Mammary Stem Cells and Mammapoiesis

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Abstract

The isolation and characterization of mammary stem cells is fundamental to understanding mammary gland development and tissue homeostasis as well as breast oncogenesis. Recent studies have led to the prospective isolation of pluripotential stem cells from the mouse mammary gland through the identification of specific cell-surface markers and transplantation of cells into the mammary stromal microenvironment. A single cell was sufficient to reconstitute a fully developed mammary gland in vivo, indicating that combinatorial activity between independent stem cells is not essential to generate an outgrowth. Here we review the characteristics of mouse mammary stem cells, their estrogen receptor status, and the potential cellular hierarchy that exists within the mammary gland.

Introduction

Almost 50 years ago, DeOme et al. (1) developed the technique of tissue fragment transplantation into mouse mammary fat pads that had been cleared of their endogenous epithelium. This transplantation system has provided a remarkable and robust in vivo model for investigators to explore the existence of mammary stem cells. These cells are presumed to be important for maintaining homeostasis in the mammary gland and for providing its enormous regenerative capacity that is evident during successive reproductive cycles. De Ome et al. (1) showed that virtually any portion of the mammary gland could reconstitute the entire mammary ductal tree on transplantation into the mammary fat pad, while Smith and Medina (2) revealed that repopulating mammary cells exist throughout the life span of the adult mouse. Further studies by Kordon and Smith (3) demonstrated the existence of stem cells using retrovirally marked mammary epithelial fragments. In the human breast, the presence of large contiguous fields of cells within mammary ducts that exhibit clonal derivation implies the existence of breast stem cells, in addition to the distinct phenotypes that arise during culture of primary breast epithelial cells (4–7). In both mouse and human, however, purification of mammary stem cells has proved elusive due to the paucity of defined markers.

Isolation and Properties of Mammary Stem Cells

The mouse mammary stem cell (MaSC) has recently been prospectively isolated using similar methods to that employed to identify the hematopoietic stem cell. The isolation methods used by Shackleton et al. (8) and Stingl et al. (9) involved the depletion of hematopoietic and endothelial cells from freshly dissociated cell preparations and definition of different mammary cell subpopulations based on their cell-surface expression of CD24 (heat-stable antigen) and either CD29 (β1-integrin) or CD49f (α6-integrin). Interestingly, these integrins are likely to form a functional α6β1 integrin heterodimeric complex that mediates interaction between epithelial cells and the mammary stroma. Subsets that expressed highest levels of CD29 or CD49f and CD24 (termed the "double-positive" population) were found to be enriched in MaSCs by transplanting these cells at limiting dilution into cleared fat pads. These cells also expressed low levels of the stem cell antigen Sca-1 and seemed to have a basal position within the mammary epithelium (8, 9). Although this subset predominantly expressed markers of myoepithelial cells, a small percentage of cells in this population were low or negative for the myoepithelial lineage markers keratin 14 and smooth muscle actin and could conceivably correspond to MaSCs (8, 10). Cells from the double-positive population gave rise to substantial outgrowths composed of luminal and myoepithelial cells and exhibited complete developmental potential during pregnancy, with the formation of lobuloalveolar structures. This multilineage differentiative capacity fulfills one of the hallmark properties of stem cells.

The most defining feature of a stem cell is its ability to self-renew. This function was shown by secondary transplantations of primary outgrowths, and in some experiments, using primary outgrowths derived from a single microscopically visualized cell. The presence of multiple secondary outgrowths revealed that self-renewal occurred in the original primary outgrowth. Stingl et al. (9) further estimated that the stem cells must have executed 10 or more symmetrical divisions using secondary limiting dilution assays. The proportion of stem cells in the "double-positive" fraction was estimated to be 1 in 20 corresponding to ~0.3% of Lineage cells, based on data from the single-cell injection experiments (8). More precise calculations will require further purification of stem cells through the discovery of additional markers and, ultimately, tracking of these cells within mammary tissue itself.

