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Cancer

Models of breast cancer

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The intention of this review is to provide a succinct overview about the availability and relevance of the major categories of mouse models for breast cancer. The review concentrates on the latest achievements in developing genetically engineered mice with conditional knockout alleles or models that allow the inducible expression of oncogenes in mammary epithelial cells. In particular, we discuss the applicability of these models for drug target validation. Furthermore, we critically evaluate experimental designs for modeling cancer prevention and therapeutic intervention by genetic means *in vivo*.

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Introduction

The pharmaceutical industry uses model organisms, in particular the laboratory mouse (*Mus musculus*), for preclinical studies and toxicity testing. Besides testing drugs to ascertain their safety, researchers are now seeking animal models that target particular pathways and authentically replicate specific human diseases such as breast cancer. Experts repeatedly emphasize that inadequate animal models are one of the major hurdles in drug discovery and development. Identifying models for diseases like breast cancer, therefore, is a priority for many laboratories. The majority of human ailments are, however, polygenic or multifactorial diseases. Breast cancer is no exception in this regard because individual cases differ significantly in their morphology, histopathology, dependence on endogenous growth factors, their activation/inactivation of specific genes and, most of all, in their clinical outcome. Hence, there cannot be only one model for breast cancer but rather a myriad of models, each being unique to a different subtype or a particular aspect of the disease.

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Main categories of breast cancer models

Wild-type mice do not develop mammary tumors during their lifetime unless they are inbred strains that carry the mouse mammary tumor virus or other selected mutations. In all types of mouse models, mutations are introduced to initiate and speed up neoplastic transformation. Currently available mouse models for human breast cancer can be categorized into three main groups: (a) xenograft models; (b) chemically induced, virally induced or ionizing radiation-induced models; and (c) genetically engineered mice (GEM) such as transgenics and knockouts. More complex models rely on a combination of particular methodologies used to generate these three main types of mammary cancer models. For example, transgenic mice are being treated with ionizing radiation or chemical carcinogens to accelerate mammary tumorigenesis. Animal models from each of the main groups have their advantages and shortcomings that we discussed in more detail in a recent commentary [1].

Xenograft models

Conventional xenograft models are still widely used in preclinical trials. For a list of available breast cancer cell lines used in xenograft modeling, please refer to a recent article by Kim *et al.* [2]. Xenograft models are relatively inexpensive, easy to generate, and tumors appear after a relatively short

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latency. Unlike the majority of neoplastic lesions from chemically induced mouse models or GEMs, several human breast cancer cell lines are estrogen receptor (ERα)-positive. Unencumbered by intellectual property concerns, they are currently indispensable for preclinical testing of inhibitors of steroid receptor signaling and drug resistance studies. Nevertheless, these models are generally poor predictors of response to therapy in humans. Virtually nothing is known about the inciting genetic events in the parental tumors, from which these cell lines were derived. Therefore, xenograft models are less useful for proof-of-principle tests for molecularly targeted therapies. Also, it is unreasonable to assume that the genome of these cell lines is stable. In fact, additional mutations and cell selection (genetic drift) frequently occur *in vitro* under variable culture conditions. In addition, doubts as to their actual tumor of origin are factors that question the validity of such models [2]. It is surprising to see many breast cancer studies still using MDA-MB-435 cells (402 published articles in 2004 alone[1]), although it has been repeatedly shown that these cells and their derivatives express melanoma markers [3,4]. This issue is currently passionately debated. Because some other breast cancer cell lines are also suggested to express melanocyte-specific markers [5], it would be interesting to see how many primary tumors actually express these markers and whether cell culture conditions artificially amplify subtypes of cancer cells expressing melanoma markers.

A continuous need for chemically induced breast cancer models

Since the 1940s, many research laboratories have been utilizing chemical carcinogens, in particular polycyclic hydrocarbons (e.g. DMBA) and alkylating agents (e.g. MNU, ENU), to study mammary tumorigenesis in mice. Early studies demonstrated that there are strain differences in the susceptibility to particular agents. A comprehensive review by Medina and Thompson [6] describes in detail the effects of particular carcinogens on specific molecular alterations as well as the role of hormones and dietary factors as modulators for chemically induced mammary tumorigenesis.

