

Stem/Progenitor Cells in Mouse Mammary Gland Development and Breast Cancer

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Breast cancer is a genetically and clinically heterogeneous disease. It is unclear whether different target cells contribute to this heterogeneity and which cell types are most susceptible to oncogenesis. Stem cells are speculated to be the cellular origin of at least a subset of human breast cancers. To begin to address these issues, we have isolated and characterized cell populations enriched in normal mammary stem/progenitors and have studied the expression of putative stem/progenitor markers in tumors derived from genetically engineered mice. Specifically, transgenic activation of Wnt signaling in the mammary gland induces tumors comprised of epithelial and myoepithelial cells harboring the same genetic defect implying that the tumor arose from transformation of a bipotent progenitor cell. On the other hand, transgenic activation of Neu signaling induces tumors comprising cells of more limited lineage capacity. Thus, the heterogeneity of different breast cancers may reflect the activation of different oncogenic pathways, different cellular targets in which these genetic changes occur, or both.

KEY WORDS: mammary gland; stem/progenitor cells; breast cancer; Wnt; ErbB2/HER2/Neu.

INTRODUCTION

The existence of adult mammary stem cells was established several decades ago when DeOme and his colleagues (1) observed that epithelium isolated from several different regions of mammary gland was able to generate normal mammary outgrowths containing ductal, alveolar, and myoepithelial cells. Further transplantation studies by Smith and Medina (2) demonstrated that these stem cells existed throughout the life-span of the mammary gland. Limiting dilution transplantation experiments using mammary epithelial cells tagged with the mouse mammary tumor virus (MMTV) indicated that clonal progenitors were capable of generating complete, functional,

mammary outgrowths when transplanted into the cleared mammary fat pads of recipient mice (3,4). Estimates of the frequency of these cells range from 1 per 1000 to 1 per 2000 mammary epithelial cells, and it has been suggested that the proportion of these cells remains relatively constant throughout mammary gland development (5). Thus, mammary stem cells must undergo both symmetric as well as asymmetric cell division, since the total number of mammary epithelial cells expand dramatically during pregnancy.

It has been speculated that stem cells may represent the cellular origin of cancer, since they exist quiescently over long periods of time, and could, therefore, accumulate multiple mutations over the life-span of an organism, ultimately giving rise to tumors when stimulated to proliferate (6). Women

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Abbreviations used: SP, side-population; Sca-1, stem cell antigen-1; MMTV, mouse mammary tumor virus; LRC, label-retaining cell; BCRP1, breast cancer resistance protein-1; MEC, mammary epithelial cell; TEB, terminal end bud; PyMT, polyoma middle T antigen; WAP, whey acidic protein.

exposed as teenagers to ionizing radiation are more susceptible to breast cancer than those exposed as adults, and these women developed breast cancer several decades following their initial exposure (7). These results, as well as the observation that almost 40% of breast cancers recur after 10 years following the diagnosis and removal of the primary tumor (8), suggest that a population of cells exists with an extremely long half life, similar to stem cells, which may be important in the etiology of breast cancer.

Highly tumorigenic cells with properties consistent with those of stem/progenitor cells have been isolated from cancers of several tissue types (9,10). Recently, such cells have been identified from human breast cancers (11). Transplantation of as few as several hundred of these cells, but not other cells from these cancers, into mammary fat pads of NOD/SCID etoposide-treated mice recapitulated the original tumors (11). These data suggest that cancer stem cells may exist in human breast cancer. This has led to the hypothesis that conventional therapies might not successfully remove all of these tumor stem cells, possibly as a consequence of the increased expression of members of the ABC family of drug transporters. This may ultimately result in recurrence or metastasis even when remarkable initial responses are observed clinically (12).

The following discourse presents a brief overview of the characterization of normal mammary stem/progenitors and their role in the etiology of a number of different genetically engineered mouse models of mammary cancer, with an emphasis on the studies from our own laboratories.

