ANIMAL MODELS OF BREAST CANCER

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ABSTRACT

Rodent models for breast cancer have for many decades provided unparalleled insights into cellular and molecular aspects of neoplastic transformation and tumorigenesis. Despite recent improvements in the fidelity of genetically engineered mice, rodent models are still being criticized by many colleagues for not being 'authentic' enough to the human disease. Motives for this criticism are manifold and range from a very general antipathy against the rodent model system to well-founded arguments that highlight physiological variations between species. Newly proposed differences in genetic pathways that cause cancer in humans and mice invigorated the ongoing discussion about the legitimacy of the murine system to model the human disease. The present commentary intends to stimulate a debate on this subject by providing the background about new developments in animal modeling, by disputing suggested limitations of genetically engineered mice, and by discussing improvements but also ambiguous expectations on the authenticity of xenograft models to faithfully mimic the human disease.

KEYWORDS: breast neoplasms, gene targeting, genetically engineered mice, genetic models, genetic technique, xenograft.

INTRODUCTION

Breast cancer is not a single disease but a diverse set of diseases characterized by heterogeneity in histology, genomic aberrations, and protein expression that influence treatment response and patient outcome. Importantly, this heterogeneity cannot be precisely defined through the traditional parameters of histopathology, tumor size, grade, nodal involvement, and biomarker expression that are currently used to guide treatment decisions. Although survival rates following diagnosis have improved in recent years, patients with recurrent disease are almost invariably treatment resistant, highlighting the need for
identifying new therapeutic strategies. The heterogeneity of breast cancer is a significant stumbling block for the application of personalized medicine approaches. For this strategy to be successful, a complete set of clinically relevant and validated biomarkers is required, along with the development of companion diagnostic tests to evaluate treatment responses.[1]

To date, these platforms do not exist for breast cancer. Nevertheless, a more refined breast cancer classification system has been developed over the past 15 years, integrating information based on gene expression arrays. Five intrinsic clusters were initially defined – luminal A, luminal B, basal-like, human epidermal growth factor 2 (HER2) over-expressing, and the normal breast-like subtypes. The precise characteristics of the latter group remain unclear. These subtypes can predict clinical behavior including overall survival, patterns of metastasis, and response to treatment.[2]-[5] More recently, other subtypes have been defined, notably the claudin-low tumors, which are predominantly triple-negative and exhibit mesenchymal features[2] and a stem cell-like expression signature.[2],[6] The different tumor subtypes are likely to result from distinct cells of origin, unique differentiation blockades, and different repertoires of mutations.[7] It is essential to decipher the molecular and cellular differences amongst the subtypes in order to develop a personalized medicine approach.

**A DIALECTIC LIASION:** various types of human breast cancer differ significantly in their morphology, their histopathology, their dependence on endogenous growth factors, their activation/inactivation of specific genes, and, most of all, their clinical outcome. For example, the latest studies distinguish at least four or five breast cancer types based solely on hierarchical clustering of gene expression profiles.[3],[4] The term 'breast cancer' thus does not stand for a specific disease, which is genetically and phenotypically uniform. It is, therefore, a misapprehension to envision a single model system that could mimic all features of breast cancer. Based on this fact alone, it is likely there will be more than just one 'authentic model' to recapitulate human breast cancer(s).

The general definition of a model is that it reflects only certain aspects of the original. From an ideal animal model for human breast cancer we expect that it faithfully reflects the human disease on various levels such as etiology, pathology, and genetics, that cancer originates only in the mammary gland, that neoplasia occur with a 100% incidence in treated or modified animals, whereas control animals do not develop tumors, and that tumorigenesis should have a relatively short latency.
THE MODELS: The animal models described in this issue are organized under five general sections representing the major groups of animal models currently available. Special attention is brought to models that are relatively new or novel and that are likely to receive increasing attention in the future, and to the application and relevance of these models to the study of the human disease. For example, a delineation of the early events in mammary tumorigenesis, and the cell types within the mammary gland that are susceptible to transformation, are critical for our understanding of the role of mammary biology in the process of carcinogenesis.

