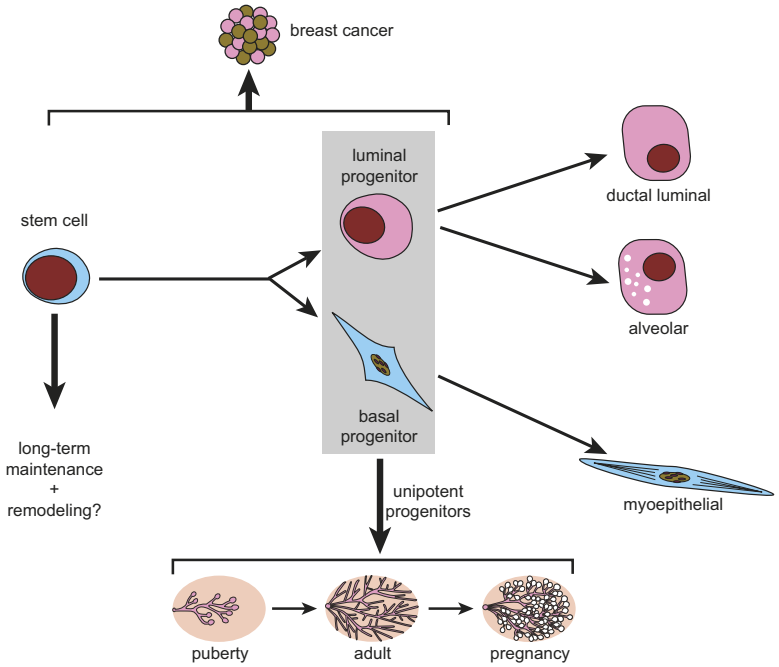
Physiological Reviews Review Article

STEM CELLS AND THE DIFFERENTIATION HIERARCHY IN MAMMARY GLAND DEVELOPMENT

GRAPHICAL ABSTRACT



AUTHORS

Nai Yang Fu, Emma Nolan, Geoffrey J. Lindeman, and Jane E. Visvader

CORRESPONDENCE

visvader@wehi.edu.au

KEYWORDS

breast cancer; mammary gland development; progenitors; stem cells; transcriptional regulators

CLINICAL HIGHLIGHTS

This review summarizes recent advances in understanding normal cell types in the mammary gland (breast), their potential lineage relationships, and the roles of molecular regulators in fate specification and differentiation. The mammary epithelial hierarchy provides an important framework for deciphering the cell types necessary for normal physiological function as well as understanding perturbations that lead to breast cancer. The prospective isolation of stem and progenitor cells, informed by both in vivo transplantation and lineage tracing experiments, has helped to shed light on the cell of origin for distinct subtypes of breast cancer. For example, a hyperproliferative RANK$^+$ luminal progenitor cell appears to be the culprit cell that gives rise to triple-negative breast cancer in women harboring a germline mutation in the *BRCA1* gene. This has revealed a targetable cell for chemoprevention. Female steroid hormones (estrogen and progesterone) and key regulators (e.g., Notch pathway, GATA-3) are instrumental for normal development, and their deregulated expression is implicated in breast cancer. The microenvironment is also emerging as an important regulator of mammary gland development and neoplasia. Finally, the reactivation of embryonic developmental programs and emergence of cellular "plasticity" appear to play important roles in breast cancer.

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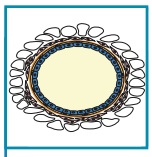
STEM CELLS AND THE DIFFERENTIATION HIERARCHY IN MAMMARY GLAND DEVELOPMENT

Nai Yang Fu, Emma Nolan, Geoffrey J. Lindeman, and Jane E. Visvader

Cancer and Stem Cell Biology Program, Duke-NUS Medical School, Singapore, Singapore; Tumour-Host Interaction Laboratory, Francis Crick Institute, London, United Kingdom; Cancer Biology and Stem Cells Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia; Department of Medicine, The University of Melbourne, Parkville, Victoria, Australia; Royal Melbourne Hospital and Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; and Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia



Fu NY, Nolan E, Lindeman GJ, Visvader JE. Stem Cells and the Differentiation Hierarchy in Mammary Gland Development. *Physiol Rev* 100: 489–523, 2020. First published September 20, 2019; doi:10.1152/physrev.00040.2018.—The mammary gland is a highly dynamic organ that undergoes profound changes within its epithelium during puberty and the reproductive cycle. These changes are fueled by dedicated stem and progenitor cells. Both short- and long-lived lineage-restricted progenitors have been identified in adult tissue as well as a small pool of multipotent mammary stem cells (MaSCs), reflecting intrinsic complexity within the epithelial hierarchy. While unipotent progenitor cells predominantly execute day-to-day homeostasis and postnatal morphogenesis during puberty and pregnancy, multipotent MaSCs have been implicated in coordinating alveologenesis and long-term ductal maintenance. Nonetheless, the multipotency of stem cells in the adult remains controversial. The advent of large-scale single-cell molecular profiling has revealed striking changes in the gene expression landscape through ontogeny and the presence of transient intermediate populations. An increasing number of lineage cell-fate determination factors and potential niche regulators have now been mapped along the hierarchy, with many implicated in breast carcinogenesis. The emerging diversity among stem and progenitor populations of the mammary epithelium is likely to underpin the heterogeneity that characterizes breast cancer.

breast cancer; mammary gland development; progenitors; stem cells; transcriptional regulators

This review summarizes recent advances in understanding normal cell types in the mammary gland (breast), their potential lineage relationships, and the roles of molecular regulators in fate specification and differentiation. The mammary epithelial hierarchy provides an important framework for deciphering the cell types necessary for normal physiological function as well as understanding perturbations that lead to breast cancer. The prospective isolation of stem and progenitor cells, informed by both in vivo transplantation and lineage tracing experiments, has helped to shed light on the cell of origin for distinct subtypes of breast cancer. For example, a hyperproliferative RANK$^+$ luminal progenitor cell appears to be the culprit cell that gives rise to triple-negative breast cancer in women harboring a germline mutation in the *BRCA1* gene. This has revealed a targetable cell for chemoprevention. Female steroid hormones (estrogen and progesterone) and key regulators (e.g., Notch pathway, GATA-3) are instrumental for normal development, and their deregulated expression is implicated in breast cancer. The microenvironment is also emerging as an important regulator of mammary gland development and neoplasia. Finally, the reactivation of embryonic developmental programs and emergence of cellular "plasticity" appear to play important roles in breast cancer.

I. INTRODUCTION

The mammary gland is a highly specialized organ whose primary function is to produce milk for the nourishment of offspring. Unlike other organs, morphogenesis of the mam-

mary gland predominantly occurs in the postnatal period, where it undergoes dramatic ductal morphogenesis in puberty to produce a mature ductal tree, hormone-regulated budding and regression with estrus cycling, and dynamic cycles of alveolar expansion, differentiation, and cell death with each round of reproduction (51). The underlying processes are orchestrated by intricate molecular and cellular mechanisms instigated by local and systemic cues. Architecturally, the mammary gland comprises a complex epithelial ductal tree that is surrounded by a stromal matrix containing adipocytes, endothelial cells, fibroblasts, and immune cells **(FIGURE 1)**. In addition to providing structural support, signaling between the epithelium and stroma is essential for coordinating proper mammary gland development (99, 187). Two main cellular lineages constitute the mammary epithelium: luminal epithelial cells that surround a central lumen and an outer layer of elongated myoepithelial cells that lie adjacent to the basement membrane. Together these cells constitute a branched, bilayered ductal system that undergoes extensive morphogenesis and regeneration throughout the lifespan of a mammal **(FIGURE 1)**. The prime function of luminal cells is to generate milk secretory cells in lactation, while the contractile property of myoepithelial cells enables the expulsion of milk.

Here we review the recent literature pertaining to the normal epithelial constituents of the mammary gland and the unfolding differentiation hierarchy. We discuss the origin, specification, and differentiation of mammary stem cells (MaSCs) and unipotent progenitor cells through development, and the delineation of key molecular regulators of the hierarchy, with emphasis on the murine mammary gland. The highly dynamic nature of the mammary epithelium and its responsiveness to hormones and contextual cues from neighboring cells likely render stem and progenitor cells susceptible to breast oncogenesis. The heterogeneity uncovered within the normal epithelial compartment has implications for understanding breast tumor subtypes. Although

