Delineating the Epithelial Hierarchy in the Mouse Mammary Gland

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Reconstitution assays have shown that mouse mammary stem cells reside within the mature mammary gland *in vivo*. Single cells could be prospectively isolated and shown to regenerate an entire mammary gland that exhibited full developmental capacity. The more recent identification of luminal progenitor populations has indicated that the mammary epithelium is organized in a hierarchical manner. Further definition of epithelial cell types in both mouse and human mammary glands will provide insight into the “cells of origin” in the different subtypes of breast cancer, as well as the nature of cancer-propagating cells. Here, we review the known characteristics of mammary stem and progenitor cells, their steroid receptor status, and the pathways that have thus far been implicated in regulating their self-renewal and differentiation.

Development of the mammary gland occurs predominantly after birth and is largely governed by the concerted action of steroid and peptide hormones together with locally produced cytokines (Hennighausen and Robinson 2005). During puberty, the mouse mammary gland undergoes extensive growth, resulting in the formation of a network of branching ducts that fills the mammary fat pad. In the adult, the mammary epithelium undergoes cycles of proliferation and differentiation with each estrus cycle, during which alveolar buds arise. These develop into alveolar structures during pregnancy and undergo terminal differentiation, thus allowing milk production in lactation. Involution represents the final stage of mammary gland morphogenesis and involves substantial structural remodeling of the gland, resulting in a near prepubertal (virginal) appearance. The massive expansion of mammary epithelium during puberty and pregnancy, together with the polyregenerative capacity apparent upon successive reproductive cycles, suggests the existence of long-lived stem cells in the mammary gland.

Pioneering studies in the mammary gland biology field led to the development of an *in vivo* transplantation assay in which the mammary fat pad (MFP) can be cleared of endogenous epithelium while retaining an intact stromal environment (De Ome et al. 1959). Small mammary tissue fragments (explants) (Hoshino and Gardner 1967; Daniel et al. 1968) or cell suspensions prepared from whole mammary tissue (Smith 1996) were shown to reconstitute functional mammary epithelial structures and were capable of serial transplantation, although regenerative senescence was induced after seven generations. Direct support for the stem cell concept came from transplantation studies using retrovirally marked donor tissue fragments, which suggested that a single stem cell was capable of repopulating the entire mammary epithelium (Kordon and Smith 1998). In human breast tissue, there is also evidence for a popula-

tion of stem cells. This includes the presence of large contiguous fields of cells within mammary ducts and lobules that exhibit clonality (Tsai et al. 1996) and the presence of distinct progenitor cell phenotypes in colony-forming assays (Stingl et al. 1998, 2001).

Three distinct epithelial cell types reside within the mammary gland: ductal, alveolar, and myoepithelial cells. The ductal and alveolar cells belong to the luminal epithelial cell lineage and line the lumina or constitute the alveolar units that emerge during pregnancy, respectively. The myoepithelium surrounds the luminal epithelium and contacts the basement membrane. In the lactational phase, these cells provide contractile properties to facilitate milk expulsion. It is presumed that a hierarchy of stem and committed progenitor cells give rise to mature luminal and myoepithelial cells, analogous to that in the hematopoietic compartment. Moreover, breast cancer is a very heterogeneous disease, and the observation of multiple subtypes based on gene expression profiling studies further suggests the existence of a hierarchy of epithelial cells. At least six distinct subtypes have been described (Perou et al. 2000; Sørlie et al. 2001; Sotiriou et al. 2003; Herschkowitz et al. 2007), and these are thought to originate in different breast epithelial cell types that serve as the “cell of origin.” Elucidation of the molecular pathways involved in regulating the self-renewal and progressive differentiation of mammary stem and progenitor cells is critical for understanding the mechanisms that underpin mammary carcinogenesis.

PROSPECTIVE ISOLATION OF MAMMARY STEM CELLS

The mouse mammary stem cell (MaSC) has been prospectively isolated using the “gold-standard” transplantation assay developed for the mammary gland (De Ome et al. 1959). Using optimized methods of tissue dissociation to obtain a single cell suspension, we used a combination of cell surface markers and fluorescence-activated cell sorting