CHOICE OF MODEL: Each of the animal models described in this issue has specific advantages and disadvantages. It is important, therefore, for investigators to critically assess these factors in the light of the hypotheses they wish to test. For example, the chemically induced rodent models are excellent for looking at early events in the process of chemical carcinogenesis and for studying malignant progression. However, the choice of carcinogen can be of considerable importance.\textsuperscript{[4]} For example, 7,12-dimethylbenz(a)anthracene (DMBA) requires hepatic activation, with metabolites remaining in the animal for several days. The co-administration of treatments with DMBA requires careful consideration, since an agent could alter tumorigenicity by changing the pharmacokinetic properties of DMBA in a manner that produces potentially confounding results. N-nitrosomethylurea (NMU;MNU) is a direct acting carcinogen with a short half-life. However, DMBA-induced tumors have a relatively low incidence of activated ras expression (~25%), whereas up to 75% or more of NMU-induced tumors express activated ras.\textsuperscript{[13]} The use of NMU-induced tumors could thus tend to slant in vivo mechanistic/signal transduction studies towards ras-mediated events, even though mutation of ras is extremely rare in human breast cancer, with activation through amplification occurring in less than 25% of human breast tumors.\textsuperscript{[14]}

The human xenografts have the advantage of being human in origin, but are already transformed, and therefore have limited value for looking at very early events, e.g., initiation. The adaptation of the cell lines to in vitro growth could, ultimately, prove to be a further limitation of these models.\textsuperscript{[9]} The endocrine responsiveness of some of the transfection\textsuperscript{[15]} and xenograft models\textsuperscript{[16,17]} has "flip-flopped" from estrogen-dependent/antiestrogen responsive to estrogen-inhibited/antiestrogen resistant. The precise relevance of this pattern of endocrine responsiveness to the human disease remains to be definitively established. However, tamoxifen withdrawal responses are observed in some
breast cancer patients\textsuperscript{[18-20]}, providing a potential human corollary. The ability of endocrine agents, e.g., estrogens, antiestrogens, or aromatase inhibitors\textsuperscript{[21-26]}, to alter immune function in immune-deficient rodents requires careful consideration in experimental design and choice of model.\textsuperscript{[9]} The choice of site for inoculation also is of some importance for the use of xenografts\textsuperscript{[9]}, particularly for metastatic models.\textsuperscript{[11]}

It is likely that the transgenic mouse models will prove to be of considerable use, but their application can be limited. For example, the use of constructs that do not target the appropriate organ, e.g., with secreted gene products, can make differentiation between autocrine, paracrine, intracrine, and endocrine effects difficult. Where expression is constitutive throughout life, the time when the most critical events occur can be difficult to establish, particularly where there are no obvious morphologic changes associated with these events. The level of expression of the transgene can produce, or fail to produce, bio-logically relevant alterations because the level of expression is too high or too low.

The most critical concern is often the extent to which the model accurately reflects the human disease. Thus, the selection of the model must fit the purpose of the investigation. This is not always as widely appreciated as might be expected. Cost, availability, time constraints, and/or the experience/expertise/preference of the investigator can lead to the choice of a suboptimal model.

With such apparent diversity of choice, it is entirely possible that no single model will adequately address all the aspects of breast cancer biology. However, it is equally likely that, for any specific biological property, there will be at least one model that is adequately suited to the task. It is often more convincing when a particular observation is reproduced in more than one model, although with the rising cost of animal experimentation and the inevitable movement to-wards reducing the use of animal models, it is becoming more difficult to use multiple in vivo models. Whether the use of a single model is appropriate will depend upon the nature of the question, the availability and characteristics of the model(s), and the investigator's evaluation of the appropriate scientific concerns.

**THE APPROPRIATE USE OF ANIMALS:** The use of animals in biomedical research is becoming more closely scrutinized by both government and lay organizations. While
there are interest groups on both sides, the public perception is most effectively influenced by the activities of the vociferous opponents of the use of animals in biomedical research. The lack of a well defined "middle ground" on this divisive issue, which has not gone unnoticed, places the individual investigator in an often difficult position.

It is apparent that, were it at all possible, a significant number of individuals, both within and without the scientific community, would prefer that we reach a point where animal experimentation is no longer necessary. This is probably as widely held to be a laudable goal by scientists as it is by the lay community. It also seems likely that, at some point in the future, we will have developed our technologies to a degree where this is possible. Unfortunately, the realities of our current technologies clearly leave its achievement sometime in the future. There are still many biological processes that can only adequately be modeled/studied in vivo. For example, there are no ways to accurately predict tissue/organ toxicity, bioavailability, metabolism, elimination, or accumulation for all classes of experimental anti cancer agents purely from in vitro studies or by computer modeling. Screening drugs for safety prior to their administration to humans still requires animal models. Almost all tumor cell lines are inhibited by 100% serum, yet this is the environment into which systemically administered drugs are introduced. All tumor cells in the body exist in a complex and continuously changing environment comprised of multiple compartments, e.g., tumor-stromal interactions, infiltration of immunologic effectors, and cyclic changes in normal hormones occurring over minutes, days and months. The function and regulation of many of these compartments are poorly understood in both healthy and diseased tissues. The interactions among compartments, e.g., neurologic-endocrine, or immunologic-endocrine, are even less well understood. Until we have a better understanding of these complex systems, we have no way to de- velop in vitro models or computer simulations, let alone validate such alternative approaches. Para- doxically, it will likely be necessary to use data obtained from animal studies to validate and define the limitations of any ex vivo modeling systems generated.