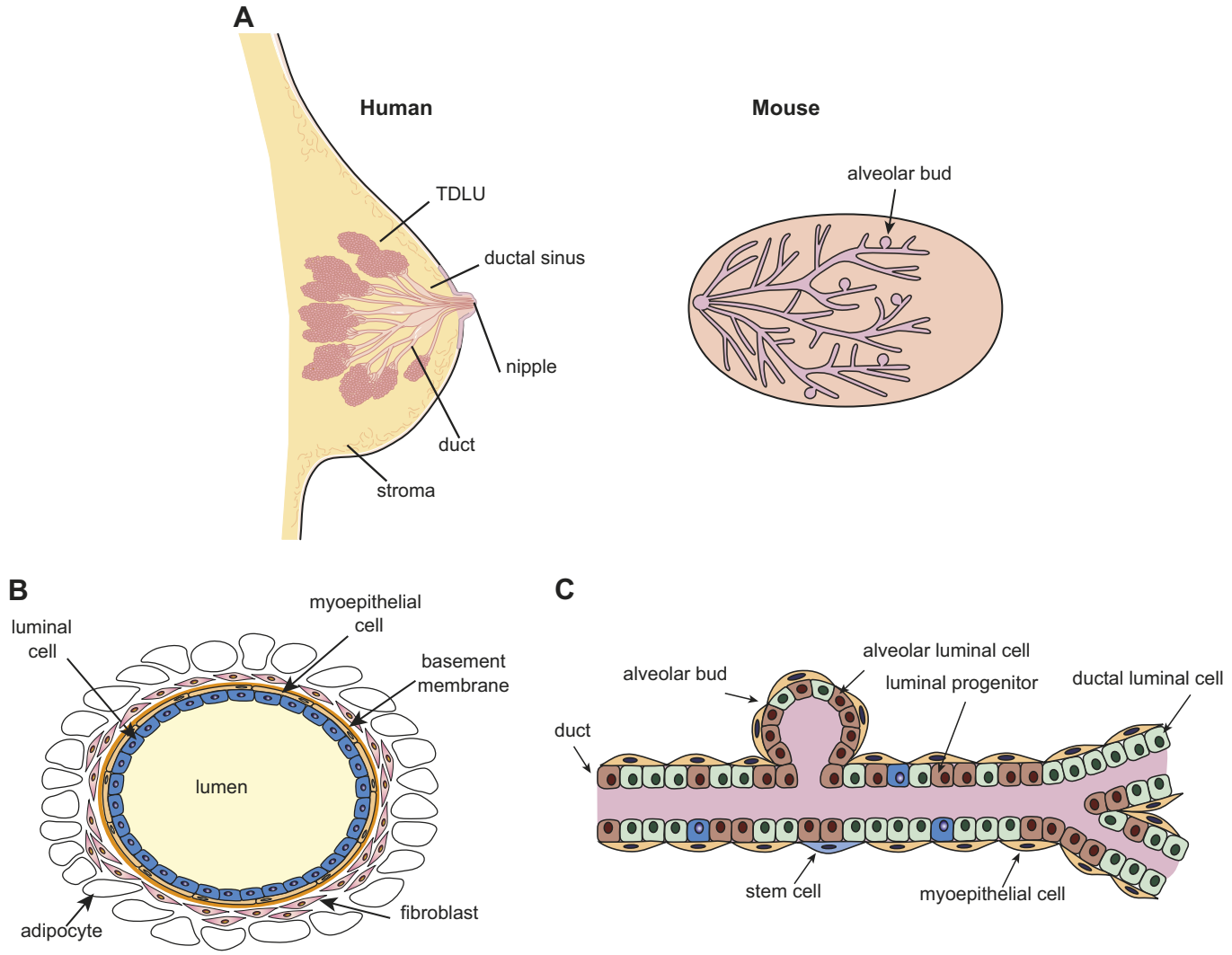


FIGURE 1. Schematic representation of the human and mouse mammary glands and ductal structure. *A*: human and mouse mammary glands. TDLU, terminal duct lobular unit. *B* and *C*: different epithelial cell types resident in mammary ducts.

the hierarchy appears to be unidirectional under steady-state conditions, plasticity is evident in perturbed states such as neoplasia and likely contributes to intratumoral diversity.

II. MAMMARY GLAND DEVELOPMENT

A. Embryonic Development

There are three major stages of mammary gland development: embryonic, pubertal, and reproductive. The precise processes underlying mammary specification and patterning during embryogenesis remain obscure, but inductive signals from the mesenchyme are likely to instruct local migration or aggregation to form the anlage. Mammary morphogenesis is initiated at embryonic day (E) 10.5 in mice with the formation of ectoderm-derived bilateral mammary milk lines that extend from the anterior to posterior limb bud **(FIGURE 2)** (94). By E11.5, migration and aggregation of ectodermal cells at specific locations along the mammary line give rise to five pairs of multilayered structures called placodes (174). The subsequent invagination of placodes into the underlying mesenchyme leads to the formation of mammary epithelial buds, which continue to descend and generate a stalk that connects the mammary bud with the epidermis.

Mesenchymal cells surrounding the epithelial bud condense and differentiate to form the mammary mesenchyme, a compact layer of fibroblastic cells. Importantly, factors secreted by the mesenchyme exert significant influence on the mammary line, stimulating differentiation towards the mammary epithelial lineage. Indeed, recombinant transplantation experiments between mesenchymal and epithelial tissue demonstrated that the tissue origin of the mesenchyme dictates the morphology of the resulting epithelium (47, 186). At E15.5, the epithelial bud begins to proliferate, and a single sprout extends into the fat pad precursor mesenchyme, which is composed of a cluster of preadipocytes located in the dermis. At this point, ductal branching morphogenesis is initiated, yielding a rudimentary ductal tree comprising a primary duct and 10–15 secondary branches, which is present at birth and remains largely quiescent until puberty (94). This process is generally conserved in humans, in which mammary lines arise during the first trimester and then resolve into a single pair of placodes (100). However, multiple sprouts extend from the epithelial bud and create numerous ductal trees emanating from the nipple, culminating in 8–15 patent ducts radiating from the nipple to the lobes.

B. Puberty

Driven by hormonal cues and growth factors, extensive ductal branching and elongation occur at the onset of puberty and result in an elaborate epithelial ductal tree that extends through the entire mammary fat pad (the

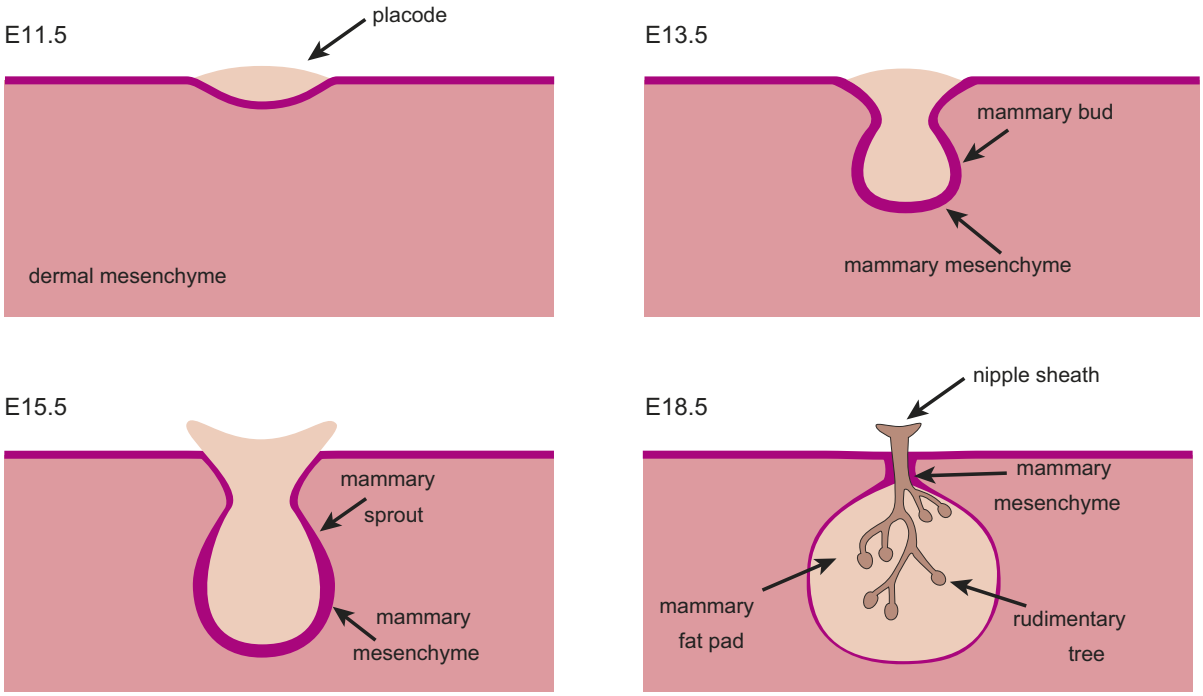


FIGURE 2. Schematic diagram of mammary ontogeny in the embryo. In the mouse embryo, the placodes (visible at E11.5) develop into mammary buds that penetrate the underlying mesenchyme around E13.5. These buds sprout by E15.5 and form a lumen. By E18.5, a small arborized gland invades the mammary fat pad.

nonepithelial elements of the mammary gland) (FIGURE 3). Cell proliferation predominantly occurs within terminal end buds (TEBs), which are specialized club-shaped structures located at the tips of growing ducts. TEBs are composed of two distinct cell types, a compound inner layer of central body cells surrounded by a single distal layer of cap cells that give rise to the differentiated luminal epithelium and myoepithelium, respectively (FIGURE 3). Cap cells are morphologically undifferentiated, which led to the hypothesis that this layer may contain progenitor cells that generate both myoepithelial and luminal cells of the subtending ducts (238). Progression through the mammary fat pad is largely driven by the highly proliferative TEB structures, which penetrate the stromal pad at a rate of ~0.5 mm/day (50). Bifurcation of the TEB leads to the formation of primary ducts, while central body cells within the TEB undergo apoptosis to form a lumen in a process that requires a precise balance between cell proliferation and cell death (102). Once ductal elongation extends to the boundaries of the mammary fat pad, TEBs are no longer present (68). The fully developed epithelial tree comprises a system of primary ducts and secondary branches plus short tertiary branches that emerge laterally to fill the interductal space (50).