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(FACS) to resolve four distinct mammary populations. Cells were depleted of leukocytes and endothelial and erythroid precursor cells, using CD45, CD31, and TER-119, respectively, thus defining a Lin^- population. Cells were then sorted on the basis of expression of CD24/HSA (heat-stable antigen) and CD29/ $\beta 1$ -integrin and transplanted into cleared mammary fat pads at limiting dilution to enable evaluation of the repopulating cell frequency (Bonnefoix et al. 1996). Only $\text{CD29}^{\text{hi}}\text{CD24}^+$ cells were able to regenerate a mammary epithelial tree, at a frequency of 1 in 64 cells (Shackleton et al. 2006). Similarly, Stingl et al. (2006) showed that 1 in 62 $\text{CD49f}^{\text{hi}}\text{CD24}^{+/\text{mod}}$ cells could yield mammary outgrowths. It is relevant that CD49f ($\alpha 6$ integrin) is known to form heterodimers with CD29 in the mammary gland. Costaining revealed that the vast majority of CD29^{hi} cells were also CD49f^{hi} (Fig. 1, top right), although there were some CD29^{hi} cells that did not express high levels of CD49f . It seems probable that this integrin heterodimer mediates interactions between the basement membrane and stem cells, which likely reside in a basolateral position. CD24 appears to serve as a pan-epithelial cell surface marker in the mouse mammary gland (Shackleton et al. 2006; Sleeman et al. 2006; Stingl et al. 2006). Sleeman et al. (2006) have described three levels of CD24 expression in mouse mammary tissue: CD24^{neg} , CD24^{mod} , and CD24^{hi} subpopulations, corresponding to nonepithelial, myoepithelial/basal, and luminal cells, respectively. Only the CD24^{mod} cells, which are also negative for Sca-1, were capable of regenerating mammary outgrowths. It should be noted that different levels of fluorescence have been obtained with different anti-CD24 antibodies and with varying antibody concentrations, leading to differing CD24 nomenclature. However, there is full agreement on the emerging phenotype of the MaSC, which is CD29^{hi}

$\text{CD49f}^{\text{hi}}\text{CD24}^{+/\text{mod}}\text{Sca-1}^-$. This population, however, is not pure and contains MaSCs ($< 10\%$) together with mature myoepithelial cells and likely basal progenitors.

Analogous to the hematopoietic stem cell, a single microscopically visualized cell was demonstrated to reconstitute an entire functional mammary gland. The isolated stem cells exhibited full developmental potential (Fig. 1, bottom left), giving rise to extensive outgrowths comprising milk-producing alveolar units in pregnant recipients (Fig. 1, bottom middle and right). Furthermore, the regenerated structures contained daughter cells with the same *in vivo* repopulating activity as the original stem cell (Shackleton et al. 2006). *In vivo* serial transplantation experiments demonstrated that clonal primary outgrowths can be passaged for up to 10 generations (Vaillant et al. 2008), revealing that the MaSC is capable of extensive self-renewal. Indeed, Stingl et al. (2006) estimated that the MaSC can execute 10 symmetrical self-renewing divisions. Thus, the prospectively isolated mammary repopulating cells fulfill the two defining features of a stem cell: multilineage differentiation and self-renewal. Although sorted MaSCs were shown to be cycling (Stingl et al. 2006), the $\text{CD29}^{\text{hi}}\text{CD24}^{+/\text{mod}}$ population is enriched for bromodeoxyuridine (BrdU)-label-retaining cells *in vivo*, suggesting that a pool of quiescent stem cells may reside in the mammary gland (Shackleton et al. 2006).

IDENTIFICATION OF A LUMINAL EPITHELIAL PROGENITOR CELL

In the mouse mammary gland, the $\text{CD29}^{\text{lo}}\text{CD49f}^{\text{lo}}\text{CD24}^{+/\text{hi}}$ population is enriched for luminal epithelial cells that are characterized by expression of cytokeratin 18 but lack smooth muscle actin, a myoepithelial marker. Using an

Figure 1. (Top panels) Expression of CD29, CD24, and CD49f in Lin^- mammary cells. Flow cytometric analysis of Lin^- cell suspensions costained with CD29 and CD24 (left panel) and CD49f and CD24 (middle panel). (Right panel) CD29 and CD49f expression in $\text{Lin}^- \text{CD29}^{\text{hi}}\text{CD24}^+$ cells. (LUM) Luminal epithelial cells; (MaSC) mammary stem cells. (Bottom panels) X-gal-stained outgrowths derived from single transplanted Rosa-26 cells in virgin (left panel) and pregnant (middle panel) recipients. Bar, 250 μm . (Right panel) Immunofluorescent staining for milk proteins in a mammary duct derived from a single cell and harvested during pregnancy.