Because we are now able to engineer mutations at precise locations within the mouse genome (see next paragraph), chemically induced tumor models seem to be outdated to many researchers in our field. It is, however, misleading to assume that only technological advances determine the superiority of one model over another. This is certainly not the case. For instance, like in humans, a full-term pregnancy significantly reduces the incidence of mammary tumorigenesis in chemically induced breast cancer models [7]. In the vast majority of transgenic breast cancer models generated over the last two decades, however, pregnancy considerably shortens the latency of mammary tumorigenesis. Therefore, many genetically engineered mice might be suitable to study particular aspects of pregnancy-associated mammary tumorigenesis but they are unable to recapitulate the long-term protective effects of a full-term pregnancy on breast cancer. Although this phenomenon is one of the best-studied epidemiological findings on breast cancer in human populations, the cellular and molecular basis for this observation has not been identified. In conclusion, to study the protective effect of pregnancy on breast cancer, chemically induced models are currently highly relevant. For more information on this subject, please refer to the summary report of the 2003 workshop on Early Reproductive Events and Breast Cancer (http://www.nci.nih.gov/cancerinfo/ere).

Genetically engineered mice (GEMs) for modeling breast cancer

Transgenic mice that express oncogenes under the mammary tumor virus long terminal repeat (MMTV-LTR) or other mammary-specific promoters such as the whey acidic protein gene (*Wap*) were the first generation of GEMs for modeling breast cancer. Since the pioneering work conducted by Leder and co-workers 20 years ago [8], hundreds of transgenic strains have been generated to test the biological relevance of several oncogenic pathways for the initiation of neoplastic transformation of mammary epithelial cells. An entire edition of the journal *Oncogene* published in January 2000 was dedicated to review some of the paramount breast cancer models. In addition, the consensus report of the Annapolis Meeting highlighted individual histopathological features present in the first generation of GEMs [9]. The most important lesson that transgenic mice taught us was that tumorigenesis is indeed a multistep process involving different signaling pathways. Again, Leder and co-workers led the way by demonstrating first that two oncogenes can act in synergism to accelerate neoplastic transformation [10].

Conventional knockouts with targeted mutations of tumor susceptibility genes represent the second generation of GEMs. For example, gene targeting since 1992 has generated more than 20 different germline mutations of the Trp53 gene alone. However, the tumor spectrum in these mice often differs from humans with inherited mutations. For instance, most p53-deficient mice succumb to lymphoid neoplasia before they develop carcinomas. The transplantation of mammary epithelia from knockout donors containing multipotent stem cells into epithelia-divested mammary fat pads of wild-type recipient mice is an elegant technique to bypass the shortcomings of conventional knockouts and to establish mice lacking tumor suppressor genes specifically in the mammary gland [11]. The introduction of missense mutations into tumor suppressor genes (i.e. the generation of knock-in mutants) is another approach to more faithfully mimic human genetic diseases. For example, mice heterozygous for a p53 mutant allele (R175H substitution) differed from

[1] PubMed search for 'MDA-MB-435 AND 2004'.

conventional p53 knockout mice in their tumor spectrum. They exhibited a significant increase in the number of carcinomas and a slight decrease in the number of lymphomas [12].

Thus far, GEMs of the first and second generation are used primarily to study the biological function of genes during normal development and tumorigenesis. The US Food and Drug Administration is considering altering the guidelines on preclinical testing for the carcinogenicity of pharmaceuticals and specific strains are now being used in selective chemoprevention and chemotherapy trials (for examples, please refer to a more comprehensive review by Van Dyke and Jacks [13]).