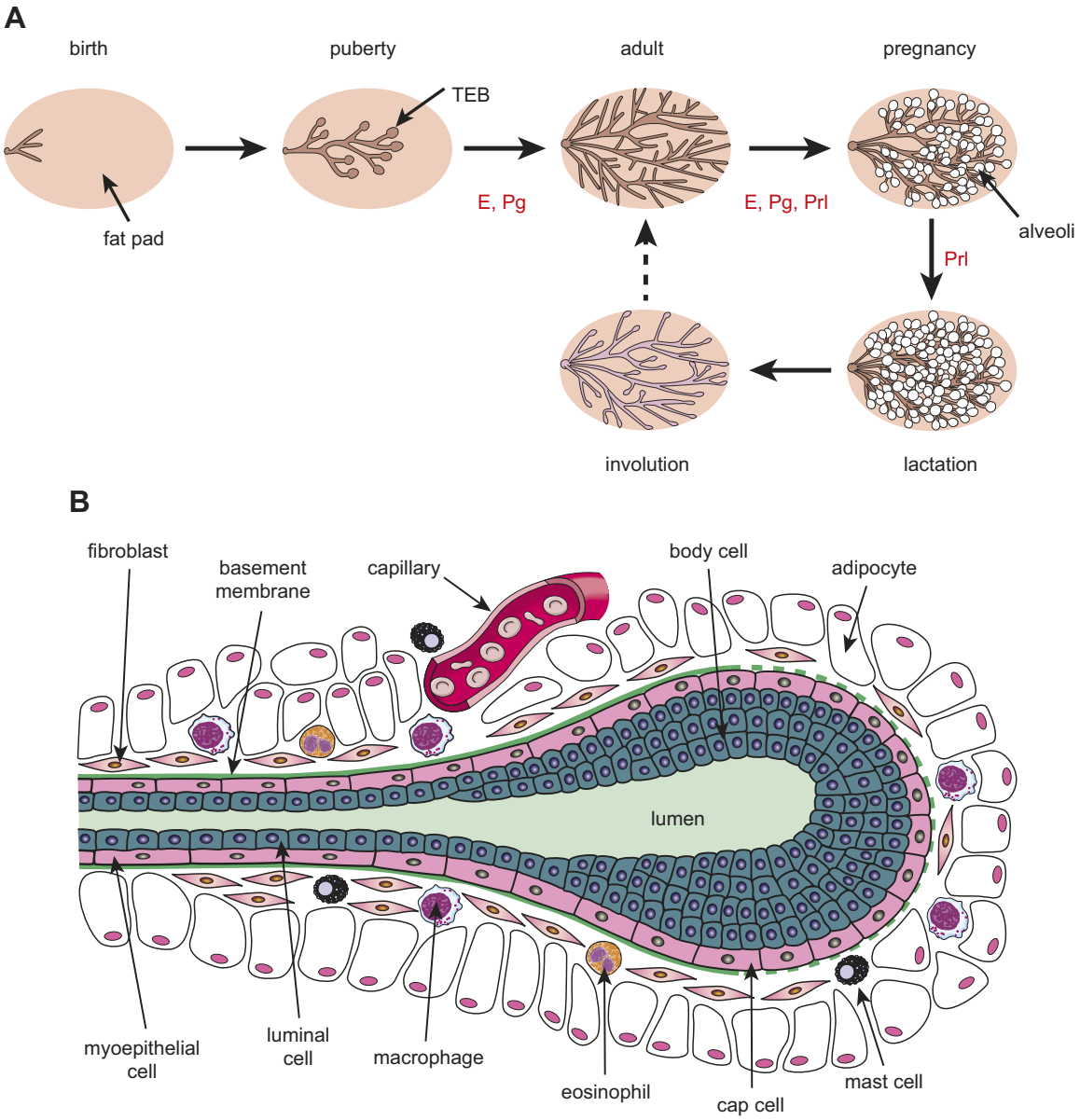


FIGURE 3. Diagram of postnatal mammary gland development. *A*: in the postnatal animal, the early mammary gland grows in an allometric fashion and remains relatively dormant until the onset of puberty. At this stage, dramatic morphogenesis occurs, largely under the control of estrogen (E). In the young adult, progesterone (Pg) regulates side-branching, while in pregnancy, the steroid hormones E, Pg, and prolactin (Prl) exert roles in expansion of the alveolar units. In the late stages of pregnancy and during lactation, the peptide hormone Prl plays a key role in establishing the secretory state. After lactation, the gland involutes and returns to a resting state. *B*: representation of a terminal end bud in a pubertal mouse mammary gland.

In human breast, puberty-induced arborization leads to the formation of a considerably more complex ductal tree compared with the mouse, with primary ducts branching into segmental and smaller subsegmental ducts (100). Further subdivision of subsegmental ducts leads to terminal ducts, which culminate in a collection of blind-ended ductules termed acini **(FIGURE 1)**. A cluster of acini extending from one terminal duct, and encased in loose intralobular connective tissue, is referred to as a terminal duct lobular unit (TDLU). The degree of lobular complexity within a TDLU has been classified into types I–IV, which reflects their differentiation status, as described in detail by Russo et al. (185). Type I lobules are the least differentiated and consist of short terminal ducts ending in a collection of acini, while the more complex type II and III lobules encompass numerous ductules branching from the terminal duct, resulting in large numbers of acini. Interestingly, type IV lobules are only present in lactating women, and after menopause, there is substantial regression to the type I TDLU. Of note, the TDLUs rather than the ducts are the major sites in which breast cancers arise (237). Significant variation in both the epithelial and stromal content of the nulliparous human breast has been documented between individuals and within the breast tissue of the same individual (63, 100). In contrast, the mouse mammary gland lacks TDLUs but develops alveolar units during pregnancy. Another key morphological difference between human and mouse mammary tissue is the stroma. In humans, the stroma is enriched for fibrous connective tissue compared with the adipocyte-rich environment in the mouse mammary gland (161). This difference may relate to the decreased requirement for physical support particularly during lactation in the mouse mammary gland, given its flattened position under the skin (99). Despite these differences, the cellular composition of the epithelial compartments within human and mouse mammary tissue is remarkably similar.

C. The Reproductive Cycle

During homeostasis in the adult mammary gland, additional changes to ductal morphology occur with each estrus cycle. Cyclical proliferation and differentiation give rise to side branches (tertiary branches) as well as alveolar buds that wax and wane during the cycle (210). Notably, these buds develop further and differentiate under pregnancy stimuli. The architecture of the mammary gland is markedly transformed in pregnancy through the formation of complex alveolar units composed of alveolar cells that undergo terminal differentiation into specialized secretory cells in late pregnancy **(FIGURE 3)**. The prime function of these secretory alveolar cells is the synthesis and secretion of milk for lactation. The morphogenic changes in pregnancy are largely driven by progesterone and prolactin (reviewed in Refs. 25, 27). In the initial phase of pregnancy, progesterone induces a profound surge in cellular proliferation, leading to extensive secondary and tertiary branching of the ductal tree. In human breast tissue, substantial heterogeneity is apparent during pregnancy, in which acini undergoing dramatic proliferation can lie adjacent to acini in a quiescent state (100). Progesterone and prolactin coordinate alveolar development during the next phase of pregnancy, characterized by a decline in proliferation in lieu of epithelial differentiation and secretory lineage commitment. Concurrent angiogenesis leads to the alveolar buds becoming encased in a network of capillaries (57). Interestingly, cytokinesis failure results in most secretory alveolar cells adopting a binucleated state in the transition from pregnancy to lactation (180). This appears to be an evolutionarily conserved mechanism to maximize milk production and thus promote the survival of offspring. Substantial stromal remodeling also occurs during this period, and by late pregnancy, adipose tissue is highly compressed due to the expanded alveoli (98). Interestingly, adipocytes are not regenerated during the reproductive cycle, and expansion occurs through hypertrophy of existing adipocytes (252). During the secretory phase in late pregnancy, the expression of milk proteins (such as whey acidic protein and α-lactalbumin) is markedly upregulated, and lipid droplets begin to form (2). The production of vast quantities of protein and other nutrients by secretory alveolar cells imposes considerable stress upon the cellular machinery. To ensure the survival of these apically oriented luminal cells and the production of milk, a mechanism involving epidermal growth factor (EGF)-mediated induction of the pro-survival protein Mcl-1 at the lactogenic switch has evolved (76).

Following weaning, the mammary gland undergoes another period of drastic remodeling, in which alveolar cells are removed by cell death and the epithelial tree returns to a state similar but not identical to the resting virgin mammary gland. In a tightly regulated series of events, 80% of the mouse mammary epithelium is lost within 3 days. In mice, there are two key phases of involution: the first phase is largely regulated by local signaling cues, in contrast to the second phase, where systemic hormones exert significant influence (236). Collectively, changes occurring in the mammary gland during the reproductive cycle are remarkable examples of tissue remodeling and dynamics that allow the mammary epithelium to continually adapt throughout life to meet its physiological requirements.

III. IDENTIFICATION OF STEM CELLS IN THE ADULT MAMMARY GLAND BY TRANSPLANTATION

A. Definition of Tissue-Resident Stem Cells

Adult stem cells, irrespective of tissue of origin, are classically defined as unspecialized cells with the capacity for almost indefinite self-renewal and for multipotent differentiation to generate all cell types present within a tissue or organ. Progenitor cells, in contrast, have more restricted differentiation capacity but lack the extensive self-renewal capacity of stem cells. A number of tissue-specific designs appear to have been engineered into stem cell hierarchies to accommodate their function, as re-

cently reviewed (231). It has also become evident that stem cell activity is context dependent, thus warranting definition according to the functional assay.

