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Human breast cancer cell line xenografts as models of breast cancer — The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines

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Summary

The ability to maintain and study human tissues in an *in vivo* environment has proved to be a valuable tool in breast cancer research for several decades. The most widely studied tissues have been xenografts of established human breast cancer cell lines into athymic nude mice. Human breast tumor xenografts provide the opportunity to study various important interactions between the tumor and host tissues, including endocrinologic, immunologic, and tumor-stroma interactions. The nude mouse is not the only immune-deficient recipient system in which to study xenografts. Additional single and combined mutant strains have been used successfully, including mice homozygous for the severe combined immune deficiency mutation (*scid*), both the beige (*bg*) and nude (*nu*) mutations in combination (*bg/nu*), and mice bearing the combined *bg/nu/xid* mutations. The differing immunobiologies are discussed, with particular reference to the immunobiology of breast cancer, as are the characteristics of several of the more frequently utilized breast cancer xenografts and cell lines. The ability of several endocrine treatments to modulate effectors of cell mediated immunity, *e.g.*, estrogens and antiestrogens, and the effect of site of inoculation on tumor take and metastasis, also are described.

Introduction

The incidence of breast cancer mortality has continued to rise over the past thirty years, despite the innovation of various cytotoxic and endocrine therapies. It is clearly important to generate new and innovative therapies based upon our developing knowledge of the cellular and molecular factors driving breast cancer growth and progression. In this regard, the application of human xenograft models has much to offer both for increasing our understanding of malignant progression, and for the development and screening of novel therapies.

While there are several classes of rodent models for breast cancer, human tumor xenografts provide the unique opportunity to study the regulation of human cell growth and metastasis in an *in vivo* environment. While breast tumor biopsies can be established directly in nude mice, the take rate is generally low [1]. However, it is clear that a significant proportion of the established human

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breast cancer cell lines are tumorigenic in nude mice. Several have recently been determined to be metastatic in immune-compromised rodents. Human breast cancer xenografts have been widely used to study breast cancer progression, the effects of gene expression on tumorigenicity, and acquisition of antiestrogen resistance, and to screen new endocrine and cytotoxic agents. However, a major restriction has been the lack of a diverse panel of estrogen receptor (ER) positive and hormone-dependent cell lines. There are only three major hormone-dependent cell lines available, MCF-7 (the most widely used breast cancer cell line) [2], T47D [3], and ZR-75-1 [4]. The majority of breast cancer cell lines, and almost all of those with a significant inherent metastatic ability, as opposed to transfected cell line variants, are ER negative and hormone-unresponsive [5].

The development of additional breast cancer models is clearly of some importance. However, the nude mouse is not the only immune-compromised rodent available in which to establish xenografts. The additional immune-compromised systems will be briefly introduced in the following sections, and an indication of their diverse immunobiologies provided. The effects of endocrine manipulations on immunity and their importance for the use of breast cancer xenografts also will be addressed. Since the immunobiology of the rodent systems and the immunobiology of breast cancer are central to the use of xenografts, a brief discussion of these systems also is included. It is hoped that these discussions will encourage investigators to broaden the use of different immune-deficient systems, and to establish novel human breast cancer xenograft models.

Immunosurveillance of neoplastic cells: general comments

The precise role of immunosurveillance in breast and other cancers is unclear. In severely immune-compromised individuals, *e.g.*, patients with AIDS, there is evidence for an increased cancer risk. In normal women, both humoral and cell-mediated immunities likely contribute to the suppression of malignancy. The humoral aspects are difficult to study in experimental animal models, since almost all fully immune-competent animals reject xenografts. However, the role of cell-mediated immunity (CMI) can be modeled *in vivo*, at least partly, by using the diverse immune-compromised rodent models described below. While this is not the primary use of immune-compromised models, an understanding of the role of CMI in breast cancer, and of the immunobiologies of the different animal models, is important for the use of xenograft models of breast cancer.

Immunosurveillance of neoplastic cells: breast cancer

Cell-mediated immunity has been implicated in the pathogenesis of breast cancer, but its precise role remains to be established. For example, the skin window procedure, which provides an estimate of the extent of CMI, correlates inversely with metastatic disease [6,7]. Low levels of natural killer (NK) cell activity are associated with familial breast cancer [8], and with patients with stage III/IV disease [9-11]. NK cell activity is generally low or absent in the axillary lymph nodes of patients with demonstrable metastatic disease [12,13], although lymphokine-activated killer (LAK) precursor cells are often present [13]. There appears to be an inverse relationship between ER expression and NK activity [14,15]. ER-positive tumors have fewer T-cells when compared with ER positive tumors [9]. Aminoglutethimide reduces serum estrogens and increases NK activity in breast cancer patients [16].