GEMs that allow an inducible overexpression of oncogenes

The first and the second generation of GEMs allow us to examine whether oncogenes or tumor suppressor genes are involved in tumor initiation. For the development of cancer drugs, in particular for drug target validation, it would be essential to know whether cancer-initiating or cancer-promoting genetic alterations are essential for the survival of neoplastic cells within progressing lesions. The scientific challenge of determining whether a multistage cancer process is reversible fueled the development of novel mouse models that overexpress oncogenes in a temporally and spatially controlled manner. There are several inducible systems that can be employed to express transgenes conditionally *in vivo* (for an overview on available techniques, please refer to a review by Mills [14]). Thus far, only tetracycline (tet)-based systems have been utilized successfully to regulate the expression of oncogenes in an inducible fashion in the mammary gland and other epithelial cell types. In a nutshell, a tetracycline-transactivator system [15] has three components: (I) a transgene that directs the expression of the tet-responsive transactivator protein (tTA) to a particular cell type, (II) a second transgene that controls the expression of the oncogene using the tet-operon linked to a minimal promoter derived from the human cytomegalovirus immediate early gene 1 (tet-op) and (III) a tetracycline derivative such as doxycycline (Fig. 1). The transactivator protein is a hybrid composed of the tetracycline repressor protein from *E. coli* transposon TN10 fused to the viral protein 16 (VP16) activation domain from the herpes simplex virus. The transgene expression of the oncogene under control of the tet-op sequence is suppressed by the administration of tetracycline (Tet-Off system). A mutated tetracycline repressor domain was utilized to generate a reverse transactivator (rtTA or Tet-On system) [16]. In this system, the rtTA binds operator sequences and activates the oncogenic transgene only when tetracycline is administered to the animal. While on sabatical at the laboratory of Peter Gruss at the Max-Planck-Institute in Goettingen (Germany), Priscilla Furth (University of Maryland) and Lothar Hennighausen (NIDDK, NIH) were first to adapt the tet-inducible system to transgenic mice [17]. Subsequently, these researchers developed transgenic mice that express the transactivator under the LTR of the mouse mammary tumor virus (MMTV-tTA; JAX® Stock #002618) [18]. These mice were bred to a transgenic strain carrying the simian virus 40 (SV40) T antigen (TAg)-coding sequence

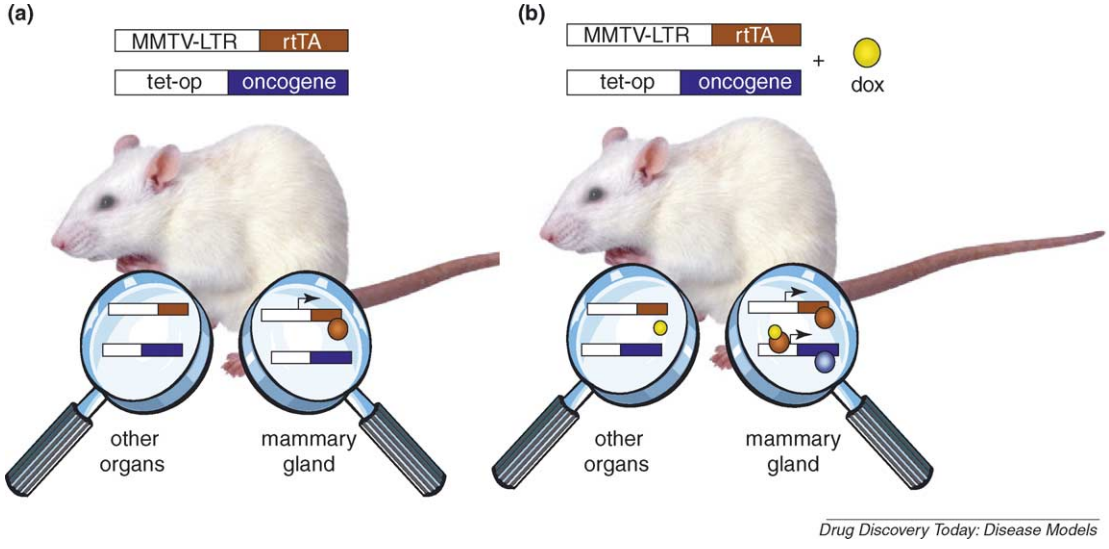

Figure 1. The bi-transgenic tetracycline-inducible system (Tet-On) allows a temporally and spatially controlled expression of oncogenes in the developing mammary gland of genetically engineered mice. (a) The tissue specificity of the tet-inducible system is mediated by regulatory elements (here the MMTV-LTR) that control the targeted expression of the reverse transactivator protein (rtTA). (b) The administration of a tetracycline analog (doxycycline, dox) regulates the binding of the transactivator protein to operator sequences within the promoter of the transgene, which regulates the expression of the oncogene. Withdrawal of doxycycline leads to a deactivation of the tet-op-driven oncogene.

linked to a tet-op promoter [19]. This animal model and the resulting landmark publication in the journal *Science* provided for the first time experimental evidence suggesting that tumorigenesis is reversible at an early stage of neoplastic transformation and that progressing tumor cells can become independent from the tumor-initiating event. Unfortunately, the expression of the transactivator protein in this particular MMTV-tTA strain exhibited less expression in the mammary gland as compared with other organs, and the analysis of tumorigenesis remained restricted to the salivary gland.