To some extent, the requirements of a rigorous experimental design and application of the scientific method impose their own limitations on the use of animals. For example, reducing the number of animals in an experimental design to the point where significant statistical power is lost may invalidate the data and require the use of even more animals in subsequent
studies. Alternatively, the appropriate use of pilot studies, e.g., using a small number of animals to determine the dose range for a toxicity analysis, can significantly reduce/eliminate the number of animals subsequently exposed to what would otherwise prove to be lethal or highly toxic doses of an experimental agent or regimen.

The inability to currently use ex vivo systems, e.g., to adequately screen new agents for biological activity, assess toxicity, or understand the tumor host interactions and how these are influenced by therapies, is at the center of the issue concerning the use of animal models for breast cancer research. Currently, the alternatives to not using animals include either [a] using humans as an experimental model, [b] not screening compounds beyond ex vivo systems and assuming that these will predict human toxicity [c] perhaps accepting that our current cancer treatments, many of which are based on studies performed in animal models, are good enough. For many cancer patients and their families, lay people, scientists, and physicians, these are unrealistic/unacceptable choices. Hence the decision is made by many scientists/physicians, often but not always a difficult decision, to use animal models for cancer research. The final experimental design adopted for animal use will reflect each investigator's personal interpretation of the balance between the ethical and appropriate use of animals, the governmental and institutional requirements of the research environment, and the scientific constraints required to obtain valid data that ultimately will have a significant impact upon disease.

Once taken, the decision to use animal models in no way abrogates the investigator's obligation to responsibly use animals. For example the U.S., U.K., and many other governments already have enacted legislation that accepts the principle that the use of animals requires specific justification, a demonstration that ex vivo techniques are inapplicable, that studies are not unnecessarily duplicative, that appropriate anesthesia and analgesia are provided to limit pain and stress, and that the number of animals used should be clearly justified and minimized. These are frequently specified as prerequisites for obtaining approval to use animal models; they are entirely appropriate, as is their effective implementation. For some individuals, current legislation is merely considered insufficient, for others the use of animals will always be considered indefensible. Until we have technologies that enable us to eliminate the need to use animals, it is realistic to assume that the scientific/medical breast cancer research community, with society's broad-based approval, will likely continue with their use. In the interim, what is likely to change
is the way and extent to which animal models are used. The nature of these changes, and both how and when they occur, will reflect the continuing modification in attitudes and legislation emerging from the debate within our societies.

**DMBA INDUCED BREAST CANCER:** Numerous studies have shown that 7,12-dimethylbenz(a) anthracene (DMBA) can be used to induce experimental breast carcinomas in rats and that this process involves disruption of tissue redox balance; in turn, this suggests that biochemical and pathophysiological disturbances may result from oxidative damage.[16,17] Under normal physiological conditions, any free radicals generated in subcellular compartments would subsequently be scavenged by antioxidant defence systems of the corresponding cells.[18] However, such protective mechanisms can be broken easily by chemicals, such as DMBA, which disrupt the pro-oxidant–antioxidant balance, leading to cellular anomalies. Furthermore, owing to their high content of poly-unsaturated fatty acids, cellular membranes are highly susceptible to lipid peroxidation, and adverse alterations of the cell membrane can result in a pathological outcome.[19]

**Chemically-induced mammary tumorigenesis:** The two most widely used experimental systems for the study of mammary tumorigenesis are the models in which tumors are induced by 12-dimethylbenz(a)-anthracene (DMBA) or by N-methylnitrosourea (NMU). Tumor latency is, in general, inversely related to carcinogen dose. Tumor histology is influenced by carcinogen dose. There can be considerable variation in tumor incidence and latency between laboratories, and between experiments in the same laboratory. The susceptibility of the mammary gland to DMBA or NMU-induced carcinogenesis is strongly age-dependent, and is maximal when the carcinogens are administered to animals between the ages of approximately 45 and 60 days, that is, the age of sexual maturity.[23,24] Active organogenesis and high rate of proliferation of the glandular epithelium are characteristics of that period[15,24]; DMBA activation in the gland is also high, but it may not be a significant factor, since NMU, which is similarly most effective at that age, does not require activation.[25] The age-related changes in susceptibility are independent of dietary fat content; increased tumorigenesis occurs in rats fed high-fat diets at all ages tested.[25] In virgin rats treated with DMBA, tumors that develop are largely carcinomas, although the proportion can be altered by carcinogen dose and dietary fat.[25] The administration of DMBA to virgin rats of different ages induces tumors with an incidence which is directly proportional to the
density of highly proliferating terminal end buds (TEBs). A 100% incidence of carcinomas is obtained when DMBA is administered to rats aged 30 to 55 days, but the highest number of tumors per animal is observed when the carcinogen is given to animals when they are 40 to 46 days of age, a period when TEBs are most actively differentiating into alveolar buds (ABs). The sharp decrease in the number of TEBs observed in animals older than 55 days is also accompanied by a lower incidence of tumors as well as a lower number of tumors/animal.