The precise role of CMI and its effector cells in regulating the growth/tumorigenicity of breast cancer is unknown. The use of Matrigel (Collaborative Research Inc., Bedford MA) to increase xenograft take rate [17,18] also suggests that an effective barrier could protect cells from

locally infiltrating CMI effectors. The evidence from clinical studies implicates CMI effectors, at least to some degree, in the control of breast cancer growth. The diversity of immune-deficient models currently available provides a potential means to more adequately determine, in an experimental animal model, the likely contribution of some aspects of CMI to the control of neoplasia and metastasis of human breast cancer cells *in vivo*.

Immunosurveillance of neoplastic cells: Natural Killer (NK) and Lymphokine-Activated Killer (LAK) cells

Both cellular and humoral immunities participate in the immunologic response to neoplasia. Most of the immune-deficient animal models used in breast cancer research lack T-cell-mediated immunity. T-cell independent immunosurveillance, *e.g.*, aspects of cell-mediated immunity, is thought to consist of a number of lymphoid cells, most notably NK cells, LAK precursor cells, and macrophages [19]. Both NK and LAK cells are distinct from cytotoxic T-lymphocytes, lysing cells lacking significant expression of the MHC genes. NK and LAK cells can infiltrate solid tumors and malignant effusions [20].

NK cells make up approximately 1-2.5% of peripheral lymphocytes and have been widely demonstrated to possess antitumor activity [19]. Since NK activity may also contribute to control of metastasis [19,21-23], the poor metastatic potential of most human xenografts growing in nude mice may reflect their elevated NK cell activities [19,22-24]. Some tumors appear to be able to suppress NK activity [25].

LAK cells are clearly distinct from NK cells, a determination initially derived from studies of mice bearing different immune-deficiency mutations, *i.e., nu* and *bg* [26]. LAK cells are capable of killing neoplastic cells, and can kill tumor cells resistant to NK cytolysis [27]. Some tumors produce material capable of blocking the development of LAK cells from their precursors [28].

Immunosurveillance of neoplastic cells: macrophages

Macrophages are widely observed to infiltrate solid tumors [9,29,30] and can kill tumor cells by both phagocytic and non-phagocytic processes [30]. Non-phagocytic cytolysis may include the release of lysosomal enzymes by exocytosis. Macrophages may recognize some tumors on the basis of their abnormal growth [31] or by surface modifications [30] and can produce a non-specific cytolysis. The tumoricidal properties of macrophages are acquired following activation by contact with either the target cell and/or secreted products or by soluble lymphokines, *e.g.* interferon-γ [32].

The biology of macrophage-induced cytotoxicity is independent of the sensitivity of the target cells to lymphocyte or NK mediated cytolysis [33]. Tumor cells do not appear to acquire resistance to the cytotoxic effects of macrophages, in marked contrast to their ability to develop resistance to NK-mediated cytolysis [32-34]. The limiting factor in macrophage control of neoplasia appears to be effector:tumor cell ratio [32]. The sera from some cancer patients possess macrophage inhibitory activity [35], while some tumors secrete a macrophage colony-stimulating-like factor [30,36]. Macrophage infiltration is associated with tumor progression rather than inhibition, implying that some macrophages may secrete factors mitogenic for tumor cells [37].

Loss of immunosurveillance

Tumors proliferating successfully in the presence of cytotoxic host cells clearly indicate that the cells have evaded cytolytic effectors. The ability to become resistant to immunologic inhibition is thus a central problem in cancer biology and immunology. The precise mechanisms involved remain unknown, but modification or masking of surface antigens, the secretion of factors that inhibit NK, LAK, or macrophage activation/function, and an altered sensitivity to the direct cyto-