Six years later, the laboratory of Lewis Chodosh (University of Pennsylvania) generated a mouse strain that expresses the reverse transactivator under the control of the MMTV-LTR (MMTV-rtTA) [20]. Its efficient expression in the mammary gland was determined by reporter genes (luciferase, LacZ) fused to the minimal promoter/tet-op sequences. Although reporter gene expression and activity could be controlled in the mammary gland, its activation was also detected in several other tissues, including the salivary gland, thymus and seminal vesicle. Since its inauguration, this strain has been utilized in several experiments to generate tumor models that overexpress various oncogenes such as *ErbB2* [21], *Wnt-1* [22] and *c-Myc* [23]. Interestingly, the downregulation of *ErbB2* resulted in reversible pulmonary metastasis, whereas a sustained regression of *c-Myc*-induced mammary lesions following brief or prolonged *c-Myc* inactivation was not observed. These observations might suggest that, unlike *c-Myc*, targeting *ErbB2* could be therapeutically relevant for advanced stages of breast cancer. Regarding drug target validation, the tet-inducible system is clearly superior to the first generation of transgenic tumor models. Unfortunately, four years after their introduction, these tool mice are still not available to the broad scientific community through non-for-profit distributors such as the Jackson Laboratory or the Mouse Model for Human Cancer Consortium (MMHCC).

Another interesting technology to study signal transduction in transgenic breast cancer models was published recently by the laboratory of Jeff Rosen (Baylor College of Medicine). A drug-mediated dimerization of the fibroblast growth factor receptor 1 (Fgfr1), which acts independent of its natural ligand, induced the formation of mammary tumors [24]. Although this system does not affect the transcriptional regulation of the oncogene, it modulates signal transduction pathways through protein–protein interaction. In this regard, such models might better validate drugs in a pharmacological setting, in which small molecule inhibitors affect only particular functions of a protein. Additional functions of a protein, including a role as a scaffold for signal transduction, might not be affected by this approach.

GEMs with conditional knockout alleles

Both inducible systems (i.e. Tet-On and ligand-dimerization) described above require that inducible ligands are administered continuously to the animals to induce tumor formation. Also, current experimental designs that utilized these technologies only studied the importance of transforming oncogenes in progressing tumors. Thus far, they did not manipulate tumor suppressor proteins through, for example, the overexpression of dominant negative molecules or antisense constructs. Also, these techniques are not designed to deregulate the expression of downstream mediators or effectors of tet-inducible oncogenes. These limitations can be overcome in GEMs that carry conditional knockout alleles.

Conditional knockout mice on the basis of the Cre-lox technology were originally developed to bypass embryonic lethality observed in several conventional knockout mice. This includes mouse models that lack tumor suppressor genes implicated in breast carcinogenesis such as *Brca1*. Cre is a site-specific recombinase, which allows for a cell-type-specific deletion of floxed target genes in genetically engineered mice (Fig. 2). Again, it was the pioneering work of the laboratory of Lothar Hennighausen (NIH), which generated the first transgenic mouse strains (Wap-Cre and MMTV-Cre mice; JAX® Stock #003551-003553) that allow a mammary epithelial-specific deletion of genes at various stages during mammogenesis [25]. Both transgenic lines were employed shortly thereafter by the groups of Chuxia Deng and Lothar Hennighausen (both investigators were postdoctoral fellows at the Leder laboratory) to generate the first mouse model for hereditary human breast cancer by deleting the *Brca1* gene conditionally in mammary epithelial cells [26]. These