additional cell surface marker, CD61/ β 3-integrin, the luminal $CD29^{lo}CD24^{+}$ population could be subdivided into two subpopulations ($CD61^{+}$ and $CD61^{-}$ fractions). The $CD61^{+}$ subset represents about 30% of the luminal population and has extensive colony-forming capacity in both two-dimensional and Matrigel cultures, yielding colonies that stain exclusively with luminal lineage markers. In contrast, the $CD61^{-}$ subset is highly enriched for mature luminal epithelial cells (Asselin-Labat et al. 2007). In this more mature population, a small fraction of cells can form colonies in vitro, indicating that $CD61^{-}$ cells also comprise a progenitor population (Asselin-Labat et al. 2007). In separate studies, CD133/prominin-1 was found to fractionate the luminal cell population into $CD24^{+hi}CD133^{-}$ and $CD24^{+hi}CD133^{+}$ cells enriched in progenitor and differentiated cells, respectively (Sleeman et al. 2007). CD61 expression, however, does not directly correlate with CD133 expression in the luminal cell population defined by $CD29^{lo}$ and $CD24^{+}$ (Fig. 2, top). This observation suggests that these markers may be identifying distinct progenitor cells. During mammary gland ontogeny, the percentage of $CD61^{+}$ progenitor cells was highest in pubertal mammary glands, a stage involving extensive ductal branching and elongation to generate the mature ductal tree, and declined thereafter in the adult gland. Pregnancy was accompanied by a dramatic decrease in the proportion of $CD61^{+}$ progenitor cells, compatible with alveolar differentiation occurring during this stage. Taken together, these results suggest that $CD29^{lo}CD24^{+}CD61^{+}$ cells can act as progenitors for both ductal and alveolar

luminal cells, dependent on the hormonal milieu. The side population (SP) defined by Hoechst 33342 dye efflux, originally thought to be enriched in MaSCs (Welm et al. 2002), has subsequently been shown to be depleted of $CD29^{hi}CD24^{+}$ (MaSC-enriched) cells (Shackleton et al. 2006; Stingl et al. 2006). In fact, the SP contains sixfold more luminal progenitor cells than differentiated luminal cells, and 70% of SP cells are found in the luminal progenitor fraction ($CD29^{lo}CD24^{+}CD61^{+}$) (Fig. 2, bottom).

There are at least two potential hierarchical models for mammary stem cells giving rise to progressively restricted intermediate and differentiated epithelial cells in the mammary gland. In the model that we currently favor (Fig. 3), mature luminal and myoepithelial cells arise from unipotent progenitor cells. Although there appears to be a common luminal progenitor cell ($CD61^{+}$) for ductal and alveolar epithelial cells, this model does not exclude the existence of further intermediate cell types downstream from the common luminal progenitor cell, with distinct precursors for ductal and alveolar luminal cells. The generation of these will presumably be dependent on the hormonal environment and developmental stage. Markers of the myoepithelial progenitor cell are yet to be determined, but this cell is presumed to reside in the MaSC-enriched fraction, and the presence of myoepithelial cell-only colonies in cultures of human breast epithelial cells (Stingl et al. 1998) provides support for their existence. In an alternative model, bifurcation of the ductal and alveolar lineages may occur before luminal versus myoepithelial cell-fate decisions. Separate

Figure 2. (Top) Prominin-1 (CD133) and CD61 costaining in MaSC-enriched ($CD29^{hi}CD24^{+}$) and luminal ($CD29^{lo}CD24^{+}$) populations. Cells were depleted of $CD31^{+}CD45^{+}TER119^{+}$ cells (Lin $^{-}$). (Bottom) Enrichment in the Hoechst side population (SP) cells of luminal progenitor cells ($CD29^{lo}CD24^{+}CD61^{+}$) but not MaSC-enriched ($CD29^{hi}CD24^{+}$) or mature luminal cells ($CD29^{lo}CD24^{+}CD61^{-}$).

Figure 3. Schematic model of the mouse mammary epithelial hierarchy. The cell surface immunophenotypes and keratin expression profiles of the stem and intermediate cell types are shown. Genes and pathways that have been implicated in regulating cells in the hierarchy are indicated. (MaSC) Mammary stem cell.

progenitors for the ductal and alveolar lineages may exist as well as a novel alveolar myoepithelial cell. Some support for this hierarchical organization comes from the observation that transplantation of cultured mammary cells into cleared fat pads produced epithelial outgrowths with either a ductal-only or lobule-only morphology (Smith 1996). Further studies using additional cell surface markers and freshly isolated cells at clonal dilution will be required to unravel the epithelial hierarchy and to allow lineage tracing to determine the relationship between these cell types. One potential question that arises is Can the different hormonal influences impose a degree of plasticity on stem and progenitor cells in the mammary gland?