Two types of in vivo assays have been used to define MaSCs at a functional level: transplantation and genetic lineage tracing. While stem cells have been classically defined by their potency, recent studies have focused on defining the fate of cells under steady-state conditions. The transplantation assay reads out the inherent lineage potential (or potency) of a cell to function as a stem cell in its normal anatomical site, albeit in a perturbed environment **(FIGURE 4)**. Only cells that harbor multipotent capacity can repopulate a mammary ductal tree. In a powerful and independent approach to understand the contribution of stem and progenitor cells to development and homeostasis in situ, lineage tracing in mice enables the fate of a given cell to be tracked at a given time point in its native environment **(FIGURE 5)**. The distinction between the "potency" versus the "fate" of a cell is noteworthy, and both properties should be considered when constructing lineage relationships. In this review, we refer to mammary repopulating cells identified in transplantation assays as stem cells. For lineage-marked cells in vivo, we primarily define as progenitor cells, unless extensive capacity for self-renewal was established.

B. Historical Evidence for Mammary Stem Cells

The dynamic nature of mammary epithelial cell proliferation, differentiation, and turnover, coupled with the remarkable regenerative capability through successive cycles of pregnancy, suggested the existence of a renewable stem cell population. Indeed, the concept of a stem cell entity within the mammary gland was first implied through the pioneering work of DeOme et al. (54), who showed that transplantation of normal mammary tissue fragments into the cleared (de-epithelialized) mammary fat pads of 3-wk-old recipient mice generated ductal outgrowths that mimicked the structure of the normal epithelial tree **(FIGURE 4)**. The ductal outgrowths could be serially transplanted, thus demonstrating self-renewal capacity (49, 96, 97, 203). The mammary epithelium, however, has finite proliferative capacity and can be serially passaged in vivo for up to seven generations before the onset of senescence (49). Further studies demonstrated that tissue fragments harvested from donor mice at any age, developmental stage, or location within the mammary gland could give rise to fully functional mammary outgrowths (203). Early cell tracing experiments using retrovirally-marked mammary cells first suggested that the progeny of a single multipotent stem cell could reconstitute a mammary gland following transplan-

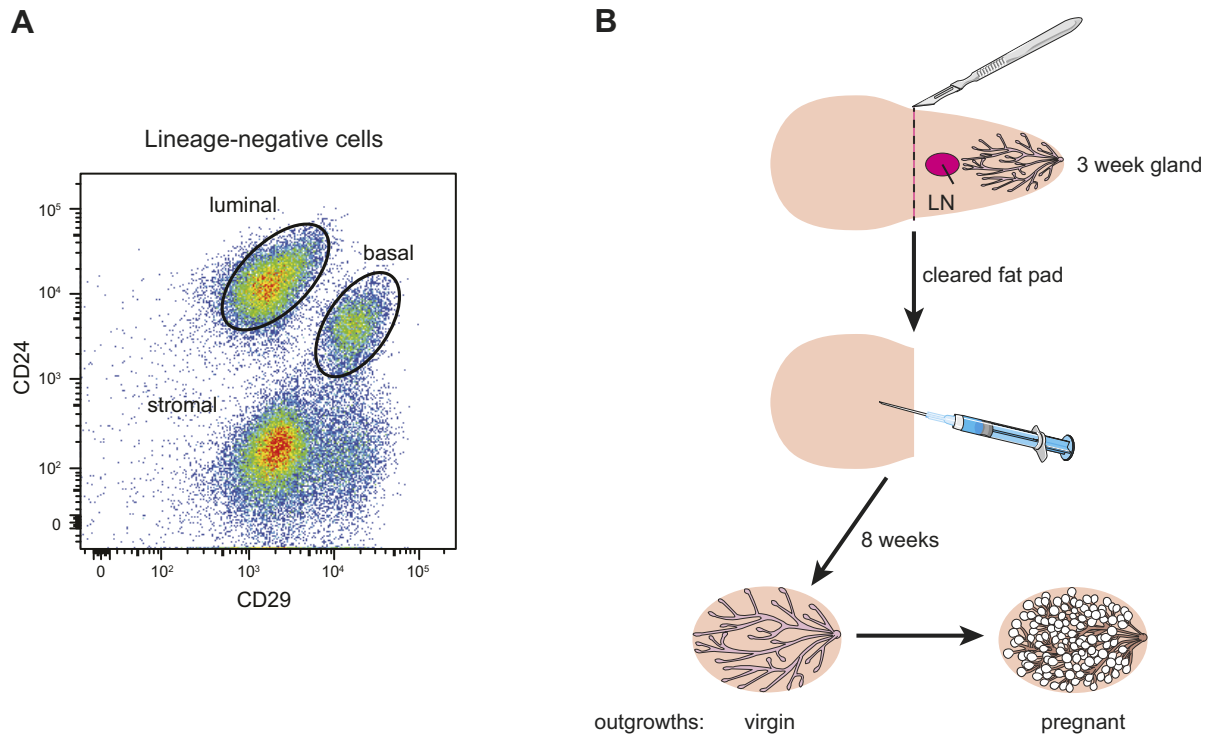


FIGURE 4. Identification of stem cells by transplantation into the mammary fat pad. *A*: antibodies against the markers CD24 (heat-stable antigen) and CD29 (β1-integrin) can be used to fractionate lineage-negative cells into basal and luminal epithelial compartments by flow cytometry. *B*: cells are implanted into the cleared mammary fat pads of 1-wk-old mice and harvested from either virgin or pregnant hosts. It is challenging to produce highly viable mammary cell suspensions; more recent optimization has yielded a repopulating frequency of $<1/100$ for single-sorted cells in the basal compartment, implanted in 25% Matrigel (76, 172).

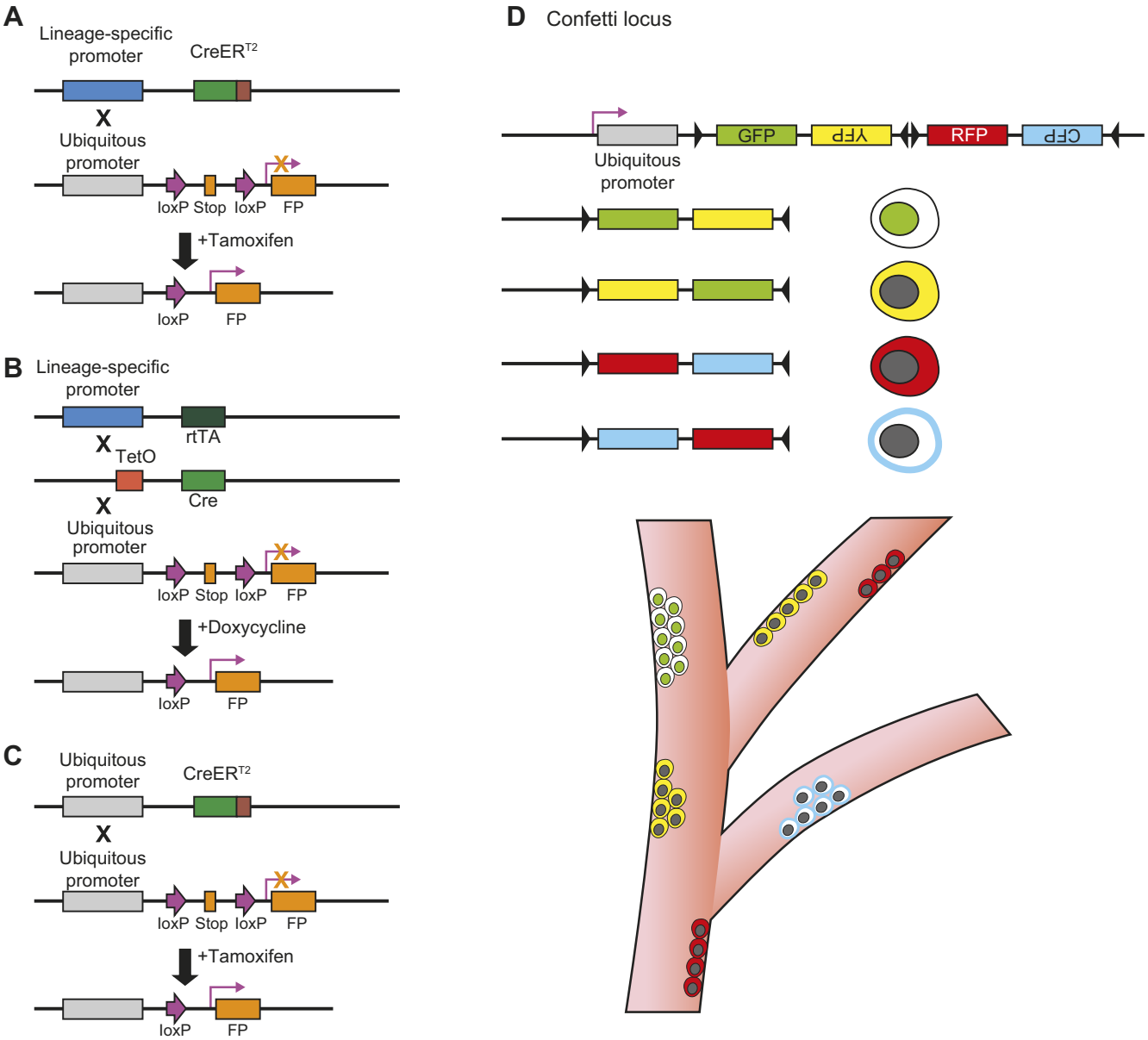


FIGURE 5. Genetic lineage tracing strategies in mice. *A*: lineage tracing using the tamoxifen-regulated creER^{T2} system together with a lineage-specific promoter. *B*: lineage tracing using the doxycycline-rtTA-Tet operon-regulated system together with a lineage-specific promoter. *C:* neutral or stochastic labeling of cells using a ubiquitous promoter (e.g., Rosa26) and creER^{T2} system. Fluorescent protein expression can occur in any cell. *D:* the Rosa26-confetti reporter model, leading to stochastic expression of a single fluorescent protein (FP) in a given cell (nuclear GFP, cytosolic YFP, cytosolic RFP, or membranous CFP). Examples of labeled clones in branching ducts are shown.

tation and self-renew upon serial passaging in mice (112). Collectively, these studies implied that stem cells are positioned throughout the epithelial tree and contribute to both ductal maintenance and development.