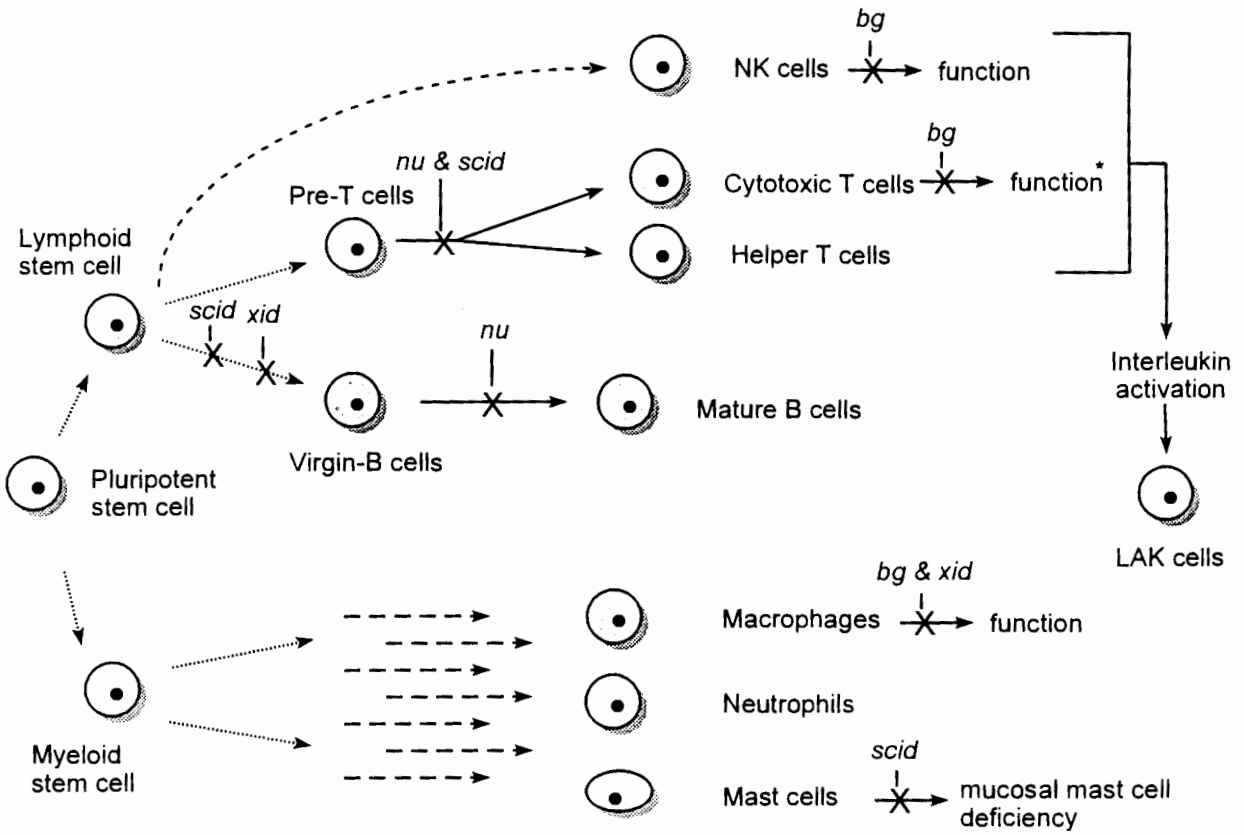

Figure 1. General representation of the likely defects in hematopoiesis associated with the different mutations. The figure is meant to be neither complete (*e.g.*, there are several myeloid lineages not shown) nor specific in terms of the precise/relative locations of the defects. For example, the precise origins of NK cells, and of the cells generating LAK activities, remain to be definitively established. The reader is referred to the text for more specific details. *The deficiencies should not necessarily be considered to represent a complete ablation of activity/function, *e.g.*, not all cytotoxic T cell functions are affected by the *bg* mutation.

lytic effects of effector cells, are probably involved [30]. The isolation of tumor cell variants that exhibit different biological properties in the presence of different immune effector systems should greatly facilitate the study of these complex tumor/immune interactions.

Immune-deficient rodent models

There are approximately 30 loci at which mutations can alter immune function in mice [38]. However, not all are amenable to human tumor xenograft studies. The most widely used immune-deficient rodent model is the nude mouse, mice homozygous for the *nu* mutation. In more recent years, other single and combined mutation bearing strains have become available and are gaining acceptance. These include mice bearing the beige (*bg*) and/or X-linked immunodeficiency (*xid*) mutations, also in combination with the *nu* mutation, and the single gene severe combined immunodeficiency (*scid*) mutation. The hematopoietic defects associated with these mutations are summarized in Figure 1.

Immunobiology of mice homozygous for the nude (*nu*) mutation

The nude phenotype (the mice have no hair; not to be confused with the hairless mutation) was

first described in Glasgow, Scotland, in 1962, with Pantelouris subsequently describing the predominating immune deficiency [39]. It was not long before Rygaard and Povlsen reported the ability to sustain human tumor xenografts in nude mice [40]. Since these early reports, the use of homozygous nude mice as recipients of tumor xenografts has become almost ubiquitous in cancer research. Breast cancer research is no exception, with a significant proportion of human breast cancer cell lines exhibiting tumorigenicity in nude mice [5]. While over 70 congenic strains of nude mice have been generated over the last 30 years, the respective immunologies and abilities to support xenografts appear strain dependent [41].

The nude mutation (*nu*) is located on mouse chromosome 11. Homozygous mice are essentially athymic [39], although a rudimentary thymus can be detected at necropsy in individual mice. The lack of a fully functional thymus is primarily responsible for the poor responses to T-dependent antigens [42], T-lymphocytes being generally at or below the limit of detection. These deficiencies may be reversed by reconstitution with T-cells [43-45]. B-cell maturation also is defective, at least that component dependent upon a fully functional thymus. The production of earlier forms of B-cells appears normal, since nude mice possess apparently normal virgin B-cells [46].

Despite the severe immunodeficiencies apparent in homozygous *nu/nu* mice, they are remarkably robust. This is almost certainly due to their remaining immune-competence. For example, *nu/nu* mice frequently exhibit an essentially normal response to T-cell-independent antigens [47]. Perhaps most notably, nude mice possess levels of NK cell activity that significantly exceed those present in normal and heterozygous *nu/+* mice of the same background [48,49]. The poor metastatic potential of most human xenografts growing in nude mice has been partly attributed to this elevated NK activity [19,22-24]. Splenocytes from *nu/nu* mice are capable of generating LAK cells at levels similar to normal mice [50]. The levels of tumoricidal macrophages are essentially equivalent in *nu/nu* and *nu/+* mice [51]. Serum IgM levels are similar to [47] or higher than [46] those in *nu/+* littermates, and while there is a significant decrease in the number of cells making IgG and IgA, both of these immunoglobulins can be detected in individual animals [44].