ISOLATION AND CHARACTERIZATION OF NORMAL MAMMARY STEM/PROGENITOR CELLS

Several methods are currently being utilized to isolate and study putative mammary stem/progenitor cells, including long-term bromodeoxyuridine (BrdU) labeling to identify label-retaining cells (LRCs), Hoechst dye efflux to identify side population (SP) properties, and potential stem cell-surface markers, such as stem cell antigen-1 (Sca-1, 13). In the hematopoietic system, cells that efflux Hoechst 33342 dye and segregate into a spur or "side-population" on flow cytometry analysis have been shown to represent a small fraction of the bone marrow. The efflux of the Hoechst dye is due to the

existence of the BCRP1 (breast cancer resistance protein-1)/ABCG2 transporter on the SP cells (see below). These cells are capable of recapitulating the bone marrow in irradiated mice, establishing their functional capacity as hematopoietic stem cells (14). A comparable SP population derived from mammary epithelial cells also has been demonstrated in both the mouse and human mammary glands (13, 15–17). Human SP cells have been shown to comprise about 5% of human breast epithelial cells defined by BER-EP4. They are enriched for ER α /PR+ cells relative to non-SP cells, and they co-express putative stem cell markers, including p21, cytokeratin 19, and Musashi, a translational regulator important in controlling asymmetric cell division in stem cells (17,18). Other studies have identified a bipotent, K18⁺/K14⁺ primitive precursor population in SP cells (16).

The BCRP1 pump was first identified in breast cancer cells resistant to topoisomerases and may be responsible for the mechanism of drug resistance in many types of cancer (19). An increase in SP cell survival following chemotherapy has been attributed to the expression of ABC transporters that have high drug-efflux capacity, including ABCG2 (BCRP1) and ABCA3, as highlighted by a recent study in solid tumors (20). Interestingly, the BCRP1 pump has been demonstrated to confer a survival advantage to hypoxic SP cells not through its function as a pump, but through interactions of the pump with heme (21).

The SP fraction in the mouse mammary gland is enriched for long-term BrdU retaining LRCs as well as Sca-1 positive cells (13). Despite the toxicity of the Hoechst dye, transplantation of mouse mammary SP cells at limiting dilution into cleared mammary fat pads generates epithelial ductal and alveolar structures (13,15). However, enrichment in stem/progenitor activity has not been observed in the isolated SP cells, as compared to the non-SP cell population.

It is difficult to directly estimate the frequency of SP cells in the normal mammary gland, since this may be influenced by the method of isolation, the intrinsic toxicity of the Hoechst dye, and changes which may occur even during short-term primary culture of epithelial cells (see Smalley and Clarke, this issue). With these caveats, the SP population appears to represent approximately 0.5% or 1 per 200 cells. Thus, the SP population is 5- to 10-fold larger than the estimate of the frequency of stem cells determined by limiting dilution experiments, raising the need for

other molecular markers for isolating and identifying mammary stem cells.

Approximately 8% of the cells in the SP population retain BrdU, but even this subpopulation of SP cells may not be totally quiescent (54). Thus, the SP phenotype appears to be a useful surrogate for stem-like/progenitor cells, and may be comprised primarily of a “transient amplifying” progenitor population. It is interesting to note that our recent studies have indicated that the SP population of primary mouse mammary epithelial cells (MECs) are also more resistant to clinically relevant doses of radiation than non-SP cells (W. Woodward and J.M. Rosen, unpublished observations).

The mammary gland originates from the embryonic epidermis. While mammary and epidermal stem/progenitor cell populations are probably different, it is interesting to compare and contrast these stem/progenitor cell populations. For example, it has been reported that the mouse and human epidermis also contains SP cells, and that these cells constituted a subpopulation of the $\alpha 6$ integrin-positive basal cells of the mouse epidermis that are positive for Sca-1 (22,23). However, the epidermal SP cells did not express particularly high levels of $\beta 1$ -integrin, another marker of epidermal stem cells. In addition, they were not identical to the label-retaining population, but were cycling cells. Furthermore, keratinocytes positive for Sca-1 were located outside the stem cell-containing bulge area of the hair follicle.