HUMAN BREAST STEM AND PROGENITOR CELLS

There is substantial evidence for the existence of a bipotent epithelial progenitor cell in human breast tissue. This includes a MUC1⁺CALLA^{lo/hi}ESA⁺(EpCam⁺)CD49f⁺ progenitor cell (Stingl et al. 1998, 2001), an immortalized bipotent human mammary epithelial cell line derived from MUC1⁺ESA⁺ cells (Gudjonsson et al. 2002), and multipotent epithelial cells that preferentially form mammospheres (Dontu et al. 2003). Using several postulated stem cell markers including stage-specific embryonal antigen-4

(SSEA-4); keratins 15, 5, and 6a; chondroitin sulfate; and Bcl-2, Villadsen et al. (2007) described putative stem cells that resided within ducts and were virtually absent from lobules. In vitro culture assays further showed that the cells with clonogenic capacity and bipotency were localized to ducts (Villadsen et al. 2007).

Different types of unipotent progenitors have also been described, including two types of luminal-restricted progenitors and a myoepithelial-restricted progenitor (Stingl et al. 2001; Villadsen et al. 2007). Luminal-restricted progenitors with the EpCAM⁺CD49f⁺ phenotype express the luminal marker MUC1 and can be further subdivided into two subsets, K19⁺K14⁻ and K19⁻K14⁻ cells (Villadsen et al. 2007), but the physiological relevance of these two populations is not yet understood. Differentiated luminal (EpCAM⁺MUC1⁺CD49f⁺) and myoepithelial (EpCAM^{lo}MUC1⁻CD49f⁺) cell populations have also been identified (Stingl et al. 2001; Stingl and Caldas 2007). Thus, there appear to be striking parallels in the cell types that reside in mouse and human mammary epithelium.

Studies on human mammary stem and progenitor cells have been hampered by the challenges of xenotransplantation into the mouse mammary fat pad. Toward this end, Kuperwasser et al. (2004) "humanized" the mouse mammary fat pad of immunocompromised NOD/SCID mice by injection of irradiated and unirradiated immortalized

human fibroblasts in an attempt to recreate a stromal environment characteristic of that in human breast tissue. Breast organoids were transplanted into this microenvironment and were able to generate ducts and lobular units. More recently, Ginestier et al. (2007) identified a subpopulation of cells with stem/progenitor cell activity in normal human mammary tissue based on aldehyde dehydrogenase 1 (ALDH1) activity using the ALDEFLUOR assay. High ALDH1 activity is a feature of both stem and progenitor cells in the neural and hematopoietic systems (Armstrong et al. 2004; Hess et al. 2004; Matsui et al. 2004). ALDH1⁺ breast epithelial cells were shown to be enriched for cells capable of forming mammary epithelial ducts *in vivo*. Although serial transplantation presents a major challenge for human stem cell work, it will ultimately be required to prove that these cells correspond to breast stem cells.

HORMONAL REGULATION OF MAMMARY STEM CELLS

The steroid hormones estrogen and progesterone have essential roles in mammary gland development, and both estrogen receptor α (ER α) and progesterone receptor (PR) are important prognostic markers in breast cancer. Estrogen exerts its activity by binding to nuclear ER α , and ER α knockout mice display profound defects in ductal development (Bocchinfuso and Korach 1997; Mueller et al. 2002). Interestingly, we have found that the mouse MaSC-enriched population CD29^{hi}CD49^{thi}CD24^{+/mod} does not express ER α or PR, which is also a target gene of ER α (Asselin-Labat et al. 2006). The CD24^{mod} population described by Sleeman et al. (2007) also lacked expression of these receptors. Notably, only a small proportion (6%) of the luminal progenitor population expressed ER α and PR, in contrast to the differentiated luminal cell population, in which 40% expressed these receptors (Asselin-Labat et al. 2006). Thus, the luminal progenitor cell may be the first cell type along the hierarchy that expresses ER α , and these cells may be the initial target of transformation in individuals that develop ER α ⁺ breast cancer. Other studies have reported that label-retaining mammary epithelial cells in the mouse mammary gland (Booth and Smith 2006) and side population cells in human breast tissue are enriched in ER α -positive cells (Clarke et al. 2005). These findings are compatible with the observation that some progenitor cells express ER α and lend support to the notion that a hierarchy of stem cells may exist, reminiscent of that occurring in the hematopoietic compartment.