PRENEOPLASTIC MODELS OF BREAST CANCER DEVELOPMENT: Most studies of preneoplasia and early progression of mammary tumors have utilized mouse HAN. Medina described a number of HAN lines that progress to carcinoma at different rates. When HAN tissue is transplanted into epithelium-free mammary fat pads, the transplanted epithelium expands to fill the mammary gland and resembles the normal mammary gland epithelium of pregnant mice. These HAN lines are not cultured cell lines, but rather tissue must be transplanted serially. The preneoplastic stage may be maintained indefinitely by serial transplantation but, if allowed to persist in situ, foci of carcinoma arise and rapidly growing tumors develop.

Although the HAN models are the basis for much information regarding the basic biology of mammary cancer, a number of differences in the histology and biology of mouse and human lesions exist. Unlike the HAN models, in which homogeneous lobuloalveolar lesions consistently give rise to rapidly growing adenocarcinomas within a few months, the breasts of women who are at high risk for proliferative breast disease are heterogeneous, and early breast cancer grows slowly.

The MCF10AT system is a xenograft model of progressive human proliferative breast disease. In this model the progression of a T24-Ha-ras-transformed derivative of normal-appearing MCF10A cells (ie MCF10AneoT) can be followed from a histologically precancerous stage to development of frank invasive carcinoma. In contrast to MCF10A cells, MCF10AneoT cells form persistent lesions in immunodeficient mice when 1 × 10^7 cells suspended in Matrigel are inoculated subcutaneously. MCF10AneoT cells and lines derived by alternating in vivo transplantation and in vitro culture (MCF10ATn) are collectively known as the MCF10AT system. MCF10AT cells grow in immunodeficient mice, in which, over a period of several months, a percentage of lesions undergo a sequence of progressive histologic changes. These changes mimic those observed in the breasts of women.
who are at high risk for breast cancer, and culminate in a significant proportion of grafts with frankly invasive carcinoma. The lesions formed by lines of the MCF10AT system are composed of a heterogeneous spectrum of ductular tissues with a range of morphology that includes mild hyperplasia, moderate hyperplasia, atypical ductal hyperplasia (ADH), carcinoma in situ, moderately differentiated and undifferentiated carcinoma, and histologically normal ducts.

Although it may be argued that the presence of mutant Ha-ras gene, a rare mutational event in human breast cancers, may have contributed to the transformation process by initiation and/or selection of a subpopulation of MCF10A cells, the presence of mutant Ha-ras is clearly not sufficient for histologic progression of MCF10AT cells. Indeed, MCF10AneoT clones that express high levels of protein encoded by mutant Ha-ras have been shown to lack the ability to form lesions.\(^8\) Furthermore, 50\% of human breast carcinomas express elevated levels of the protein encoded by normal Ha-ras.\(^9,10\)

An important feature that distinguishes MCF10AT cells from parental MCF10A cells is the presence of a functional wild-type estrogen receptor (ER). MCF10AT cells are able to respond to estrogen treatment in vitro with increase in size and ability to form colonies on soft agar\(^11,12\) and in vivo with rapid morphological conversion to ADH and ductal carcinoma in situ (DCIS).\(^13\) The effects of estrogen on histologic progression to ADH and DCIS appear to be ER-mediated, because treatment of animals with tamoxifen causes specific suppression of progression to ADH and DCIS.\(^14\) Also, the highest levels of ER in human breast tumors are generally observed in atypia and nonhigh-grade DCIS.\(^15\)