Sca-1, also designated as Ly-6a, is a GPI-anchored membrane protein and a member of the Ly-6 family. It is expressed by murine bone marrow and muscle stem cell populations, and may function in T-cell activation or cell adhesion (24). Sca-1-positive mammary epithelial cells are capable of producing mammary outgrowths after transplantation into cleared murine mammary fat pads (13). In this case, limiting dilution reconstitution experiments have demonstrated that a population of cells in mouse mammary gland primary cultures expressing Sca-1 was required for outgrowth. One thousand Sca-1-enriched mouse primary culture cells were able to reconstitute the mammary glands of host mice cleared of the endogenous tissue (13). Moreover, transplantation of Sca-1-depleted primary culture cells resulted in poor outgrowth rates. These results established that functional mammary stem/progenitor cells can be isolated using Sca-1. Unfortunately,

while other members of the Ly-6 family are conserved between mouse and human, no Ly-6a homologue has yet been identified in the human genome.

The Sca-1 phenotype appears to have been retained in the immortalized COMMA-D mammary epithelial cell line derived originally from mid-pregnant Balb/c mice (25 and Dr. Marie-Ange Deugner, personal communication). Since Sca-1 is expressed on about 20–30% of freshly prepared mouse mammary primary cells (13), the Sca-1 positive cells in the mammary gland likely comprise a mixed population containing stem, progenitor and possibly some differentiated cells. In addition to transplantation studies, using a Sca-1-GFP knockin mouse, highly GFP-positive cells are located in the terminal end buds (TEBs). The brightest GFP expressing cells are found at the tips of growing ducts in what appears to be the highly proliferative cap cell layer, and weaker GFP-expressing cells are scattered along the mature ducts.

Interestingly, a subpopulation of cells in the TEBs appear to be doubly positive for keratin 6 as well as Sca-1 (S. Grimm and J.M. Rosen, unpublished observations). Keratin-6 expression is normally restricted to the body cells of TEBs during ductal morphogenesis and is rarely observed in the mature gland (26,27). Keratin-6 is, however, normally thought to be a marker of hyperproliferation, especially in the skin during wound healing (28). Therefore, it was somewhat surprising that keratin-6 expression was not observed in the highly proliferative mammary epithelial cells during pregnancy, but it was highly expressed in Wnt-1 induced hyperplasias (see below and 27). During embryonic mammary gland development keratin-6 expression has also been detected in the mammary anlagen (S. Grimm, personal communication). Thus, keratin 6 may also provide a marker for early mammary progenitors.

In summary, while the functional significance, if any, of BCRP1, Sca1, and K6 in mammary stem/progenitor cells remains to be established, these genes have provided useful markers for stem/progenitor cell isolation and characterization. Furthermore, as detailed in the following section, these markers have provided important insights into the etiology of a variety of different mouse models of mammary cancer with implications for understanding the heterogeneity of human breast cancer.

DO BREAST CANCERS ARISE FROM STEM CELLS?

As discussed in the Introduction, stem cells have been hypothesized to be the cells of origin of human cancers. There is strong evidence to support this hypothesis in the case of teratocarcinomas and leukemias (29,30). However, the cellular origin of breast cancer has been controversial. It is still technically challenging to directly ask whether breast cancers arise from stem cells or their more differentiated progeny. However, several outcomes can be expected for cancers that do arise from stem cells: 1) their premalignant lesions may have an expanded pool of stem cells, 2) a subset of cells within the tumors may retain markers of stem cells, 3) they may be comprised of multiple cell types, due to remnant differentiation of transformed stem cells, and, 4) deregulation of genes that normally regulate stem cell proliferation and differentiation may be found in these cancers. Although cancers that arise by dedifferentiation of differentiated cells may also have these features, the evidence for dedifferentiation is scarce for most cancers that have been studied to date (reviewed in 31).