Given the importance of estrogen to normal mammary development, how does a single ER α -negative mammary stem cell reconstitute an entire ductal tree? It seems likely that the MaSC creates its own epithelial niche through symmetrical and asymmetrical cell divisions, giving rise to both ER-positive and ER-negative progeny that respond to hormonal cues during puberty and coordinate the development of a branching ductal tree. It is known that estrogen mediates its effects in the mammary gland through paracrine signaling (Scully et al. 1997; Mallepell et al. 2006). It is therefore presumed that ER α -positive epithelial cells secrete a paracrine factor(s), in response to

activation by estrogen, which in turn activates MaSCs. Although these factors are yet to be fully established, amphiregulin, an agonist of the epidermal growth factor receptor, appears to be a critical mediator of estrogen effects in the mammary gland. Amphiregulin was recently found to be a direct target of estrogen through ER α and to regulate both mammary epithelial proliferation and ductal elongation (Ciarloni et al. 2007).

Although multiparity reduces the long-term risk of breast cancer, there is an increased risk of cancer for a number of years following pregnancy (Lambe et al. 1994). A recent meta-analysis highlighted that parity-associated risk reductions are limited to ER⁺PR⁺ breast cancers (Ma et al. 2006). It is not clear how pregnancy exerts this dual effect, but delineation of the cellular hierarchy may provide some insight. It is likely that pregnancy involves the expansion of a transit-amplifying cell population and that any cells harboring mutations would therefore be expanded, increasing the risk of developing breast cancer for a short term following pregnancy. Alternatively, the microenvironment might be transiently tumor-promoting after remodeling of the mammary gland to its prepregnant state (Schedin 2006). Of relevance, although the mammary gland returns to a near prepregnant state following weaning, it is not identical to the original gland (Medina and Smith 1999; D'Cruz et al. 2002; Master and Chodosh 2004). This finding suggests that there may be permanent changes in a specific cell population. For example, the number of MaSCs may be diminished, thus reducing the pool of long-lived cells that have a greater probability of accumulating mutations over time (Britt et al. 2007). The protective effect of a full-term pregnancy on breast carcinogenesis has also been observed in mice, indicating their utility to study the effects of pregnancy on cell populations (Sivaraman et al. 2002).

During mouse mammary gland development, the size of different epithelial subpopulations fluctuates. We have shown that the number of cells in the MaSC-enriched population CD29^{hi}CD24⁺ varies during ontogeny, reaching its maximum at midpregnancy, and then declining to the level in virgin glands during involution (M.-L. Asselin-Labat, unpubl.). In aged virgin mice, the proportion of CD29^{hi}CD24⁺ cells is increased by twofold to threefold compared to young adult mice, but multiparity only slightly reduces the number of CD29^{hi}CD24⁺ cells compared to aged-matched controls. However, *in vivo* transplantation studies are required to assess whether the observed changes in the size of the CD29^{hi}CD24⁺ population during aging and pregnancy reflect changes in MaSC number or function of MaSCs. Interestingly, a parity-identified (previously termed parity-induced) mammary epithelial cell population (PI-MECs) (Smith and Medina 2008) that persists following involution has been described using Wap-cre/Rosa-LacZ and Wap-cre/CAG-GFP double transgenic reporter systems (Wagner et al. 2002; Matulka et al. 2007). Transplantation of mammary fragments or large numbers of cultured cells showed that the PI-MECs were capable of regenerating a mammary epithelial tree, but it is not yet clear whether these correspond to definitive stem cells.

REGULATORS OF LINEAGE DIFFERENTIATION IN THE MAMMARY GLAND

Gata-3 has emerged as a key regulator of differentiation along the luminal lineage. This transcription factor was found to be an essential regulator of mammary gland morphogenesis at several different stages: It is required for the formation of primary mammary buds in the embryo, for ductal growth in the adult, and for alveolar development during pregnancy (Kouros-Mehr et al. 2006; Asselin-Labat et al. 2007). Significantly, Gata-3 is expressed exclusively in luminal epithelial cells, and its absence results in failed differentiation and a marked expansion of luminal progenitor cells. Interestingly, Gata-3 can induce milk protein gene expression in the MaSC-enriched population in the absence of a lactogenic stimulus. Its critical role in differentiation is consistent with the gradient of Gata-3 expression that is apparent along the hierarchy: The MaSC-enriched population expresses low levels of Gata-3, with moderate expression in luminal progenitor cells and highest levels apparent in differentiated luminal cells (Asselin-Labat et al. 2007). Expression of Gata-3 in the MaSC-enriched population implies a role in stem cell function, but this is yet to be determined. Costaining for ER and Gata-3 revealed that a substantial proportion of ER⁺ cells in the mature luminal cell subset (CD61⁺) coexpress Gata-3 (Asselin-Labat et al. 2007), compatible with recent findings that ER α and Gata-3 directly coregulate each other's expression (Eeckhoutte et al. 2007).