C. Prospective Isolation of Adult Mouse Mammary Stem Cells

Cell fractionation based on cell surface markers and flow cytometry, together with transplantation studies, led to the prospective isolation of mouse MaSCs more than a decade ago **(FIGURE 4)**. These cells harbored multilineage differentiation, which included the generation of functional milk-producing alveolar units, and long-term self-renewal potential, thus fulfilling the two hallmark properties of stem cells (193, 201, 213). Of note, the terms *multipotent* and *bipotent* are often used interchangeably in the literature as there are two primary mammary epithelial cell lineages, but the luminal lineage can be further subdivided into the ductal and alveolar sublineages.

On the basis of flow-sorting and transplantation studies, mammary repopulating cells with an immunophenotype of $Lin^-CD24^+/CD29^{high}/CD49f^{high}/Sca1^{low}$ were found to specifically reside within the basal epithelial compartment (adjacent to the basement membrane). In contrast to basal cells, the luminal compartment ($Lin^-CD24^+CD29^{low}$-$CD49f^{low}$) lacks repopulating potential. Transplantation assays (172, 193, 213) suggest that MaSCs comprise ~2–5% of the basal compartment, with mature myoepithelial cells and presumptive basal progenitor cells representing the bulk of the population **(TABLE 1)**. The mammary repopulating frequency for the flow-sorted basal subset is ~1/60 to 1/100 cells, but more refined single-cell transplantation studies incorporating microscopic visualization of individual cells indicated that 1 in 18 basal cells (~5%) harbor this capacity (193). EpCAM plus CD49f-based flow cytometry is also commonly used for the separation of epithelial populations and provides comparable enrichment of mammary repopulating cells (194). Taken together, only a small fraction of freshly sorted basal cells harbor repopulating capacity in the cleared mammary fat pad. It remains to be determined whether MaSCs are predetermined or reflect a stem cell state. Although it is important to use uncultured mammary cells for reconstitution studies, it is interesting to note that basal/myoepithelial cells cultivated in the presence of ROCK inhibitor (disrupts actin-myosin interactions) show enhanced mammary repopulating potential (172).

Gene expression profiling and in vitro clonogenic assays confirmed that the $Lin^-CD24^+CD29^{high}CD49f^{high}$ population expresses typical myoepithelial/basal markers including cytokeratins 5 and 14 (K5 and K14) and smooth muscle

Table 1. *Cell surface markers for isolation of epithelial cell subpopulations from the mouse mammary gland (fresh, uncultured cells)*

Markers	Population Definition	Stage	Reference Nos.
CD31, CD45, Ter-119, CD24, CD29	**MaSC/basal**: $Lin^-CD24^+CD29^{hi}$ **Luminal**: $Lin^-CD24^+CD29^{lo}$	Adult	Shackleton et al. (193)
CD31, CD45, Ter-119, CD140a, CD24, CD49f	**MRU**: $Lin^-CD140a^-CD24^{med}CD49f^{hi}$ **Luminal**: $Lin^-CD140a^-CD24^+CD49f^{lo}$ **Myo**: $Lin^-CD140a^-CD24^{lo}CD49f^{lo}$	Adult	Stingl et al. (213)
CD45, CD24	**Basal**: $CD45^-CD24^{med}$ **Luminal**: $CD45^-CD24^{hi}$	Adult	Sleeman et al. (200)
CD31, CD45, Ter-119, CD24, CD29, CD61	**MaSC/basal**: $Lin^-CD24^+CD29^{hi}$ **Luminal progenitor**: $Lin^-CD24^+CD29^+CD61^+$ **Mature luminal**: $Lin^-CD24^+CD29^+CD61^-$	Adult	Asselin-Labat et al. (4)
CD45, CD24, Sca1/CD133	**Basal**: $CD45^-CD24^{med}Sca1^-CD133^-$ **ER/PR$^+$ luminal**: $CD45^-CD24^{hi}Sca1^+CD133^+$ **ER/PR$^-$ luminal**: $CD45^-CD24^{hi}Sca1^-CD133^-$	Adult	Sleeman et al. (201)
CD31, CD45, Ter-119, CD24, CD29, CD14, cKit	**MaSC/basal**: $Lin^-CD24^+CD29^{hi}$ **Luminal progenitor**: $Lin^-CD24^+CD29^{lo}CD14^+cKit^+$ **Mature luminal**: $Lin^-CD24^+CD29^+CD14^-cKit^-$	Adult	Asselin-Labat et al. (5)
CD45, CD24, CD49f, Sca1, cKit	**MaSC**: $CD45^-CD24^{+/lo}CD49f^{hi}cKit^-$ **Myo**: $CD45^-CD24^{+/lo}CD49f^{+/lo}cKit^-$ **ER/PR$^+$ luminal progenitor:** $CD45^-CD24^{+/hi}Sca1^+cKit^+$ **ER/PR$^-$ luminal progenitor:** $CD45^-CD24^{+/hi}Sca1^-cKit^{+/-}$ **Mature luminal**: $CD45^-CD24^{+/hi}Sca1^+cKit^-$	Adult	Regan et al. (177)
CD31, CD45, Ter-119, BP-1, EpCAM, CD49f, Sca1 and CD49b	**MaSC/basal**: $Lin^-BP\text{-}1^-EpCAM^{med}CD49f^{hi}$ **ER/PR$^+$ luminal progenitor**: $Lin^-BP\text{-}1^-EpCAM^{hi}CD49f^+Sca1^+CD49b^+$ **ER/PR$^-$ luminal progenitor**: $Lin^-BP\text{-}1^-EpCAM^{hi}CD49f^+Sca1^-CD49b^+$ **Mature luminal**: $Lin^-BP\text{-}1^-EpCAM^{hi}CD49f^+Sca1^+CD49b^-$	Adult	Shehata et al. (194)
CD31, CD45, Ter-119, CD49f, CD24, EpCAM	**Fetal MaSC**: $Lin^-CD24^{hi}EpCAM^{hi}CD49f^{hi}$	E18.5	Makarem et al. (137), Spike et al. (208)
CD31, CD45, Ter-119, CD24, CD29, CD1d	**Slow cycling MaSC**: $Lin^-CD24^+CD29^{hi}CD1d^+$	Adult	dos Santos et al. (60)
CD45, CD31, Ter119, CD24, CD29, Procr	**Cycling MaSC**: $Lin^-CD24^+CD29^{hi}Procr^+$	Adult	Wang et al. (233)
CD31, CD45, Ter119, CD29, CD24, Tspan8	**Quiescent MaSC**: $Lin^-CD24^+CD29^{hi}Tspan8^{hi}$ (Lgr5-GFP$^+$)	Adult	Fu et al. (75)

MaSC, mammary stem cell; Myo, myoepithelial.

actin (SMA). It is noteworthy that basal (and luminal) cells are faithfully maintained without promiscuous differentiation in short-term colony-forming assays either in two dimensions on a fibroblast feeder layer or in three-dimensional assays, in which cells are embedded in the extracellular matrix Matrigel (193, 213). When basal cells were subjected to a lactogenic stimulus, however, they gave rise to a small percentage of acinar luminal colonies, consistent with a subset harboring multipotent capacity. Recently, organoid or "mini-organ" assays, first developed for the intestine (189), have been adapted for the mouse mammary gland (103, 104, 247). Only basal cells could generate complex structures comprising an inner compartment of luminal cells surrounded by a network of elongated myoepithelial cells, even at the single-cell level. Luminal cells, on the other hand, yielded luminal-restricted organoids on short-term passage (103, 247).

There are notable parallels between the evolving mammary hierarchies in mouse and human. Human breast epithelium has been fractionated into three populations based on EpCAM and CD49f expression: basal ($Lin^-EpCAM^{low/-}CD49f^+$), luminal progenitor ($Lin^-EpCAM^+CD49f^+$), and mature luminal cells ($Lin^-EpCAM^+CD49f^-$) **(TABLE 2)**. Interestingly, these two markers only distinguish two populations in the mouse, basal and total luminal cells. In vivo repopulating capacity has been demonstrated for human $Lin^-EpCAM^{low/-}CD49f^+$ cells based on transplantation into humanized mammary fat pads (126) or under the renal capsule of immunocompromised mice (64). These data are consistent with cellular assays that support the concept of bipotent breast epithelial cells and a differentiation hierarchy in human breast tissue (84, 212, 228).