Several signaling pathways are implicated in regulating mammary stem cells. Examples of these pathways are Wnt, Notch, Hedgehog, and TGF- β . Dysregulation of these signaling pathways in the mammary gland has been demonstrated to induce mammary tumors in genetically engineered mice (32,33). Stem cell studies of other tissue types have demonstrated that Wnt signaling plays a critical role in proliferation of stem cells and maintenance of their "stemness" (34–37). Therefore, we and others asked whether alteration of Wnt signaling in the mammary gland may cause an expansion of stem cells and consequently predispose them to tumors. Indeed, cells expressing keratin 6 are expanded in hyperplastic glands in both MMTV-Wnt-1 and MMTV- β -catenin transgenic mice, and cells expressing Sca-1 are also increased in mammary glands in MMTV-Wnt-1 transgenic mice (27). Furthermore, SP cells are increased by 15- and 3-fold in mammary glands in MMTV- β -catenin and in MMTV-Wnt-1 transgenic mice, respectively, in comparison to littermate controls (38). In addition, the potential of mammary cells from these transgenic lines to regenerate mammary glands after transplantation into cleared-fat pads is increased compared to that of the cells from littermate controls (38 and Zhijun Du and

YL, unpublished), although it is not known if this is unique to mammary cells in these lines or a general feature of all oncogene-stimulated mammary cells of transgenic mice.

In addition to the expansion of putative stem cells in these transgenic mice, stem cell markers are also detected in tumors in these mice. Keratin 6 is found in mammary tumors induced by either MMTV-Wnt-1 or MMTV- β -catenin, and Sca-1 is expressed in tumor cells in MMTV-Wnt-1-induced tumors (27). Consistent with observations in human cancers that only a small subset of tumor cells are tumor stem cells, only a small fraction of tumor cells in these models are keratin 6-positive, and the SP fraction of tumor cells in both Wnt and β -catenin transgenic mice is similar to that of normal mammary gland. However, Sca-1 is present in a larger fraction (60%) of tumor cells, probably reflecting its expression in both stem cells, progenitor cells, and some differentiated cells. In contrast to tumors induced by deregulated Wnt signaling, tumors induced by transgenic expression of Neu or polyoma middle T antigen (PyMT) do not express either keratin 6 or Sca-1 (27 and see below), suggesting that different oncogenes may either target distinct mammary progenitors or promote expansion of distinct mammary cell lineages and/or differentiation programs during tumorigenesis.

In support of the possibility that Wnt signaling pathway-induced cancers arise from stem cells that retain the ability to continue to differentiate into multiple cell types, we have found that pulmonary metastasis may also contain transformed progenitor cells based on the staining for keratin 6 and on the presence of multiple cell types including myoepithelial cells (YL, unpublished observations). Collectively, these data suggest a hypothesis that activating Wnt signaling induces transformation of stem/progenitor cells, which can continue to self-renew and differentiate into multiple cell types in tumors.

Cellular heterogeneity within a tumor can be indicative of continual differentiation of transformed stem cells. We and others have found that tumors in these genetically engineered mice contain both differentiated epithelial cells and myoepithelial cells (27,38,39). It should be noted that these myoepithelial cells are not normal cells recruited to the tumors because they have large pleomorphic nuclei and are disorganized. A more direct evidence that both epithelial and myoepithelial tumor cells arise from differentiation of a common progenitor comes from

the following experiment: mammary tumors arising in a cross between Wnt-1 and Pten± mice are comprised of approximately equal numbers of epithelial and myoepithelial cells; however, 70% of these tumors lose the wild type Pten allele completely when assayed by Southern blotting and by immunohistochemical staining using antibodies against Pten (27). Since it is unlikely that these two cell types sustained mutations independently, the mutation probably occurred in precursors to these differentiated tumor cells.

The majority of human breast cancers do not express appreciable amounts of myoepithelial cell markers. Interestingly, a subset of human breast cancers have been identified which express myoepithelial cell markers and keratin 17, a binding partner of keratin 6. These breast cancers have also been shown to upregulate components of Wnt signaling, including c-Myc, whose transgenic expression also leads to tumors of mixed cell types including myoepithelial cells and keratin 6-positive cells (40–43). This subset of human breast cancers has also been hypothesized to arise from stem/progenitor cells (44).

DO BREAST CANCER ARISE FROM MORE DIFFERENTIATED CELLS?