The absolute level of Gata-3 in the mouse mammary gland appears to be critical, and heterozygous mice exhibit a profound defect in ductal morphogenesis. Moreover, in breast cancer, the level of Gata-3 is also a determinant of prognosis. The highest expression of this gene is observed in the "luminal" subtypes (A and B) of breast cancer (Perou et al. 2000; Sørlie et al. 2001; Sotiriou et al. 2003; Kouros-Mehr et al. 2008b), which express ER α and predict good prognosis (Voduc et al. 2008). Gata-3 mutations have also been identified in a subset of breast cancers (Usary et al. 2004), and Gata-3 polymorphisms are associated with breast cancer risk (Garcia-Closas et al. 2007). In the mouse mammary tumor virus–polyomavirus middle T (MMTV–PyMT) mouse model of tumorigenesis, Gata-3 is down-regulated during tumor progression from mammary adenomas to carcinomas (Kouros-Mehr et al. 2008a), consistent with Gata-3 acting as a tumor suppressor. Kouros-Mehr et al. (2008a) further showed that transduction of MMTV–PyMT carcinoma cells with a Gata-3-expressing lentivirus reduced tumor cell dissemination to the lung following transplantation. The role of Gata-3, however, in the initiation of mammary tumorigenesis has not yet been addressed.

Luminal cell differentiation is regulated by the Ets transcription factor Elf-5. Unlike Gata-3, Elf-5 deficiency does not lead to defects in ductal growth and morphogenesis (Zhou et al. 2005; Harris et al. 2006). However, both Elf-5^{+/-} and Elf-5^{-/-} mammary glands show defects in alveolar differentiation during pregnancy, whereas glands from transgenic Elf-5 mice exhibit precocious alveolar differentiation in the virgin state (Zhou et al. 2005; Oakes

et al. 2008). Accordingly, Elf-5^{-/-} glands harbor an increased number of luminal progenitor cells during pregnancy, and transgenic mice show a decrease in mature luminal cells, indicating that Elf-5 regulates the differentiation of progenitor to alveolar cells. We speculate that Elf-5 lies downstream from Gata-3 in the differentiation pathway, because Gata-3 is expressed earlier in the epithelial hierarchy and has a critical role in ductal morphogenesis in the peripubertal mammary gland.

PATHWAYS THAT REGULATE MAMMARY STEM/PROGENITOR CELL PROLIFERATION

A number of pathways have now been identified that have a key role in normal mammary development and oncogenesis. There are accumulating data pointing to a critical role for the Wnt/ β -catenin pathway in the mammary gland. Wnt-1 is a frequent site of integration by the MMTV (Nusse and Varmus 1982; Nusse et al. 1984) and overexpression of Wnt-1 causes mammary hyperplasia and tumors (Tsukamoto et al. 1988; Bocchinfuso et al. 1999). Evaluation of preneoplastic mammary tissue in MMTV–Wnt-1 mice revealed an expanded MaSC-enriched CD29^{hi}CD24⁺ population and a fourfold to sixfold increase in the absolute number of MaSCs compared to age-matched wild-type control glands (Shackleton et al. 2006). Other studies have suggested that the oncogenic effects of Wnt-1 in mammary epithelium are initiated in a mammary progenitor cell population, with an expansion of the SP and Sca1⁺ cell populations observed in hyperplastic MMTV–Wnt-1 glands (Li et al. 2003; Liu et al. 2004). Wnt-1 further appears to render these progenitor cells radioresistant (Woodward et al. 2007). Intriguingly, we have recently shown that luminal progenitor cells in preneoplastic Wnt-1 glands are capable of repopulating the fat pad *in vivo*, suggesting that Wnt-1 induces oncogenic activity in both stem and luminal progenitor cells. Alternatively, it is also possible that Wnt-1 predominantly affects stem cells but that their cell surface phenotype alters slightly upon commitment to a neoplastic state (Vaillant et al. 2008).