One characteristic shared by a number of stem cell types is their ability to exclude dyes such as Hoechst33342, identifying a side population of cells due to increased expression of membrane transporter proteins. For the mammary gland, Stingl et al. (9) and Shackleton et al. (8) recently showed that the side population tail is depleted of "double-positive" cells and that almost all mammary outgrowths were derived from the main population, by transplanting at limiting dilution to allow a direct comparison of the repopulating frequencies of the two populations. Although previous studies on mouse and human mammary epithelial cells with a side population phenotype indicate that they are undifferentiated and can give rise to both luminal and myoepithelial lineages (11, 12), it seems far more likely that this population is enriched for bipotent progenitor cells and not stem cells. Further evidence in support of this comes from the observation that the...
side population fraction is enriched 30-fold in human mammo-
mammospheres and yet each sphere comprises only one sphere-initiating cell,
even on continual passage. These data imply that mammospheres
predominantly contain progenitor cells or transit-amplifying cells
(4). The mammary gland field clearly requires the development of a
robust cellular assay to distinguish the activities of stem cell and
non–stem cell populations.

Somewhat surprisingly, mammary stem cells appear to be
actively cycling. Their presence within the G1 or S-G2-M fractions,
determined by Hoechst 33342 and pyronin Y staining (9), is
compatible with the recent observation that long-term label-
retaining mammary cells identified in vivo are cycling and seem
to retain their template or “immortal” DNA strands through the
process of asymmetrical cell division (13). Although in vivo bromodeoxyuridine (BrdU) labeling studies have revealed that the
stem cell–enriched subpopulation contains ~3-fold more long-
term label-retaining cells (8), the precise relationship between
MaSCs and label-retaining cells is not yet clear. The possibility
remains that there is a minor subpopulation of MaSCs that
reside in a quiescent state (G0) in vivo. Determination of the
“activation” status of MaSCs is dependent on further identification of stem cell markers and in vivo labeling of both DNA strands in
these cells by pulsing with analogues such as [3H]thymidine and
BrdU.

The identification of MaSCs has significant implications for
breast oncogenesis. In mammary tumor-prone MMTV-wnt-1
transgenic mice, the number of stem cells was found to be
markedly expanded (~6.4-fold) in the preneoplastic phase (8).
These findings suggest that wnt-1 signaling participates in
controlling the self-renewal of MaSCs, reminiscent of its function
in hematopoietic stem cells, and raise the possibility that stem cells
may be the targets of transformation in the Wnt-1 model. However,
the relationship between the expanded stem cell pool in MMTV-
Wnt-1 transgenic mice and cancer stem cells, originally described
in human breast cancers by Al-Hajj et al. (14), is yet to be
determined. Interestingly, no expansion of MaSCs was evident in
MMTV-neu/erbB2 mice, implying that a later progenitor cell is the
target of transformation in this model (8).

A Single Cell Is Sufficient to Generate an Entire
Mammary Gland

A single cell from the CD29hi/CD24+ (8) or CD49fhi/CD24+ (9)
population was found to reconstitute a functional mammary gland
in vivo. The presence of supporting cells did not affect the activity of
this cell (genetically marked with the LacZ transgene) to form an
outgrowth or its size. Further definitive evidence for the clonality
of outgrowths was derived from classic “mixing” experiments.
Differentially marked cells were mixed before transplantation and
outgrowths were analyzed in recipient mice (8) or the outgrowths
were used in colony forming cell assays (9). Both studies showed
that the vast majority of outgrowths contained one genotype only.
For example, 95 of 97 outgrowths were pure wild-type or Rosa-26
derived (LacZ+), with only two chimeric outgrowths evident (8).
These studies also addressed the question of whether combina-
torial activity between individual mammary stem cells was
necessary to initiate the formation of a mammary outgrowth.
Such combinatorial action was shown not to be essential.
Nevertheless, this finding is not incompatible with the notion
that cooperativity occurs between stem and progenitor cells
to direct expansion of the mammary epithelial network (15). The

implanted stem cell presumably undergoes asymmetrical cell
division to generate another stem cell and a daughter progenitor
cell that act in concert to produce an entire mammary gland.