Clearly, nude mice are not totally immune-deficient. The elevated NK cell activity at least partly contributes to the relatively poor take rate of primary human breast tumors [1]. Of interest is the observation that tumor take rate in nude mice is frequently increased by encapsulating cells/biopsies in Matrigel [17,18]. This artificial basement membrane capsule could provide two important functions, (i) a protective barrier from the effects of tumoricidal macrophages, NK, and LAK precursor cells, and (ii) a more natural structural/dimensional environment for the tumor with access to several attachment and mitogenic molecules, *e.g.,* laminin, fibronectin, and type IV collagen.

The beige (*bg*) mutation

The *bg* mutation is located on mouse chromosome 13. Among several phenotypic modifications induced is a reduced synthesis of pigment granules in melanocytes which results in a light coat color. The general phenotype is considered analogous to Chédiak-Higashi syndrome in humans, a multisystem autosomal recessive disease, where afflicted individuals possess both structurally and functionally defective lysosomes. The main immune-deficiency exhibited by *bg/bg* mice is the direct result of a block in NK function [52,53]. However, there also are functional defects in T-cells, macrophages, and granulocytes [38]. For example, generation of cytotoxic T-cells is impaired in beige mice. A further complication in these mice is an apparent clotting disorder resulting from platelet dysfunction. Ovariectomies and hormone pellet supplementations are frequently performed with breast cancer xenografts, and the loss of animals during or shortly after surgery occurs

more frequently for mice with the *bg* mutation than for many of the other immune-deficient models.

The very low levels of NK activity in *bg* mice contrasts with the elevation in this activity in *nu* mice. The functional significance of the reduced NK cell and other immunologic activities is evidenced by the higher acceptance rate of low tumor cell inocula when compared with their heterozygous (*bg*/+) littermates [54].

Combined *bg/nu* mutations

Since the *bg* mutation essentially eliminates NK cell activity, it was hypothesized that combining the *bg* and *nu* mutations would eliminate the problems associated with the elevation of NK cell activity in nude mice. Double congenic *bg/nu* mice have now been available for several years [48,49]. Their phenotype is largely that predicted by the known effects of both single gene mutations. Thus, the defective B-cell maturation and impaired T-cell dependent responses are apparent. While the contribution of the *bg* mutation significantly reduces NK cell activity relative to homozygous *nu* and wild type (+/+) mice, the level remains higher than that in single mutant *bg/bg* mice [55-57]. Splenocytes from these mice cannot produce LAK activity, an observation that assisted in delineating NK cells from precursors of LAK cells [26]. Reduced IgA and IgM levels can be presumed as a result of the *nu* mutation, the levels being potentially dependent upon the mouse background. While more immune-deficient than mice bearing only the *nu* mutation, the *bg/nu* mice retain the clotting disorder produced by the *bg* mutation.

The X-linked immunodeficiency (*xid*) mutation

The *xid* mutation (X-chromosome) is associated with the X-linked lymphocyte regulated gene family [38]. Thus, males (*xid*/Y) and homozygous females are affected. While there are several impaired functions contributing to the immune-deficiencies in these mice, the major contributor is an impaired development of B-cells. Thus, B-cell colonies are not detected in *in vitro* assays [58]. A specific subset of mature B-cells is absent, and this appears to be the result of an inability of otherwise normal immature B-cells to respond to early activation signals.

The general response to thymus-independent type-II antigens is low in *xid* mice [38,59]. Macrophages are at least partially defective. In affected animals, macrophages produce low levels of IL-1 following stimulation with thymus-independent type-II antigens *in vitro* [38]. Immunoglobulin levels, particularly IgM and IgG_3, are low [60]. These mice, however, are not fully immune-compromised, producing essentially normal responses to thymus-independent type-I antigens [61].

Combined *bg/nu/xid* mutations

With the successful generation of homozygous *bg/nu* mice, it became apparent that a further reduction in immune-competence could potentially be achieved by also adding the *xid* mutation. Andriole *et al.* [26] generated a strain designated NIH III, which is homozygous for all three *bg/nu/xid* mutations. These mice have intermediate levels of NK cells and low levels of LAK precursor cells [26,62], and exhibit defects in the maturation of both B- and T-cells [63,64]. Macrophage activity/function may be partly defective, due to the contribution of the *xid* mutation [38]. The clotting disorder (*bg*) also remains.

The severe combined immune deficiency (*scid*) mutation

A relatively more recent addition to the single gene deficiency models is the severe combined immune deficiency (*scid*) mutation [65]. The locus occurs on mouse chromosome 16, the equi-

valent human mutation occurring at chromosome 8q11 [66]. The *scid* mutation produces mice with significantly smaller lymphoid organs [65,67]. There is a clear defect in the differentiation/maturation of lymphocytes [68], and both pre-B and B-cells are undetectable. The few remaining T-cells appear non-functional. In contrast, the entire myeloid lineage appear normal [68,69].