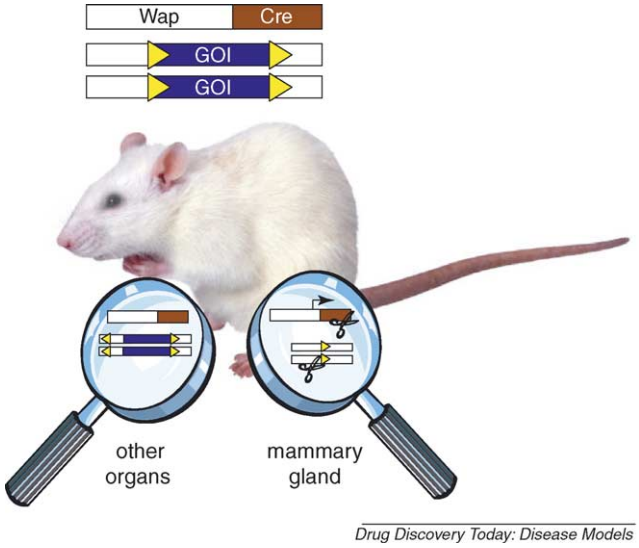

Figure 2. Wap-Cre-mediated mammary-specific deletion of two alleles of a gene of interest (GOI). The promoter of the *whey acidic protein* gene (*Wap*) targets the expression of the Cre recombinase ('molecular scissors') to the mammary gland. Cre recombinase binds to *loxP* (locus of X-ing over) recognition sites (yellow triangles). Cre is able to mediate the recombination and excision of DNA fragments located between two directly oriented *loxP* sites, leaving one *loxP* site in the chromosome.

ground-breaking experiments also addressed the genetic interaction between two tumor suppressor proteins (Brca1 and p53) during the initiation and promotion of mammary tumorigenesis. Since the Wap-Cre and MMTV-Cre mice became available in 1999 through various non-for-profit distributors, these strains were employed by several laboratories to generate a growing number of mouse models (more than 50 to date) that lack a variety of proteins regulating mammogenesis such as hormone receptors, signal transducers, as well as regulators for cell cycle and apoptosis. These conditional knockout models also taught us that some suggested breast cancer susceptibility genes are not involved in neoplastic transformation as previously reported from cell culture studies [27].

Conclusions

Originally designed to bypass embryonic lethality of conventional knockouts, Cre-lox-based conditional models are much more versatile. They can be utilized in several very diverse experimental settings. For example, conventional knockouts often cause pleiotropic effects. In particular, the ablation of hormones, their receptors, or additional downstream signal transducers frequently cause infertility or reduced fertility in females that, in turn, indirectly affect ductal elongation and mammary epithelial specification. Hence, conditional knockout models are helpful to separate systemic effects from cell intrinsic functions of genes. In addition to studying the function of genes during normal development, many conditional mutants will become important for breast cancer research. It is currently the standard to cross conventional knockouts (e.g. cyclin $D1^{-/-}$, $ER\alpha^{-/-}$, $Stat5^{-/-}$) into a variety of transgenic strains overexpressing different oncogenes to assess the effects of a gene ablation on mammary tumorigenesis [28–32]. Because tumorigenesis was absent or delayed in these complex models, the authors concluded that the functional inhibition of these targets might serve a suitable strategy for *therapy* in human lesions that express corresponding oncogenes [30]. Clearly, this is a premature conclusion. Because these females never developed mammary cancer, they might serve as models for cancer *prevention*, but these studies do not allow for conclusions about targeted cancer *therapy*. Two things are essential to model therapeutic intervention: (a) animal models need to develop progressing tumors and (b) the therapeutic target protein has to be expressed in neoplastic cells. Conditional knockout mice can be utilized to better model chemotherapy by genetic means. In its simplest experimental design, the Cre-lox technology can determine whether the genetic ablation of a particular gene is relevant for chemoprevention (deletion before tumor onset) and therapy (deletion in preneoplastic, neoplastic, or metastatic cells). It will be interesting to see how many of the suggested therapeutic targets will be validated when they are knocked out specifically in neoplastic cells using the Cre-lox technology. Whether one uses conventional and conditional knockout models to test the efficacy of particular proteins or pathways as molecular targets for prevention and therapy, the effect of the ablation of a gene on the expression of the oncogene needs to be addressed prior to the study.

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