Cancers may also arise from nonstem or differentiated cells. Cells that have lost the ability to divide may reenter the cell cycle and evolve into tumors if an oncogene can drive these cells back into cell cycle. Although their long life-span allows stem cells to accumulate multiple genetic alterations, differentiated cells may also gain a mutation that can increase their life-span or immortalize them so that they will have a chance to accumulate additional mutations and eventually evolve into cancers. Unless dedifferentiation is involved, stem cell markers and nonepithelial cells are unlikely to be present in cancers that arise from these cells.

Tumors induced by the MMTV promoter-regulated overexpression of Neu and other genes (H-Ras and PyMT) that regulate the Neu signaling pathway lack the Sca-1 and keratin 6 markers and have no myoepithelial tumor cells (27), suggesting that Neu signaling might induce tumors from differentiated cells. Consistent with this possibility, mammary glands of mice carrying abnormally expressed Neu are enriched for differentiated ductal and alveolar epithelial cells, but contain few keratin 6-positive cells (27,45). In addition, the tumorigenic potential

of Neu is severely inhibited if Neu is activated in the early phase of mammary development or in mammary cells whose differentiation fate has been impaired (45,46).

In a recent report, Wagner and colleagues (47) created triple transgenic mice that carry the MMTV-Neu and WAP-Cre transgenes and the ROSA-STOP-LacZ reporter construct, which expresses LacZ only after the Cre recombinase has deleted the intervening STOP sequence between the ROSA promoter and LacZ. Most of the preneoplastic and neoplastic lesions in this triple transgenic line express β -galactosidase (47). Similar results are noted in a similar cross between MMTV-PyMT mice and these two reporter strains (47). These observations suggest that MMTV-Neu induces expansion of and tumorigenesis from a subpopulation of mammary cells that express the differentiation marker WAP, though some of these cells also seem to have the potential to contribute to both epithelial and myoepithelial cells upon transplantation (48).

WAP-positive cells are present as a minor subset in nulliparous mice, but are enriched in primiparous and multiparous animals; consequently, tumors evolved more rapidly in parous transgenic animals for Neu and PyMT (47). However, the transcriptional activity of MMTV is also known to be enhanced during pregnancy; thus, it is not clear whether the increased tumor evolution is due to the increased pool of the cellular target, the enhanced expression of the transgene, or both. Nevertheless, consistent with the hypothesis that Neu and PyMT induce tumors from more differentiated mammary cells, these tumors express alveolar cell marker κ -casein (45 and S. Huang and YL, unpublished observations, 49), but lack the expression of ductal cell marker NKCC1, a sodium, potassium, and chloride transporter (47). In contrast, tumors arising in MMTV-Wnt-1 mice do not express κ -casein (S. Huang and YL, unpublished observations), but a fraction of the tumor cells do express NKCC1 (47), supporting the hypothesis that Wnt induces tumors from stem cells and the transformed stem cells can continue to differentiate into heterogeneous cell types.

In summary, it appears that breast cancer may arise from both stem/progenitor cells and more differentiated cells. Cancers that do arise from stem cells may exhibit cellular heterogeneity; on the other hand, cancers that arise from more differentiated cells are likely to be more uniform in their cellular makeup. The transforming function of oncogenes seems to be influenced by the differentiation status

