xenograft tumors from the various SUM cell lines that were introduced into the mammary glands of female NOD/SCID mice. Two different magnifications of the tumors (10X and 20X) are shown. The histologies vary widely among the cell lines. Well-stromalized tumors contain bands of stroma, seen as light-pink strands surrounding purple nests of tumor cells (SUM149, SUM225, SUM229, SUM190). By contrast, poorly stromalized tumors consist largely of sheets of tumor cells devoid of any stromal reaction (SUM1315, SUM159). Nevertheless, the SUM1315 and SUM159 lines both exhibit high amounts of basement membrane and extracellular matrix deposition, seen as light-pink/purple staining that is devoid of stromal cells.

Tissue-recombinants xenografts

Prostate cancer studies entered a new dimension when researchers discovered the important role of stromal cells in cancer pathogenesis. A tissue recombinant model was developed in which benign prostatic epithelium was co-mixed with either normal stromal cells or prostate cancer-derived



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Figure 3. Histology of SUM225 tumor behavior. Haematoxylin and eosin (H&E)-stained sections of the SUM225 cell line xenograft tumor. This cell line was isolated from a chest wall recurrence of ductal carcinoma in situ (DCIS) and the tumors that grow resemble the commonly found DCIS in actual human breast cancer samples (a). In addition to it growing out from the injection site, human breast cancer cells can also be readily detected within cross sections of mouse mammary ducts (b). When the tumor expands it becomes highly invasive into the adjacent mouse mammary stroma (c, d).

stromal cells, and the mixture implanted beneath the renal capsule of nude mice. The kidney capsule was chosen as a site of implantation because it provides a rich vascular environment conducive for xenograft outgrowth. Using this system, researchers were able to foster the growth of either benign prostate tissue or prostate cancer [17]. Recently, similar models have been employed to study the effects of stromal cells on breast epithelium. In one such model, investigators introduced stromal–epithelial mixtures, embedded in collagen gels, under the renal capsules of nude mice [18]. These studies demonstrated the growth of epithelial structures that resembled human ducts.

In another tissue-recombinant model, 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 [19]. This system allows for full developmental and functional outgrowth of human breast ducts and lobules, to the point of human milk production within the alveolar lumen. Using this system, investigators examined the influence of stromal cells on epithelial growth and morphology, through the engraftment of genetically modified human breast fibroblasts. These experiments indicated that appropriate stromal environments can facilitate normal breast development, and that altered environments can promote neoplastic growth.

In summary, tissue recombinant models are exciting novel systems that are ideal for experiments aimed at characterizing heterotypic interactions involved in breast cancer initiation and progression *in vivo*.

Breast cancer metastasis to bone

Breast cancer metastasis to bone is the most common event associated with the morbidity and mortality of the disease. Of all patients with metastatic breast cancer, 80%–95% of women develop skeletal metastases [1]. A recent study has shed light on the molecular pathways involved in breast cancer metastasis to bone. This work relied on a model in which MDA-MB-231 breast cancer cells are introduced into the circulation of nude mice by injection into the left ventricle of the heart [20]. The subpopulation of cancer cells that succeeded in forming metastatic nodules in the bones of the animals were re-introduced, through cardiac administration, for a second round of *in vivo* growth into a second animal. This iterative procedure enabled the investigators to select for a variant subline of the original population that could form skeletal metastases with high efficiency. This model system proved to be extremely useful in identifying some of the molecular requirements for osteotropic breast cancer metastases, and can prove similarly useful to test the efficacy of potential drugs in preventing skeletal metastasis.

Table 1. Comparison summary table

	<i>In vitro</i> models	<i>In vivo</i> models
Pros	Relatively cheap, expedient, amenable to high-throughput screening	Models multiple steps of tumorigenesis Incorporates multiple cell types, histologies, and processes
Cons	Unable to model complex aspects of the disease	Relatively expensive and time-intensive Impractical for large-scale screens
Best use of model	Molecular pathway identification and specific cellular processes	Drug development and testing and therapeutic intervention
How to get access to the model (e.g. Jackson Labs., www.cellml.org)	Literature American Type Cell Culture (ATCC) MCF10A: cat #CRL-10317 University of Michigan SUM cell lines: http://www.cancer.med.umich.edu/breast_cell/Production/index.html	Literature MMTV-cre: strain #003551 MMTV-neu: #002376 MMTV-TGFb: #002375
Relevant patents	5,846,536 6,123,941	4,736,866 5,925,803
References	[2–6]	[7–20]

In silico models

Although holding great promise for the future, *in silico* models of breast cancer are currently in their infancy, and lack any reliable predictive value. Therefore, we do not further explore this topic in this review.

Model comparison

The use of *in vitro* models allows for reliable and efficient screening of drug compounds that target specific cellular processes or signal transduction pathways. The reverse approach of identifying cellular targets of drugs that were originally discovered on the basis of *in vivo* effects can be very difficult and time-consuming. Nonetheless, numerous physiological concerns (including delivery, modification, toxicity, elimination, and permeability) make *in vivo* testing a necessary step in the drug development process.

Of relevance to both *in vivo* and *in vitro* studies, breast cancer cell lines are not all created equal. Tumor-derived cell lines that more closely replicate patient tumors are beginning to supplant pleural effusion-derived lines, the latter of which remain frequently used for largely historical reasons. For *in vitro* studies of epithelial cell morphogenesis, 3D cultures are far superior to standard 2D cultures.

The plethora of *in vivo* models of breast carcinogenesis can make choosing an appropriate model a daunting task. Mouse models of breast cancer continue to be refined, and are particularly relevant for studies of angiogenesis, metastasis, and tumor-host interactions. Novel human-mouse xenograft models capture breast cancer development within the microenvironment of reconstituted human tissues, and are

essential in situations where murine-human species incompatibilities are suspected to be relevant. See [Table 1](#).

Model translation to humans

Models are simplified approximations of a complex reality. It is important to choose models that simulate well the aspect of breast cancer intended to be studied. *In vitro* models are most appropriate for screening compounds that specifically target particular signaling pathways or observable cellular processes. The activities of compounds in such assays are often robust across *in vitro* systems. While it is generally assumed that *in vivo* models translate best to humans, there remains a large disconnect between the effectiveness of drugs in mouse models and their effectiveness in human patients.

Related articles

Debnath, J. *et al.* (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30, 256–268

Muraoka, R.S. *et al.* (2003) Increased malignancy of Neu-induced mammary tumors over-expressing active transforming growth factor beta1. *Mol. Cell Biol.* 23, 8691–8703

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Links

- http://www.cancer.gov/cancer_information/cancer_type/breast
- http://www.cancer.org/docroot/STT/content/STT_1x_Breast_Cancer_Facts_Figures_2003-2004.asp
- http://www.cancer.med.umich.edu/breast_cell/Production/index.html
- <http://mammary.nih.gov>

Next-generation transgenic and tissue recombinant models, as well as improved cell lines for orthotopic xenografts, can improve the translatability of mouse models to the clinic.

Conclusions

Numerous novel *in vitro* and *in vivo* models have and continue to be developed for breast cancer research. As research advances our ability to replicate cancer as it occurs in human patients, it is hoped that the effectiveness of drugs using *in vivo* models will be a good predictor of their utility in the clinic.

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Outstanding issues

- Improving the translatability of responses to therapies in animal models to the clinic.
- Incorporating various cell types in 3D cultures to better model heterotypic interactions *in vitro*.
- Improving and developing more xenograft-based tumor models that metastasize from an orthotopic site.

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