The *scid* mutation produces a deficiency in the rearrangement of genes encoding antigen-specific receptors on both B- and T-cells [67]. Several *scid* models are "leaky", generating small numbers of functional B- and T-cells. However, the majority of scid mice become "leaky" with age, *i.e.*, by 10-14 months of age [70]. It appears that the precursor lymphocytes are unable to adequately join the cleaved variable region segments as catalyzed by the immunoglobulin V(D)J recombinase [71]. In general, immunoglobulin levels are at or below the limit of detection. IgGs 2a, 2b, and 3a [38], IgM, and IgA [65,69] are rarely detected. However, in common with all immune-deficient models, there is individual variation, and some mice may produce detectable levels of two or more IgG isotypes and/or IgM [65]. Macrophages, NK cells [72-75], and LAK precursor cells [50] are essentially normal in *scid* mice. However, the mucosal mast cells appear deficient, perhaps due to a lack of specific lymphokines required for their development from progenitors [76].

In general, *scid* mice bearing this mutation are more severely immune-compromised than any of the other viable single gene mutation models. Nevertheless, the standard SPF environment is usually considered sufficient for their maintenance. It seems likely that, as these mice become more readily available and less expensive, they will be more widely utilized.

Impact of the different immunobiologies on xenografting

The different immunobiologies of these other rodent models provide viable and important alternatives to the nude mouse, with several occasionally supporting the growth or metastasis of xenografts that are considered either "non-tumorigenic" or "non-metastatic" based on their lack of growth in nude mouse models. The issue of which immune-deficient model is most appropriate for maintaining xenografts is somewhat controversial [77]. There is no clear and compelling evidence that *scid* or *bg/nu/xid* mice have a reproducibly higher overall take rate than *nu/nu* mice, with most investigators finding these models essentially equivalent [77-81]. However, the overall take rate can be a misleading indicator when an investigator wishes to evaluate the tumorigenicity or metastatic potential of *specific* cell lines/biopsies. An individual cell line may be tumorigenic or metastatic in one immune-deficient model and not in another, an observation that may or may not be a direct reflection of the different immunobiologies of the recipient mice. While this seems most likely to occur when assessing metastatic potential [70,80,82], individual variability among cell lines for tumorigenicity also cannot be easily discounted. We have suggested that, particularly where *in vivo* growth is assessed as part of the characterization of a new cell line, more than one model be used [83]. While this may be less important for cell lines of mammary origin than for other cell lines, the designation of a cell line as "non-tumorigenic in the nude mouse" or "non-metastatic in the nude mouse" seems most appropriate, at least until its lack of tumorigenicity/metastatic potential is confirmed in other models [83].

Use of immune-deficient rodent models for endocrinologic studies

Endocrinologic studies of hormone-responsive xenografts are frequently performed with breast cancer xenografts. Mice can be purchased ovariectomized directly from each of the major vendors, including Taconic (Germantown NY), Charles River (Wilmington MA), Harlan Sprague Dawley (Frederick MD) and Jackson Laboratories

(Bar Harbor ME). Adrenalectomized and hypophysectomized mice can generally also be obtained in this manner, although viability of the mice requires supplementation with glucocorticoids/mineralocorticoids.

There are several relatively straightforward approaches for studying additive endocrine treatments. We have routinely used the sustained release pellets provided by Innovative Research of America (Toledo, OH). For hormone-dependent cells including MCF-7, we routinely use the 60-day release 0.72 mg 17β-estradiol pellets. These are easily placed *s.c.* between the shoulder blades using a sterile 10 or 12 gauge trocar. Many experimental agents also can be administered by implantation of silastic pellets or mini osmotic pumps. We have found the Alzet osmotic pumps from Alza Corp. (Palo Alto, CA) effective for administering growth factors and other agents. The smaller sizes (100 µl and 200 µl) can be introduced into a small *s.c.* pocket, although we also have introduced these *i.p.* into NCr *nu/nu* mice with no adverse effects. The larger pumps are for use only in rats, and can be used successfully in nude rats (*rnu/rnu*).

The hypogonadal/*scid* (*hpg/scid*) mouse as an ovarian-ablation model

Ovariectomies are frequently performed on nude mice to provide an endocrinologic environment equivalent to that of postmenopausal women [84]. This produces additional cost to the investigator and stress to the animal. The Jackson Laboratories have recently generated a novel model that could alleviate some of these concerns. Mice bearing the hypogonadal (*hpg*) mutation [85] express a non-functional (truncated) LHRH protein [86]. Thus, these mice are hypogonadic, with almost undetectable levels of LH and FSH, and have serum estrogen and progestin levels essentially equivalent to those detected in ovariectomized mice [87]. By combining the *hpg* and *scid* mutations, it has been possible to generate an immune-compromised strain with a postmenopausal endocrinologic environment [88]. It seems likely that this model may become more widely used in the future, both as a potential model of postmenopausal breast cancer, and to support the growth of breast tumor xenografts.