Hedgehog (Hh) signaling has been implicated in the self-renewal of multiple types of stem cells (Molofsky et al. 2004). Activation of human breast epithelial cells with the Hedgehog ligands Shh (sonic hedgehog) and Ihh (Indian hedgehog) enhanced mammosphere-initiating capacity (Liu et al. 2006). However, *in vivo* transplantation assays using mammary cells isolated from transgenic mice expressing the smoothened receptor (smo) showed a decrease in MaSC activity (Moraes et al. 2007). Because sphere formation appears to be more indicative of progenitor rather than stem cell activity, these data are compatible with Hedgehog signaling causing an increase in progenitor cell activity. In addition, recent findings have shown that loss of one copy of Patched-1, a negative regulator of Hedgehog, results in enhanced progenitor cell formation by stem cells in the mammary gland (Li et al. 2008). Hh can also mediate its effects via the polycomb gene *Bmi-1*. Indeed, overexpression of *Bmi-1* in human breast epithelial cells increased both the number and size of primary and secondary mammospheres, whereas knock

down of *Bmi-1* levels reduced sphere formation (Liu et al. 2006), suggesting that *Bmi-1* promotes the proliferation of progenitor cells. The Notch pathway also has a central role in breast oncogenesis, and both Notch 1 and Notch 4 were identified as frequent sites of proviral insertion and activation in mouse mammary tumors (Kiaris et al. 2004; Raafat et al. 2004; Hu et al., 2006). We have recently shown that the Notch pathway promotes luminal cell commitment in the mammary gland and that deregulated Notch signaling leads to the uncontrolled expansion of luminal progenitor cells (Bouras et al. 2008). Consistent with increased progenitor activity, enhanced mammosphere formation was observed upon addition of Notch ligand to human mammary epithelial cell culture (Dontu et al. 2004).

Negative regulation of proliferation is also critical for normal development. Transforming growth factor- β 1 (TGF- β 1) regulates both mammary ductal morphogenesis and alveolar development (Daniel et al. 2001). Transgenic mice overexpressing an activated form of TGF- β 1 displayed reduced lateral branching in their mammary glands, consistent with the proposal that TGF- β 1 inhibits MaSC and/or progenitor cells located along the ducts (Pierce et al. 1993). In support of this, Smith and colleagues have reported that mammary expression of TGF- β 1 results in senescence of MaSCs (Boulanger and Smith 2001; Boulanger et al. 2005; Kordon et al. 1995). TGF- β 1 has also been implicated in tumorigenesis and metastasis in different organs including the mammary gland (Moses and Serra 1996; Siegel and Massagué 2003). Interestingly, the TGF- β 1 pathway appears to be activated in a putative human breast cancer stem cell population (CD44⁺CD24⁻) isolated from pleural effusions of breast cancer patients (Shipitsin et al. 2007).

CELL OF ORIGIN OF BREAST CANCER

The MaSC-enriched population (CD29^{hi}CD24⁺) expresses the basal markers keratin 14, p63, and epidermal growth factor receptor (EGFR) and lacks expression of ErbB2 (Asselin-Labat et al. 2006). These characteristics are reminiscent of the phenotype of the basal subtype of breast cancer and suggest that the stem cell may be the “cell of origin” for this cancer subtype. There is evidence indicating that ovariectomy prevents the formation of both ER α -positive and ER α -negative breast cancers, suggesting that ER-negative breast cancers are dependent on ovarian hormones for their formation (Nissen-Meyer 1964a,b; Gupta et al. 2007). Interestingly, despite no change in the size of the CD29^{hi}CD24⁺ mammary population in ovariectomized mice, the repopulating capacity of these cells is reduced (M.-L. Asselin-Labat, unpubl.). This finding suggests that the MaSC-enriched population is sensitive to estrogen withdrawal and raises the question of whether cancer cells in the basal (ER-negative) subtype of breast cancer have lost their dependence on steroid hormones.

Breast carcinomas from *BRCA1* mutation carriers are ER α -negative and have a basal phenotype, suggesting that they may also originate in the basal cell compartment (Foulkes et al. 2003; Ribeiro-Silva et al. 2005). Interestingly, *BRCA1* seems to directly regulate ER α expression (Hosey et al. 2007). Knockdown of *BRCA1* expression in

primary human mammary epithelial cells results in the accumulation of undifferentiated cells with a basal phenotype, and in vivo transplantation of epithelial cells in which *BRCA1* levels have been reduced leads to abnormal outgrowths lacking luminal cells (Liu et al. 2008). In combination with studies showing that loss of heterozygosity (LOH) at the *BRCA1* locus is associated with high ALDH1 activity, these findings suggest a critical role for *BRCA1* in promoting the differentiation of mammary stem/progenitor cells. Furthermore, conditional deletion of *BRCA1* in mammary glands delayed development during puberty and impaired alveolar cell differentiation during pregnancy and lactation, eventually leading to tumor development (Xu et al. 1999). Similarly, deletion of both *BRCA1* and *p53* (frequently mutated in *BRCA1* tumors) led to highly proliferative mammary tumors that were ER α -negative and expressed basal epithelial markers (Liu et al. 2007).