Three distinct multipotent cell types have been identified in the
mouse mammary gland (15). These can be distinguished by their
ability to produce lobule-limited, ductal-limited, or complete
mammary outgrowths following transplantation. All three cell
types most likely arise from a common antecedent, but the precise
cell types that generate these ductal- or lobule-limited outgrowths
have not been identified. The nature of the epithelial hierarchy
in the mammary gland is yet to be delineated but it seems intuitive
that there will be a hierarchical population of stem and progenitor
cells, akin to that in the hematopoietic system that contains both
long-term and short-term repopulating stem cells in addition to
multiple progenitor types (see Fig. 1). Indeed, the parity-induced
mammary epithelial population described by Wagner et al. (16)
may define a distinct subset of stem/progenitor cells (with limited
self-renewal capacity) that arises during pregnancy but is different
from the CD24 CD29hi/CD49fhi MaSC described above.

Further Markers Are Required to Characterize
the MaSC

Neither CD24, CD29, nor CD49f are exclusive markers of MaSCs.
Cotingaining of the different mammary subpopulations with markers
specific for the myoepithelial and luminal lineages, together with
gene profiling studies, revealed that myoepithelial cells lie within
the MaSC-enriched population. Thus, at least two epithelial
subtypes express high levels of CD29 or CD49f. Furthermore,
CD24 seems to be expressed on almost all epithelial cells that
reside within the mammary gland. Interestingly, Sleeman et al. (17)
have shown that CD24 can be used to define luminal, myoepithe-
lium, and nonepithelial cells, and that CD24lo cells show the highest
repopulating capacity. Although a single marker was used, these
studies parallel those described above and reveal that there are
distinct subpopulations of epithelial cells that can be segregated
generating according to their level of cell-surface CD24, whereas nonepithelial
cells are CD24neg.

Another discrete epithelial population, defined by higher levels
of CD24 but low levels of either CD29 or CD49f, was shown to be
enriched in colony-forming cell progenitors (9) and to comprise
cells committed to a luminal fate (8, 9). These subsets did not
yield outgrowths when implanted into the mammary fat pad,
indicating that they do not contain stem cells. The MaSC-enriched
CD29hi/CD49fhi population was also found to exhibit colony-forming
cell activity (8), although the analogous CD49fhi/CD24hi subset did
not (9), perhaps reflecting differences in the culture conditions.
Alternatively, this finding may be due to differences between the
two populations, as some β1-integrinhi cells are not α6-integrinhi.1

Do CD29, CD49f or CD24 Have a Physiologic Role
in Mammary Stem Cells?

Do the cell adhesion molecules α6-integrin and β1-integrin
regulate aspects of mammary stem cell function? In the mammary
gland, the integrin family has important functions in mediating
attachment of epithelial cells to the stroma and in determining

1 M. Shackleton, F. Vaillant, G.J. Lindeman, J.E. Visvader, unpublished data.
Epithelial cell polarity. Conditional deletion of \( \beta_1 \)-integrin in the mammary gland does not affect ductal morphogenesis and branching but severely impairs the formation and architecture of alveolar units that arise during pregnancy. Even more interestingly, transplanted mammary tissue from \( \beta_1 \)-integrin-deficient mice failed to repopulate cleared fat pads (18). This finding suggests that \( \beta_1 \)-integrin plays a role in governing MaSC self-renewal or function. Although no overt mammary gland defects have been reported in CD24-null mice and in transplants of embryonic rudiments from \( \alpha_6 \)-integrin-deficient embryos, it is conceivable that they also contribute to MaSC function, which could be similarly addressed through the “gold standard” serial transplantation assay.