Effect of endocrine manipulations on the immune response in rodents

Estrogen and antiestrogen supplementation of rodents has been widely used to study the endocrine regulation of breast cancer xenografts. However, several endocrine agents are known to influence those effectors of CMI that remain in immune-compromised rodents. For example, pharmacological but not physiological concentrations of E2 can inhibit NK activity in athymic nude mice [89-92]. This has been invoked as a potential explanation for an apparent ability of E2 supplementation to increase the growth of estrogen receptor negative breast cancer xenografts. Clearly, it is important to consider the ability of endocrine agents to perturb several effectors of cell-mediated immunity in study design.

It could be argued that the apparent endocrine responsiveness of xenografts is actually an immunologic, rather than direct endocrinologic, phenomenon. This seems unlikely for several reasons. Firstly, it is widely acknowledged that estrogens tend to produce a biphasic effect on NK cell activity, where NK levels rise for the first 30 days with an overall reduction in NK activity not observed until later [89-92]. However, most MCF-7 xenografts produce palpable tumors, and can frequently be seen to grow, during this initial period when NK cell activity is rising above the already elevated levels in nude mice. Secondly, the concentrations of estrogens reported to suppress NK activity [89-92] appear higher than those required to support MCF-7 xenografts [84, 93-96]. Approximately 300 pg/ml/day of estradiol is released from the widely used 60-day release 0.72 mg estrogen pellets produced by Innovative Research of America, Toledo OH [97]. Finally, MCF-7 cells still require estrogen supplementation for growth in *bg/nu/xid* mice (R. Clarke, unpublished observations), despite the

very low NK activity apparent in these mice [52,53].

Also of relevance is the observed ability of tamoxifen (TAM) to stimulate NK activity *in vivo* [62]. Prolonged treatment of MCF-7 xenografts produces a TAM and estrogen dependent phenotype [98,99], as does transfection with FGF4 [100] despite the opposing effects of these agents on NK cell activity (estrogens inhibit, antiestrogens stimulate). An ability of estrogen to apparently stimulate MDA-MB-231 (ER-negative) xenografts in nude mice has been specifically attributed to a reduction in cell loss [101].

Breast cancer cell lines as xenografts

Many of the breast cancer cell lines currently available are tumorigenic in the nude mouse. These cell lines most frequently produce adenocarcinomas *in vivo*, with the degree of glandular differentiation generally greater in the ER-positive lines. As with many human tumor xenografts, the characteristics of the human breast tumors are generally faithfully reconstituted in *in vivo* experimental systems. Thus, the ER positive/negative, PGR positive/negative, hormone dependent/independent, antiestrogen responsive/unresponsive, and drug responsive/resistant characteristics of cell lines observed *in vitro* are reflected in their respective *in vivo* growth responses. The characteristics of many of the breast cancer cell lines have recently been reviewed in detail [5].

Breast cancer cell lines as xenografts: hormone-dependence and acquisition of hormone-independence

The three most widely used hormone-dependent human breast cancer xenografts are the MCF-7 [2], ZR-75-1 [4], and T47D cell lines [3]. These cell lines are require some degree of estrogenic supplementation for tumorigenesis in nude mice. The estrogen-induced growth of each is inhibited by an appropriate dose of antiestrogen. While ER-positive tumors frequently invade locally and metastasize in patients, these xenografts are poorly invasive and rarely, if ever, are metastatic in nude mice.

The requirement for estrogen is surprising, since all three cell lines were derived from malignant effusions in postmenopausal women. We wished to determine whether the application of appropriate selective pressures could generate variants that no longer require E2 for growth *in vivo*. This was readily achieved by selecting for growth in the mammary fat pads of ovariectomized nude mice [96,102]. These mice have steroid hormone levels approximately equivalent to those found in postmenopausal women [84, 103].

Cells selected by one passage (MCF7/MIII) [96] or two passages (MCF7/LCC1) [102] (Table 1), retain ER and PGR expression, sensitivity to antiestrogens [96,102], and sensitivity to LHRH analogues [104]. Both variants exhibit a significant increase in metastatic potential [105]. Since tumors with these characteristics arise frequently in women, we have suggested that the phenotypes exhibited by the MCF7/MIII and MCF7/LCC1 cells more closely reflect the major ER/PGR positive, antiestrogen responsive phenotype found in postmenopausal women with breast cancer [106].

Breast cancer cell lines as xenografts: acquisition of antiestrogen resistance

While >70% of ER/PGR positive tumors respond to antiestrogens, the great majority of these will ultimately acquire a resistant phenotype [107]. The modeling of this progression has generally taken two forms, *in vitro* selection either in a stepwise manner [108] or by treatment with a high dose of drug [109], or *in vivo* selection against a continuous drug exposure [98,99]. A prolonged *in vivo* selection almost exclusively generates cells that are TAM dependent, and at least initially E2 inhibited [98,99]. This endocrinologic phenotype may be similar to the occasional TAM-withdrawal responses observed in patients [110-112].