D. Purification of Heterogeneous MaSCs: Quiescent and Cycling Stem Cells

Quiescence is a fundamental property of many tissue-resident stem cells and likely evolved to preserve long-term regenerative potential and protect stem cells against functional exhaustion. Evidence for quiescent stem cells in the mammary gland has come from different approaches primarily based on their ability to retain synthetic DNA nucleotides. Long-term label retention experiments in vivo (32, 193, 202) and PKH26 labeling of cells ex vivo (45, 163) first suggested the existence of slow-cycling MaSCs in adult mouse or human mammary tissue. Moreover, an inducible histone 2b (H2B)-green fluorescent protein (GFP) fusion protein identified slow cycling cells in the basal population of the adult mouse mammary gland, and these cells expressed the MHC-like glycoprotein CD1d (60). This population accounted for ~0.2% of cells in the total mammary gland, but the relative activity of $CD1d^+$ versus $CD1d^-$ basal cells was not determined. Interestingly, embryonically derived long-term nucleotide label-retaining cells could seed quiescent basal cells in the adult mammary gland and were responsive to hormones (21). These properties are consistent with the tracking of long-lived stem cells as opposed to terminally differentiated ductal cells.

Recent efforts towards the prospective isolation of quiescent MaSCs have uncovered functional heterogeneity in the adult MaSC compartment. Bcl11b, a C2H2 zinc finger domain transcription factor that is a component of multiple chromatin-remodeling complexes, is expressed at high levels in ~4% of basal cells (35). This subpopulation identified a suprabasal quiescent population in the adult gland with sixfold higher in vivo reconstituting capacity than its $Bcl11b^{low}$ counterpart. As anticipated, downregulation of Bcl11b occurred concomitantly with alveolar expansion and differentiation during pregnancy (35). Bcl11b appears to govern quiescence by preventing the exhaustion of stem cells through expression of the cell cycle inhibitor Cdkn2a. Furthermore, quiescent MaSCs have been isolated based on *Lgr5* and *Tspan8* (Tetraspanin 8) expression, which is highly upregulated in *Lgr5*-expressing cells (75). A small subset of cells (<5% of basal cells) was significantly enriched for quiescent cells ($Lin^-CD29^{high}CD24^+Lgr5^+$-

Table 2. *Cell surface markers used for isolation of epithelial cell subpopulations from human breast tissue (fresh, uncultured cells)*

Markers Used	Population Definition	Reference Nos.
CD45, CD10, CD14, CD15, CD19, CD24, CD44	**Stem-like**: $Lin^-CD10^-CD24^-CD44^+$ or $Lin^-CD10^-CD24^-PROCR^+CD44^+$	Shipitsin et al. (195)
CD31, CD45, CD34, IB10, CD49f, EpCAM	**Luminal progenitor**: $Lin^-CD49f^+EpCAM^{hi}$ **Myoepithelial (multipotent)**: $Lin^-CD49f^+EpCAM^{hi}$	Villadsen et al. (228)
CD45, CD31, CD235, CD49f, EpCAM	**MaSC**: $Lin^-CD49f^+EpCAM^{-/lo}$ **Luminal progenitor**: $Lin^-CD49f^+EpCAM^+$ **Mature luminal**: $Lin^-CD49f^-EpCAM^+$	Eirew et al. (64), Lim et al. (126), Shehata et al. (194)
CD24, CD49f, DNER	**MaSC**: $CD24^{hi}CD49f^{hi}DNER^{hi}$	Pece et al. (163)
CD45, CD31, CD34, CD45, IB10, EpCAM, CD10	**MaSC/common progenitor**: $CD10^+EpCAM^-$ **Mature luminal**: $CD10^-EpCAM^+$	Bachelard-Cascales et al. (8), Keller et al. (110)

MaSC, mammary stem cell.

Tspan8^{high}), and these shared striking molecular similarity to other tissue-specific quiescent stem cells. Moreover, these cells reside exclusively in the proximal region (older portion of ductal tree) over their lifespan (75), in contrast to *Bcl11b^{high}* cells, which were found dispersed throughout the ductal network (35). The location of Lgr5^{+}Tspan8^{high} cells in the vicinity of the nipple is consistent with multiscale in situ analysis of putative MaSCs (70) and points to the presence of a reservoir of long-lived stem cells **(FIGURE 6)**.

The concept of different subsets of repopulating cells that can be prospectively isolated as phenotypically discrete populations was first described for the hematopoietic system (192). By analogy, the mammary basal compartment can be fractionated into four subsets according to *Lgr5* and Tspan8 expression (together with CD29/CD24), with three subsets harboring substantive repopulating capacity in vivo. The deeply quiescent subset was the most potent, paralleling findings for other tissue-specific stem cells (75). Conversely, protein C receptor (Procr) was found to identify cycling stem cells in the CD29^{high}CD24^{+} compartment, yielding a frequency of 1 in 12 upon transplantation (230). Procr and *Lgr5* or *Bcl11b* mark nonoverlapping subsets in the MaSC compartment, consistent with the cycling status of Procr^{+} cells versus the quiescent state of *Lgr5^{+}* and *Bcl11b^{+}* cells (35, 233). However, there is no apparent overlap between *Bcl11b^{high}*, *CD1d^{+}*, and Lgr5^{+}Tspan8^{high} cells. While it is plausible that different reporter models and cell sorting protocols have not captured the full spectrum of cells, collective data in the field imply multiplicity within the adult MaSC compartment.

It remains to be determined whether human breast stem cells display functional and spatial heterogeneity as occurs in the mouse, but the differing architecture of human breast tissue suggests that there may be distinctions. Meticulous dissection of ducts versus lobules in human tissue has led to the proposal of a candidate stem cell niche that is located specifically within the ducts. Putative stem cells comprise K14^{+}K19^{+} cells and are essentially quiescent. In contrast, proliferative lineage-restricted progenitors are thought to be located within the lobules at the ends of ducts (228). Extending on this work, analysis of the myoepithelial populations in the TDLUs and interlobular ducts of human breast has provided evidence for region-specific multipotent stem/progenitor cells (73). Similar to Lgr5^{+}Tspan8^{high} basal MaSCs in the mouse, a subset of human breast stem cells that are p63^{+}K5^{+}K14^{+} cells seem to be enriched in the nipple region of the human breast and may represent quiescent stem cells (19).

IV. DELINEATION OF BASAL PROGENITOR CELLS BY LINEAGE TRACING

A. "Population-Based" Genetic Lineage Tracing

Genetic lineage tracing in mice relies on spatial and temporal control of gene expression through the selection of a highly lineage-specific promoter to drive *cre* expression in an inducible fashion, typically with doxycycline or tamoxifen **(FIGURE 5)**. Once the *cre* reporter gene is activated, all daughter cells derived from the parental cell are indelibly marked for their entire lifespan.

Despite the stem cell activity evident in transplantation assays, it has become evident that basal cells demonstrate more restricted potential in the steady-state mammary gland. The majority of cell-fate mapping studies for the basal lineage using "generic" (*K5, SMA* and *K14*) or other promoters (*Lgr5, Axin2*) have read-out basal progeny in the adult mammary gland and shown that unipotent progenitor cells drive postnatal morphogenesis and replenishment of the basal layer during homeostasis in the adult (113, 172, 223, 226). The longevity of these unipotent basal cells over

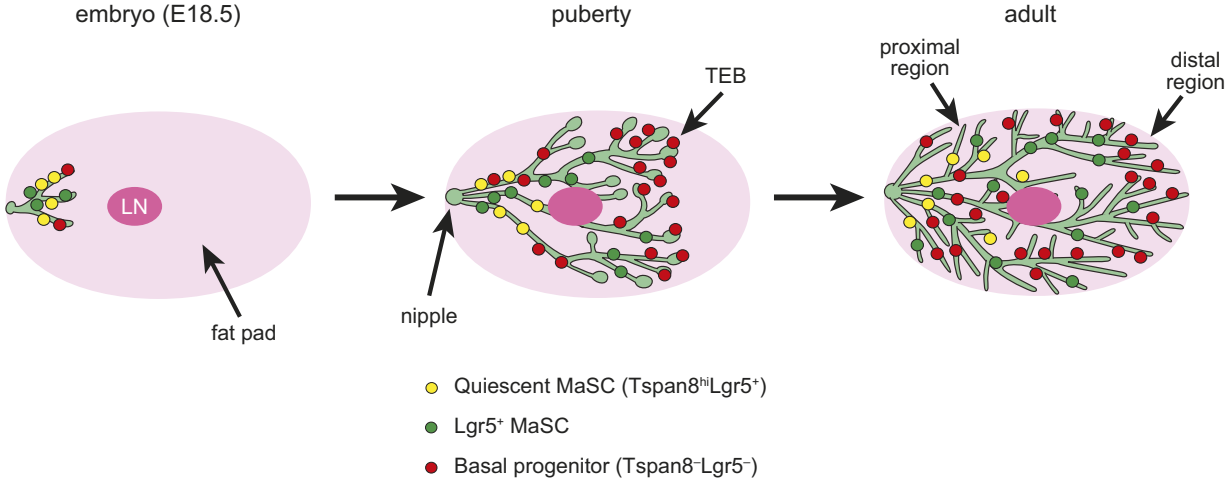


FIGURE 6. Schematic diagram summarizing the distribution of the different basal subpopulations defined by Lgr5 and Tspan8 expression at various stages of mammary gland development.

one or more cycles of pregnancy-lactation-involution is consistent with the presence of long-lived basal-restricted progenitors. While these could be coined stem cells, herein we have reserved the term *stem cell* to denote self-renewing and multipotent cells that can repopulate all epithelial lineages. Notably, lineage tracing studies at saturation, in which an average of 95% of basal compartment cells in a K14-transgenic model were labeled over 5 wk during puberty, have confirmed that the basal lineage is self-sustained by unipotent cells in both the resting and pregnant gland (226, 239). Taken together, it has been postulated that basal bipotent stem cells do not exist in the adult mammary gland and thus have negligible contribution.