Much like human breast cancers that have lengthy natural histories, the lesions produced by premalignant MCF10AT xenografts are slow growing and not yet committed to a single pathway of cancer unless they are manipulated by hormonal supplementation. Remarkable features of the MCF10AT system are the reproducible generation of pre-malignant lesions and the few cytogenetic alterations present in the various MCF10AT generations that are not already present in the parental MCF10A cells.\(^4,6,16\) Establishment of tumorigenic variants of MCF10AT xenografts has been difficult. However, serial trocar passage of small pieces of MCF10AT lesions have yielded tumorigenic variants that produce heterogeneous tumors with prominent areas of DCIS and invasive carcinoma. One of the 14 clones derived from this variant reproducibly generates tumors with predominant comedo DCIS (MCF10DCIS.com) within a few weeks.\(^17,18\) Breast cancer is a heterogeneous disease, and the heterogeneous
spectrum of disease progression exhibited by this model is indicative of its multipotentiality. The absence of commitment to a single pathway of cancer, and its easy manipulability by hormonal agents, render the MCF10AT xenograft model the only currently available human model that has been shown to exhibit the histologic stigmata identified in women who are at high risk for developing breast cancer, and furthermore to undergo preneoplastic and neoplastic progression in vivo. The invasive carcinomas generated by MCF10AT xenografts are themselves heterogeneous. Different histologic differentiation (squamous, glandular, and undifferentiated) is seen, as well as distinctive immunohistochemical staining for breast cancer-associated markers such as erbB2, ER, cyclin D1, mucins, keratins, and p53. Therefore, although the cancers that develop from this single cell line derived from a single patient may represent only a subset of human breast cancer, the model is not limited to a single cancer phenotype.

METASTATIC BREAST CANCER MODELS

Although there are a number of human breast cancer lines that will metastasize in the xenograft setting, none fully reflect the spectrum of metastatic disease in humans. Numerous laboratories have been able to obtain subpopulations from both rat and mouse mammary tumors that differ in metastatic capacity. Generally, these models consist of paired subpopulations, one of which is highly metastatic and the other not, but specific deficiencies of the nonmetastatic variants are usually unknown, and so the mechanisms of metastatic failure are obscure. The metastatic process is a sequence of steps (invasion, intravasation, transport, arrest, extravasation, and growth) that must be accomplished by cancer cells before distant metastases are established. Nonmetastatic cell lines are unable to complete one or more steps in the metastatic cascade, whereas metastatic cell lines must be able to complete all of them. In order to follow the sequential spread and replication of tumor cells, a sensitive method to determine the presence of clonogenic tumor cells in host tissue is required. This can be accomplished by using tumor cells that are resistant to specific drugs and clonogenic assays in drug-containing selective medium that quickly kills host cells but not tumor cells. Tumor subpopulation lines 67, 168, 66, and 410.4 were isolated from a single, spontaneously arising mammary tumor from a Balb/cfC3H mouse. Sublines 168 FAR(diaminopurine-resistant) and 66C14 (thioguanine-resistant) were selected from the parental populations 168 and 66, respectively, and sublines 4T1 and 4T07 (both thioguanine-resistant) were derived from the parental population 410.4. The geneticin-resistant subline 67NR was obtained by transfection of line 67. These subpopulations are phenotypically heterogeneous for a number
of characteristics but share a common origin. Subpopulations are classified as metastatic on the basis of their ability to metastasize spontaneously from the orthotopic site.\cite{22,23} One line, 4T1, metastasizes to the lung, liver, bone, and brain via the hematogenous route, whereas 66c14 metastasizes to the lung and liver via the lymphatics. Sublines 67, 168FAR and 4T07 are highly tumorigenic, but fail to metastasize at different steps. The nonmetastatic 67NR cells fail to leave the primary site; 168FAR cells reach the regional lymph nodes but fail to produce nodules and do not advance past the nodes; and, although 4T07 cells may be recovered from the blood and lungs, visible metastases never develop. Thus, this comprehensive set of sublines offers the potential to correlate specific genetic alterations with specific steps in the metastatic process, as well as to test antimetastatic therapies for their ability to interfere with known stages of the process. The metastatic lines show a distribution similar to that of human breast cancer. In addition, 4T1 is one of the very few lines of any origin that spontaneously metastasizes to bone.

CONCLUSIONS

Mouse models for breast cancer are valuable to study molecular pathways of neoplastic transformation and tumorigenesis in vivo, and they serve as tools for selected preclinical trials. Breast cancer is not genetically and phenotypically uniform, and therefore one model system will never be enough to recapitulate various forms of the disease. Whether a model system is 'authentic' to human breast cancer is determined by its capability to reflect certain features of the disease, but it is unrealistic to expect that one model can mimic all aspects of human breast cancers. The superiority of one model over another largely depends on the scientific hypothesis, on experimental design, and on the type of study that one wishes to perform.

REFERENCES