Table 1. Derivation of the MCF-7 variants.

	ER	PGR	Citation
MCF-7 → nude mouse → MCF7/MIII → nude mouse → MCF7/LCC1	116,600	29,300	[102]
MCF7/LCC1 → 4-hydroxytamoxifen *in vitro* → MCF7/LCC2	91,300	6,400	[108]
MCF7/LCC1 → ICI 182,780 → MCF7/LCC9	133,200	ND	[117]
MDA-MB-435 (ER negative) → nude mouse → MDA-435/LCC6	ND	ND	[120]

All nude mice were ovariectomized and did not receive E2-supplementation. Steroid hormone receptor data are presented as sites/cell and represent means of 3 or more determinations. ND = no data.

In vitro antiestrogen selection of hormone-dependent cells, *e.g.* MCF-7 cells, can produce resistant variants. However, these variants often are either unstable [113-116], or have lost tumorigenicity in nude mice [96]. We have taken a different approach, selecting hormone-independent cells first *in vivo*, followed by a stepwise *in vitro* selection against the antiestrogen of choice [108, 117] (Table 1). This procedure facilitates generation of stable resistant variants that retain tumorigenicity [108]. Thus, selection of the MCF7/LCC1 cells by 4-hydroxytamoxifen treatment produced MCF7/LCC2 cells (Table 1). These cells are resistant to triphenylethylenes but not to steroidal antiestrogens, an apparently clinically relevant phenotype [106,118]. Selection of MCF7/LCC1 cells against ICI 182,780 also appears to produce stable resistant cells that retain tumorigenicity [117].

Of interest is the observation that, whether selected *in vivo* or *in vitro*, antiestrogen resistant xenografts and cell lines retain ER expression, and often some degree of endocrine-responsiveness [95,102,108]. The majority of breast cancer patients also retain ER expression upon relapse on TAM therapy [119]. It seems likely that, based on these experimental models, there may be several mechanisms through which breast tumors can become antiestrogen resistant.

Breast cancer cell lines as xenografts: hormone-unresponsive models

The majority of breast cancer cell lines are ER-negative. Consequently, they are considered hormone-unresponsive both *in vitro* and *in vivo*. While this has been our general experience, and that of many others, recently Friedl & Jordan [101] have demonstrated an apparently estrogenic stimulation of the MDA-MB-231 (ER negative) cell line growing in nude mice. This response does not appear to be immunologic; rather, the authors reported an estrogen-induced decrease in cell loss. Clearly, investigators should be aware of the potential for host-treatment interactions that can influence tumor growth in a manner that may be independent of direct agent-tumor interactions.

Most of the ER-negative xenografts produce poorly differentiated adenocarcinomas, relative to the ER-positive xenografts. Several are highly locally invasive. We have recently generated an ascites variant of the MDA-MB-435 cell line [120]. These cells, designated MDA-435/LCC6, appear to have arisen from a locally invasive mammary fat pad tumor that invaded into the peritoneal cavity. The ascites can be maintained either *in vivo*, or as a monolayer culture *in vitro*, and have a highly reproducible duration of survival from cell inoculation to morbidity/death. These cells provide a novel model in which to study the pathogenesis of malignant ascites, and the effect of *in vitro* [120] or *in vivo* gene transfer by retroviral vectors [121]. The MDA-435/LCC6 ascites variants grow equally well in *nu/nu* mice and *rnu/rnu* rats [122].

Breast cancer cell lines as xenografts: drug resistance models

Probably the most widely used breast cancer model of drug resistance is the MCF-7^{ADR} cell line. These cells were stepwise selected against

doxorubicin *in vitro*, and overexpress the gp170 product (P-glycoprotein, PGP) of the human MDR1 gene. MCF-7^{ADR} cells also have become ER-negative and resistant to antiestrogens [123]. Despite its frequent use to screen gp170-reversing agents, this cell line expresses several other potential multiple drug resistance mechanisms. MCF-7^{ADR}, but not MDR1-transduced MCF-7 cells (CL 10.3), are cross resistant to Tumor Necrosis Factor [124], an observation that indicates the presence of ADR resistance mechanisms in addition to gp170. While this would include changes in manganous superoxide dismutase expression/activity [124], the activities of glutathione transferase and topoisomerase II respectively also are altered in MCF-7^{ADR} cells [127,128]. Of particular concern is the observation that differences in the potency of isomers of fluphenthixol for reversing gp170 activity in MCF-7^{ADR} cells could not be confirmed in MDR1-transfected NIH 3T3 cells [129], implying activities through mechanisms other than gp170.

To produce breast cancer cell lines where the only major resistance mechanism is gp170, we have transduced both MCF-7 [130] and MDA-435/LCC6 cells [120] with the full length human MDR1 cDNA. The MCF-7 transduced cells, *e.g.* CL 10.3, retain ER expression and sensitivity to antiestrogens [130]. The MDA-435/LCC6 transduced cells, *i.e.* MDA-435/LCC6^{MDR1} remain ER-negative, and retain the ability to produce both rapidly proliferating solid tumors and malignant ascites [120].