Nevertheless, multipotent basal cells in the adult mammary gland have been identified in lineage tracing assays using the same generic or more restricted gene promoter drivers (*K5, Procr, Lgr5,* and *Dll1*). Three-dimensional (3D) imaging of large portions of intact ductal tissue in its native stroma, together with a multicolored reporter locus for clonal cell-fate mapping studies, was instrumental in tracking stem/progenitor cells and their progeny at clonal resolution (181). K5-labeled progenitors could generate small unicolored clones (same color neighbors) comprising both luminal and myoepithelial cells, compatible with the existence of bipotent cells. No luminal-only clones were observed upon extensive 3D imaging, and labeled luminal cells were not detectable before 48 h post-induction and only in end-buds. Notably, cell-fate mapping in a knock-in K5 mouse model uncovered bipotent cells but only at a higher labeling efficiency of 60% (179) and not at lower labeling frequencies of ~15% (unpublished data and Ref. 226). The K5-knock-in model indicates a lower frequency of bipotent cells in the adult gland than the K5 transgene-based model and is likely more representative. Lineage tracing studies based on Procr, which marks a small subset of basal cells, have provided unequivocal evidence for the existence of long-lived bipotent progenitors; $Procr^+$ basal cells gave rise to bilineage clones in both the virgin and pregnant states (233). A recent lineage tracing model based on the expression of Notch ligand Dll1 also suggested that $Dll1^+$ basal cells may contribute to both the basal and luminal lineages (38). The discrepancy between these studies and the lineage tracing saturation studies are yet to be explained, but it remains plausible that a small subset of bipotent MaSCs reside in the adult gland and that these are not marked by the transgenic *K14* driver strain (239).

The mammary epithelium appears to be actively maintained through aging in the adult mouse, and this may be underpinned by multipotent MaSCs. Basal cells marked by K5 appeared to be long-lived as extensive unicolored, bilineage patches were visualized along the ducts after a 52-wk chase (178). These findings are concordant with serial transplantation studies of clonal ductal outgrowths (213) and implicate stem cells in coordinating homeostasis over the long-term through the generation of committed basal and luminal progenitor cells. The drift towards monoclonality across ducts with aging may occur through neutral competition as found for intestinal stem cells (204). In human breast tissue, the persistence of long-lived stemlike cells is implied through the findings that teenagers exposed to ionizing radiation develop breast cancer many years following exposure (120).

B. Unbiased Lineage Tracing

Neutral or stochastic lineage tracing models have been recently explored to track stem cell fate in vivo and provide a more unbiased means of cell labeling that is independent of a specific gene promoter. Labeling of cells (any cell) at clonal density enables the progeny of single cells to be clearly visualized. Using a continuous labeling approach to track a low-frequency genetic mark acquired during DNA replication (115), Davis et al. (52) only detected the presence of unipotent cells. While the data support the notion that unipotent progenitors are drivers of ductal morphogenesis and alveolar formation, the frequency of reporter activation was extremely low, on average 1.4 events/gland. Moreover, this system is dependent on DNA replication, which negates the labeling of quiescent stem cells, a feature of many tissue-resident stem cells. Nonetheless, other strategies dependent on activation of the Rosa26-driven confetti locus activation by Rosa26-$creER^{T2}$ (135, 191) lend strong support to the findings that unipotent cells predominantly function in the postnatal gland, as described in section VII.

V. COMPLEXITIES OF STEM/PROGENITOR CELL ASSAYS

The two in vivo assays employed to assess stem/progenitor cells in the mammary gland can yield differences, as highlighted by an *Axin2*-based model. Lineage tracing of *Axin2*-expressing epithelial cells in the adult showed that basal cells (~5% labeled) behaved in a strictly unipotent manner, whereas transplantation of the same cells revealed bipotency (223). These findings indicate that implantation into a transiently perturbed milieu can unleash properties that are not normally manifest under physiological conditions. It will be of interest to determine whether bipotency can be awakened upon damage or ablation of luminal cells during the course of lineage tracing.

Although lineage tracing studies provide the gold standard for assessing cell fate, limitations also apply. This technique is reliant on the specificity and efficiency of the driver promoter. Moreover, as gene promoters inevitably read-out activity of the dominant cell type, sufficient labeling of a cell population is required to capture the complete repertoire of cells within a given lineage. Labeling efficiencies in recently studied mouse mammary models have been reported in the

range of 0.2 to >60%, and thus may not encompass small subsets such as the 2–5% MaSC pool defined by regenerative capacity in vivo. The presence of large numbers of lineage-restricted progenitors does not necessarily preclude the existence of multipotent MaSCs in the adult basal compartment, and the slower turnover of myoepithelial versus luminal cells (79) may lead to the emergence of luminal-rich mixed clones, i.e., a clone that comprises both cell lineages but many more luminal than myoepithelial cells. Other parameters worth consideration are the mammary tissue toxicity associated with tamoxifen in both lineage compartments, even at relatively low doses (194), and the methods used to visualize clones. Advanced imaging techniques to track cells in 3D (52, 181) offer unprecedented views of labeled cells within large portions of the ductal tree to provide important information on the spatial distribution of stem and progenitor cells. The precise methodology utilized is also paramount since enzymatic digestion before 3D imaging results in the depletion and contraction of myoepithelial cells (179).

The developmental stage can profoundly impact mammary cell fate, often confounding interpretation. In the Wnt/β-catenin pathway, a switch in Wnt-responsive cells occurs at birth in both the *Axin2* and *Lgr5* reporter mouse models, whereby cells exhibit a luminal fate in the embryo and then switch to a basal cell fate in the early postnatal period (55, 223). In puberty, *Axin2*-marked cells gave rise to labeled clones in the basal and luminal layers of the subtending ducts (223). Although these were not always adjacent, this may be a consequence of movement of cells in the highly active TEB (238). During pregnancy, however, *Axin2*-marked cells led to the labeling of adjacent alveolar and basal cells, indicative of cells with bipotent capacity (223). Similarly, *Lgr5*-expressing cells exhibited cell-fate changes according to developmental context. Luminal cells were predominantly labeled in the prepubertal gland, while in the adult gland, basal/myoepithelial cells prevailed (55, 113, 220) in addition to rare bilineage clones (75). The latter finding suggests that basal-restricted cells may switch to bipotency in different hormonal environments. A deeper understanding of the different stage-specific outcomes observed upon lineage tracing will require more precise genetically engineered models. Furthermore, it seems that no single approach for studying stem/progenitor cells is optimal, and combined strategies will be required to resolve current questions in the field.

VI. IDENTIFICATION OF ADULT LUMINAL PROGENITOR CELLS

A. Prospective Isolation and Characterization of Luminal Progenitors

The luminal cell compartment of the mammary epithelium has been shown to encompass diverse luminal cells at the functional and morphological levels through flow cytometry and colony forming assays. Mouse luminal progenitor cells were initially identified on the basis of CD61 (integrin β3) expression: luminal progenitor cells were defined as $Lin^-CD24^+CD29^{low}CD61^+$ and differentiated luminal cells as $Lin^-CD24^+CD29^{low}CD61^-$ (4). $CD61^+$ progenitor cells are predominantly estrogen receptor-negative (ER^-), express high levels of the alveolar cell-fate determinant *Elf5* and certain milk protein transcripts, and can form milk-producing colonies in the presence of a lactogenic stimulus (155). Therefore, these progenitors likely represent the major alveolar precursor population in the adult gland. Interestingly, many $Elf5^+ER^-$ cells appear to have a distinct columnar shape within the bilayer (155). Other combinations of markers have led to the discrimination of more refined luminal subsets such as a rare and transient subset of estrogen receptor-positive (ER^+) luminal progenitors contained within the $Lin^-CD61^+CD14^+cKit^+Sca1^+$ population (5, 177, 194, 201). These cells may share similarity with the ER^{low} cells recently described (33). It should be noted that different names have been ascribed to the same subsets: hormone-sensing cells are equivalent to hormone receptor-positive (HR^+) mature luminal cells, while secretory alveolar progenitors are analogous to the predominantly ER^- luminal progenitor population.