The MaSC-Enriched Population Is Negative for Estrogen Receptor-\( \alpha \) Expression

A long-standing question in mammary gland biology has been the estrogen receptor-\( \alpha \) (ER\( \alpha \)) status of stem and progenitor cells. Estrogen plays a central role in promoting the proliferation of both normal and neoplastic breast epithelium (19). Its cognate receptor (ER\( \alpha \)) is one of the most important prognostic markers for breast cancer and is a critical determinant in the definition of the luminal A and B subclasses of breast cancer. Estrogen has a potent mitogenic activity on breast epithelial cells in vivo although expression of ER\( \alpha \) is restricted to 10% to 30% of the luminal cells in the ducts and lobules, in which the mammary epithelium has been shown to be the primary target of ER\( \alpha \) signaling (20). Most ER\( \alpha \)-positive cells seem to be nondividing cells that instruct adjacent epithelial cells to proliferate via paracrine regulatory effects (19). However, there is evidence for a small subset of ER\( \alpha \)-positive cells that are slowly cycling in vivo. Interestingly, expression of the progesterone receptor (PR), a transcriptional target of ER\( \alpha \), seems to be restricted to the same 10% to 30% of cells in the luminal mammary epithelium.

The expression of ER\( \alpha \) and PR has recently been determined in the MaSC- and luminal-enriched populations (10). Notably, the basal MaSC-enriched population had an ER\( \alpha \)-negative and PR-negative phenotype whereas the luminal population contained a substantial proportion of cells that expressed these steroid hormone receptors. Ovariectomy was shown to have little effect on the size of the mouse MaSC-enriched population but confirmed the importance of estrogen signaling to luminal cell proliferation. A role for estrogen in regulating the number and/or function of stem cells cannot be precluded, however, and may be mediated by ER\( \alpha \) cells signaling to stem cells via a paracrine mechanism.

In the context of normal development, a model for the hierarchical organization of stem, progenitor, and mature cells is
presented in Fig. 1. A hierarchy of stem cells may exist in the mammary gland, differing in their activation state and repopulating ability. The ERα− MaSC undergoes self-renewal or differentiation to yield a putative common progenitor, which gives rise to either a luminal- or myoepithelial-restricted progenitor. Under the influence of the specific hormonal milieu in the mammary gland, the luminal progenitor commits to either a ductal or alveolar fate. The luminal progenitor is predominantly ERα−, but an intermediate subset expresses ERα and gives rise to mature ERα+ ductal cells. These cells, in response to surges of estrogen during puberty or pregnancy, stimulate the expansion of ductal or alveolar epithelial cells, respectively, and may also regulate the activity of MaSCs.

It is likely that the development of ERα-positive or ERα-negative subtypes of breast cancer reflects the transformation of different stem and/or progenitor cells. The observation that stem cells are negative for ERα has important implications for understanding the cell of origin that gives rise to breast cancer and raises the possibility that the majority of breast cancers (which are ERα positive) arise in a committed progenitor population. Interestingly, the mouse MaSC is negative for ERα, PR, and ErbB2, thus sharing properties with the basal subtype of breast cancer (10).

**Conclusion**

There are several important questions that remain to be answered in this rapidly progressing field. The choice between self-renewal and initiation of differentiation by the MaSC must be tightly regulated to replenish the stem cell pool throughout life and to ensure continued production of differentiated cells. What proportion of stem cells are undergoing symmetrical versus asymmetrical division and under which physiologic conditions? Is there a small pool of quiescent stem cells that inhabits a distinct niche within the mammary gland? Where do stem cells reside in the developing mammary gland—terminal end buds within the pubertal gland and ductal branch-points in the adult gland would seem to be likely candidates. Does estrogen regulate stem cell maintenance or activation via a paracrine mechanism? How early during ontogeny is ERα expression activated and are there ERα+ luminal progenitors? Can mature luminal cells switch between an ERα− and ERα+ status? Further delineation of the linear relationships between stem, progenitor, and differentiated cells will provide important insights into understanding normal mammary gland development and breast carcinogenesis, and could ultimately lead to the identification of markers that distinguish normal and tumorigenic cells.

**References**


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