Breast cancer cell lines as xenografts: metastatic models

Two ER-negative cell lines have been reported to produce hematologic metastases from solid tumor xenografts in nude mice. The MDA-MB-231 and MDA-MB-435 cell lines produce highly locally invasive tumors, with metastases reproducibly found in the lungs and other organs [131,132]. While the time to dissemination is often long, the incidence is sufficient to provide a reproducible metastatic model. The metastatic potential of the MDA-MB-435 cells appears amenable to modification by dietary factors [133].

While the ER-positive and hormone-independent MCF-7 variants (MCF7/MIII; MCF7/LCC1) exhibit increased metastatic potential [95,102], and can generate both lymphatic and hematologic metastases [105], the incidence and reproducibility are too low to provide viable models of metastasis [105]. In contrast, MCF-7 cells transfected with FGF-4 (kFGF) have a high and reproducible incidence of hematologic metastasis, probably the result of the significant increase in angiogenic potential. These xenografts have a significantly altered endocrine responsiveness, exhibiting a TAM-stimulated/E2-inhibited phenotype [100,134] similar to the *in vivo* TAM selected MCF-7 cells [98,99]. These cells are reviewed in detail in the accompanying article by McLeskey *et al.* [135].

Site of inoculation

There is now considerable evidence supporting the importance of the choice of site for implantation. Prior to the widespread availability of nude mice, the subrenal capsule was widely used. While there are other equally effect immune-privileged sites, these are becoming less frequently used since the ability of many cell lines to grow *s.c.* in immunocompromised rodents is now well established. While the *s.c.* site is convenient, it is likely that is not the optimal site for all xenografts.

While many breast cancer cell lines will grow *s.c.*, and frequently at other sites [136], it is not clear that this is the best approach. There is increasing evidence of the importance of tumor-host and tumor-stromal interactions in the progression of many cancers, including breast cancer [137]. The most appropriate site for breast tumor xenografts is the mammary fat pad (orthotopic site), a site we use routinely [95,102,105,108]. Orthotopic transplantation can significantly increase the take rate of tumors from several can-

cers, and occasionally facilitate metastatic spread that will not occur from a *s.c.* site [138-141]. When metastases in nude mice do occur, they tend to arise at sites representative of those observed in humans [105,132].

Concluding comments and future prospects

Human breast tumor xenografts have been of considerable value in studying several aspects of breast cancer biology. Their reproducibility, stability, and reflection of their human origin are clearly considerable strengths. However, the cell lines used to generate xenografts are not without limitations. For example, at some point in their genesis these cells have adapted to growth *in vitro*. Thus, the molecular, cellular, and metabolic changes in these cell lines may, or may not, fully reflect the human disease.

Despite the potential limitations, the similarities between these models and clinical breast cancer are substantial. The histology of these xenografts frequently closely reflects the variety of human adenocarcinomas. The inverse relationship between EGF-receptors and ER, and the association with a more aggressive and less well differentiated phenotype observed in cell lines and xenografts, holds true for the human disease [142-144]. The non-crossresistance of the TAM-resistant MCF7/LCC2 cells predicted the clinical responses to ICI,182780 in patients who responded and then failed TAM [106,118]. MCF-7 cells, when exposed to analogous selective pressures in mice, progress along a pathway apparently equivalent to the progression of many human breast cancers [95,106]. These observations suggest that the physiologic/endocrinologic environment of the rodent mammary fat pad may have significant similarity to the human mammary gland. The complex tumor/host interactions also may be similar for human xenografts in rodent mammary fat pads and the human disease.

There is a need for new xenograft models to facilitate research in at least two specific areas, endocrine responsiveness and metastasis. The majority of cell lines and xenografts are estrogen-unresponsive and ER-negative. Nevertheless, greater than 50% of all breast tumors express ER, and a significant proportion of these are endocrine responsive. It seems likely that there is considerable diversity in some of the cellular and molecular events driving the growth of these tumors. Thus, it is equally likely that the few endocrine-responsive models available inadequately reflect this potential diversity.

While metastasis may have several molecular events that occur independent of the cancer site, a number of the genes/proteins associated with this cascade are estrogen-regulated in breast cancer cells, *e.g.*, laminin receptor expression [145], chemoinvasion *in vitro* [96,146], and secretion of plasminogen activator [147]. Thus, some events may be regulated differently in breast cancer than in other cancers. There are relatively few endocrine responsive and metastatic cellular models in which to study the effect of hormones and antihormones on metastasis. While there are at least two endocrine unresponsive metastatic models, these also are likely to be too few to adequately reflect the diversity of the human disease.

The development of new breast cancer xenograft models, and variants of established models, will provide the opportunity to study host-tumor interactions in greater detail. This, and the emergence of novel immune-compromised and endocrine-compromised animal models, will further our ability to establish biologically relevant xenograft models of breast cancer.

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