CANCER STEM CELLS

The “cancer stem cell” hypothesis postulates that a small subset of tumor cells with stem-cell-like properties drive tumor progression and recurrence. These cells lie at the apex of the hierarchy in tumors and give rise to more differentiated progeny with limited proliferative potential. Cancer stem cells (CSCs) do not necessarily arise from normal stem cells but may originate in progenitor cells or more differentiated cells that have acquired self-renewing properties. In human breast, Clarke and colleagues (Al-Hajj et al. 2003) first identified a population of cells with a CD44⁺CD24^{-/lo} phenotype that had significantly higher tumor-initiating capacity than CD44⁻CD24⁺ cells when transplanted into immunocompromized mice. CD44⁺CD24^{-/lo} tumorigenic cells isolated from a metastatic breast cancer xenograft model showed increased expression of Gli-1, Gli-2, and *Bmi-1* mRNA compared to nontumorigenic cells (Liu et al. 2006), consistent with the cancer stem cell population having increased self-renewal capability.

In mouse models of mammary tumorigenesis, recent data have provided direct support for the cancer stem cell hypothesis. In MMTV-Wnt-1 mammary tumors, Cho et al. (2008) found that CD24⁺Thy-1⁺ cells have a higher tumor-generating capacity than “not-CD24⁺Thy-1⁺” cells when transplanted subcutaneously into syngeneic recipients. Recently, we have shown that CD29^{lo}CD24⁺CD61⁺ cells are enriched 20-fold in tumor-initiating cells compared to the CD29^{lo}CD24⁺CD61⁻ population and can recapitulate a tumor with the same phenotypic characteristics as the parental tumor following serial transplantation (Vaillant et al. 2008). Similarly, CD61⁺ cells isolated from some tumors that developed in p53^{+/-} mice were also highly enriched for cancer stem cells. Conversely, in mammary tumors arising in doxycycline-inducible Myc bitransgenic mice, CD61 did not enrich for cancer stem cells, suggesting that this progenitor marker may not be relevant to all mouse tumor types or that the development of Myc mammary tumors follows a model distinct from that of the cancer stem cell paradigm (Vaillant et al. 2008). In MMTV-Neu mammary tumors, we were not able to identify a cancer stem cell subpopulation using a wide

range of cell surface markers. These tumors are very homogeneous and exhibit luminal characteristics, consistent with their CD61⁺ phenotype. This mouse strain seems more likely to follow a stochastic model of tumor formation. Thus, different mouse models of mammary tumors may follow distinct models of tumorigenesis.

CONCLUSIONS

The *in vivo* mammary reconstitution assay, together with the ability to sort different epithelial cell populations, has paved the way for understanding the molecular and cellular mechanisms that regulate both normal development and breast oncogenesis. With the discovery of additional markers to allow further purification of stem cells, it should be possible to answer many questions pertaining to mammary stem cells and their niche. The decision by an MaSC to self-renew rather than initiate differentiation is likely to be tightly regulated in order to maintain normal homeostasis and ensure continued production of differentiated cells. It is presumed that there is a small pool of quiescent stem cells within the mammary gland, but their location and the extracellular signals that activate them are presently unknown. The role of estrogen in regulating stem cell function is also unclear but almost certainly involves multiple paracrine factors that are yet to be identified. The earliest progenitor cell known to date to express ER and PR is the luminal CD61⁺ cell, but it is possible that there is another upstream progenitor that expresses physiologically relevant levels of these receptors. Further elucidation of epithelial cells in the mammary gland and the pathways that regulate their activity is pivotal to understanding the “cells of origin” and cancer stem cells that underlie different breast tumors and for the design of more effective therapies.

ACKNOWLEDGMENTS

This work was supported by the Victorian Breast Cancer Research Consortium, the National Health and Medical Research Council (Australia), and the Australian Stem Cell Centre. M.A. was supported by an Australian Research Council Discovery Project postdoctoral fellowship.

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Cold Spring Harb Symp Quant Biol 2008 73: 469-478 originally published online November 6, 2008
Access the most recent version at doi:[10.1101/sqb.2008.73.020](https://doi.org/10.1101/sqb.2008.73.020)

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