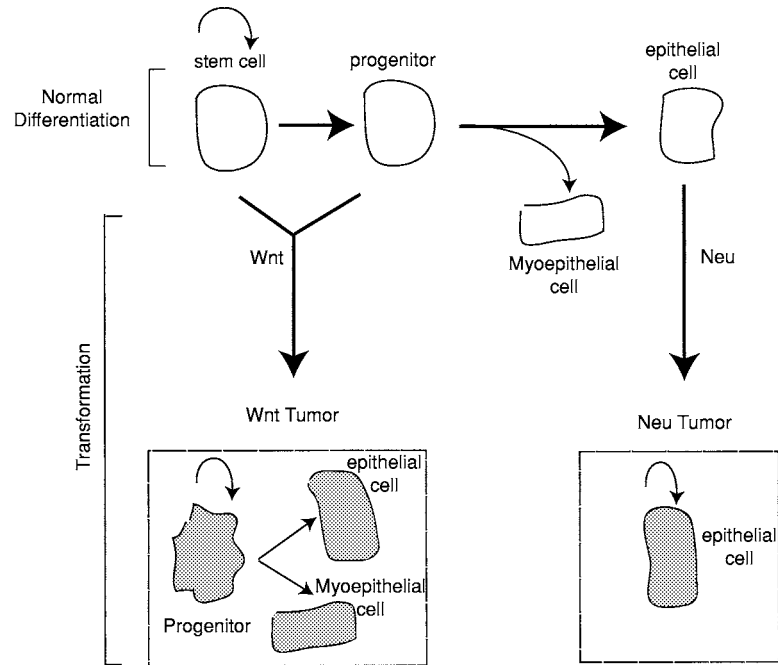


Fig. 1. Schematic model of mammary tumorigenesis induced by Wnt and Neu. Self-renewal is indicated by a half-circle with an arrowhead above the cell. Neu tumor cells are homogeneous. Wnt tumors contain transformed progenitor cells that can give rise to differentiated epithelial and myoepithelial tumor cells.

of the cell that carries the deregulated oncogenes. In another words, cells at a specific stage of differentiation may be more susceptible to transforming effects of certain oncogenic mutations, but not others (Fig. 1).

PROSPECTS

It will be important to test directly in future experiments whether the selective alterations of critical signaling pathways in stem cells will cause them to evolve into cancers comprised of both stem cells and multiple types of differentiated cells. Several potential stem cell markers are available, and more are being characterized (50); thus, it may be possible to use their promoters for selective introduction of oncogenic factors to stem cells. Spatial regulation of transgene expression will be essential, since these markers are usually expressed in other organs and tissues. Furthermore, the promoter driving the oncogene should be constitutively expressed even after the stem cells have differentiated into different cell types, including myoepithelial cells. Thus, the promoter driving the oncogene should be different from

the promoter used for targeting the oncogene selectively to stem cells. While tissue transplantation and rtTA or Cre-mediated conditional expression (45, 51) can satisfy some of these technical challenges; the TVA (tumor virus A) technology (52) offers an attractive alternative for flexible targeting of oncogenic events selectively to stem cells and maintaining the expression of oncogenes in any cell type to which the stem cell may differentiate.

The TVA gene transfer system is based on the use of a sub-group A avian leukosis virus vector (RCAS) to carry exogenous genes to specific somatic mouse cells that are made susceptible to infection by the expression of TVA, the receptor for RCAS (52). Since mammalian cells lack this receptor, they are not normally susceptible to infection by RCAS; however, ectopic expression of TVA (e.g. by the Sca-1 promoter) transforms an otherwise resistant cell type to a susceptible one. Thus, RCAS vectors may be used to infect and deliver oncogenes at any time of mammary ductal development. Since RCAS produces only the exogenous gene product, but not viral structural proteins, this virus does not spread or induce immune rejection. Importantly, exogenous genes (such as oncogenes) cloned into this vector are

expressed from the constitutively active viral LTR and independent of the promoter controlling TVA, which directs the virus to specific cells (such as stem cells).

Thus, an oncogene delivered by this method continues to be expressed even after infected cells have differentiated. However, since RCAS requires dividing TVA⁺ cells for infection, a modified HIV vector (HIV/ALV), formed by pseudotyping the HIV virus with the envelope gene of RCAS and capable of infecting all TVA⁺ cells regardless of the cell proliferation status (53), may be necessary to infect stem cells since they should, by definition, be relatively quiescent. The use of this technology may provide a more rapid approach to determine the effects of other stem cell regulators on inducing cancer. In addition, with this method, it may also be possible to ask whether stem cell regulators (such as Wnt or Notch) can induce dedifferentiation of differentiated breast epithelial cells if these factors are selectively transferred to differentiated cells using transgenic lines expressing TVA from a promoters of a differentiation marker (such as WAP). Such an approach would overcome the potential concern associated with conventional transgenic techniques that the expression of the test gene is shut off after the cell has dedifferentiated and stopped expressing the transgenic promoter.

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