Sca1 and CD49b (integrin α2) have emerged as prominent markers to fractionate the luminal compartment into distinct subsets (194, 201), whereby high levels of CD49b plus differential Sca1 expression resolve luminal progenitors into ER^+ ($Sca1^+CD49b^+$) and ER^- ($Sca1^-CD49b^+$) cells (194). Conversely, the $Sca1^+CD49b^-$ subset distinguishes nonclonogenic mature luminal cells that express ER and progesterone receptor (PR). Thus there appear to be two discrete luminal progenitor types in the mammary gland: a major ER^- subset and a minor ER^+ subset, the latter of which has a lower proliferative index. ER^+ progenitors likely represent ductal progenitors since they express high levels of *Foxa1* and other transcription factors essential for ductal morphogenesis in the mouse (15). Although luminal cells lack repopulating capacity under standard transplantation conditions, ER^+ and ER^- luminal progenitor cells could generate outgrowths at exceedingly low frequency when transplanted in the presence of Matrigel (194, 222). These cells may equally correspond to basal cells "in transit" towards the luminal lineage. Interestingly, Sca1 labels a rare pool of ER^+ bipotent cells in the basal pool in prepubertal glands, which may contribute to luminal differentiation in the early stages of puberty (48).

An analogous ER^+ progenitor population exists in human breast tissue (46, 64, 126, 194). Intriguingly, human breast comprises a much higher proportion of ER^+ luminal-restricted progenitors (~25%) than mouse tissue, which is in the realm of 5%. Moreover, luminal progenitors in human tissue express K5 (~50%), whereas their murine counterparts lack expression of this cytokeratin (126). These findings align with histopathology studies on human breast

demonstrating that luminal cells within TDLUs express basal-associated cytokeratins such as K5 and K14 (87).

B. Cell Fate Mapping of Distinct Luminal Progenitor Cells

Both long- and shorter-lived unipotent luminal progenitor cells have been identified through in situ tracing. Lineage tracing of a fraction (6%) of luminal cells indicated that $K8^+$ cells contributed to the maintenance and expansion of the luminal lineage over two rounds of pregnancy (226). Yet not all cells were found to persist long term, implying the presence of cells with differing longevities (226). Lineage tracing at saturation in another *K8*-driven model revealed that the luminal lineage is self-sustained by relatively long-lived progenitors (239). In contrast, cells marked by *Elf5,* a signature gene of luminal progenitor cells, have a shorter lifespan since only a small pool of labeled cells remain after a 20-wk chase (181). Nonetheless, these cells are encompassed within the wider $K8/K18^+$ population and contribute substantially to postnatal morphogenesis.

A plethora of lineage tracing studies have confirmed that unipotent progenitors sustain the luminal lineage. The transcriptional repressor Blimp1 demarcates a small population of $Elf5^+ER^-PR^-$ luminal progenitor cells and $Elf5^+/Blimp1^+$ cells gave rise to ER^- cells in the steady state as well as mature ER^- alveolar cells during pregnancy (1, 65). In addition, *Notch1, Notch3, Wap,* and *Sox9* mark long-lived ER^- progenitors that expand in pregnancy and are the probable precursors of mature secretory alveolar cells (117, 138, 183, 216, 232). Since $Notch3^+$ cells cycle more slowly than $Notch1^+$ cells and can generate some ER^+ cells, these Notch receptors seem to be expressed by distinct luminal subsets (117, 183). Notch-expressing cells are also likely to overlap with parity-identified mammary epithelial cells (PI-MECs) that express WAP and survive involution after activation in late pregnancy (142). Analogous to many luminal lineage tracing models, WAP-expressing cells generated self-renewing ER^- progeny, but not hormone-sensing ER^+ cells nor basal cells (41). Nevertheless, unexpected variation in cell behavior and dynamics is evident among the different luminal lineage tracing models, which likely reflects the gene promoter utilized (e.g., *Notch1, Notch3, Elf5, K8, K18, Blimp1, Sox9,* and *Wap*) as well as the spectrum of states in which a cell may exist. More sophisticated techniques will be required to draw definitive conclusions on the relationship between these marked subpopulations.

The interrelationship between the ER^+ and ER^- luminal cell subtypes remains a subject of intense interest. In addition to the ER^- lineage being independently sustained, direct evidence for ER^+ luminal cells forming a separate lineage has recently come to light. Cell-fate mapping based on Prom1/CD133 expression (232), a marker of ER^+ ductal cells, or the ER (*Esr1*) promoter itself (224) revealed that ductal ER^+ cells are exclusively sustained by ER-expressing cells in adulthood and over multiple pregnancies. Nor did ER^+ cells arise from ER^- cells during remodeling in involution (224). These data align with nucleotide analog-labeling experiments that highlight compartmentalization of different mammary epithelial cells and little flux occurring between ER^+ and ER^- luminal cells (79). Hence, these studies have defined two separate luminal branches within the hierarchy that are independently maintained.

VII. ROLE OF STEM/PROGENITOR CELLS AT DIFFERENT DEVELOPMENTAL STAGES

A. Primordial Mammary Precursors

Both transplantation and lineage tracing assays have been applied to studying stem and progenitor cells in embryonic mammary tissue. Elegant transplantation studies using limiting numbers of embryonic mammary cells indicated the presence of ancestral stem cells that were first detectable in the primordia around E16.5 (137, 208). Mammary repopulating capacity peaked at E18.5, when the rudimentary ductal tree emerges, and then declined at birth. Notably, embryonic MaSCs were about fivefold more potent than their adult counterparts in the repopulation assay (137, 208). The embryonic MaSC population expressed high levels of EpCAM, CD49f, and CD24 (208), thus exhibiting an intermediate phenotype between adult basal and luminal cells, and *Lgr5* enriched for a highly clonogenic subset of embryonic cells in vitro (220). Intriguingly, these cells also expressed *Elf5* and *Aldh1a3*, markers of committed luminal progenitor cells in the adult. Long-term label retention experiments have provided evidence that embryonic mammary cells directly seed the quiescent MaSC pool in the adult gland (21, 75). Furthermore, ~10% of mammary primordial epithelial cells at E18.5 express markers of quiescent adult MaSCs. These cells appear to be proliferative in the embryo before switching to quiescence in the postnatal period.

Even though cell-fate mapping of embryonic cells at a population level based on *K14-, Lgr5-, Notch1-,* and *Procr*-expressing cells initially suggested that all mammary lineages derive from embryonic precursors (75, 183, 220, 226, 233), the relative contribution of multipotent versus unipotent cells to formation of the embryonic mammary rudiment has recently come into question. To distinguish between the labeling of multipotent cells versus the colabeling of independent basal- and luminal-specific progenitors warrants a multicolor lineage tracing strategy at clonal density. Indeed, only luminal-restricted or basal-restricted progenitor cells were detectable from late embryogenesis in a *K14*-driven model, while multipotency was evident at E13 (240). Similarly, *Notch1*-creER^{T2} lineage tracing revealed that a subset of *Notch1*-expressing cells was multipotent between

E12.5 and 13.5 and then switched to a luminal-restricted cell fate by E15.5 (125). Neutral lineage tracing of cells at E16.5–17.5 using a Rosa26-cre/R26-confettti model also supports the concept of lineage-biased progenitors predominating in late embryogenesis and then driving formation of the postnatal mammary gland (134). *Axin2* is also highly expressed in most cells within the mammary bud and *Axin2*-expressing cells induced at E12.5, E14.5, or E17.5 exhibited luminal-biased behavior (223). Together, these findings suggest that segregation of the basal and luminal lineages occurs before birth and may occur as early as E15.5. The biological rationale for such a narrow developmental time-frame in which stem cells exhibit multilineage potential, and the discordance between embryonic transplantation and lineage tracing studies, remain perplexing.

B. Stem/Progenitor Cells in Ductal Morphogenesis During Puberty

Although it has been presumed that activated MaSCs orchestrate expansion of the mammary epithelium in puberty, recent data indicate that this may not be the case. As anticipated, TEBs are notably devoid of quiescent MaSCs (75). In an early cell tracing model (albeit not inducible), resident cap cells of the TEB were found to be marked by expression of *s-Ship*-GFP (short isoform of the inositol phosphatase *Ship*) and harbored striking mammary reconstituting potential (10). Moreover, *s-Ship*$^+$ cells localized exclusively to the basal cell layer of the alveolar units in pregnancy and not to the ducts. Their highly proliferative status in puberty and pregnancy suggested that these cells represent activated MaSCs. It will be of interest to determine whether *s-Ship*-expressing cells behave as basal-restricted progenitors under physiological conditions. Pertinently, cap cells reside in a Wnt-rich microenvironment, which may govern the proliferative status of *s-Ship*$^+$ and other precursor cells (114, 182).

Recent cell-fate mapping studies have underscored an important role for unipotent progenitor cells in driving ductal elongation in puberty. In vivo tracking of p63-expressing cells indicated that expansion of the basal and luminal lineages occurs independently during this phase (209). Although cells in both lineages were labeled in the proximal region after a short chase in puberty, the distal region comprised labeled basal cells only, indicating that p63$^+$ cap cells predominantly generate the basal lineage. Similarly, *Lgr6*-expressing cells behave in a unipotent manner, with low-frequency colabeling of luminal and basal cells that expand in puberty before declining in the adult (17). The small basal clones emanating from *Lgr6*-expressing cells at the distal tips of ducts were quiescent and did not contribute to homeostasis. Furthermore, mathematical modeling has suggested that migrating cap cells do not contribute to the luminal lineage but rather are eliminated by apoptosis (157). Thus unipotent rather than multipotent progenitor cells appear to execute ductal elongation and branching via the TEB structures in puberty. Nonetheless, it is plausible that rare long-lived MaSCs are located within the TEB and are left behind in the newly formed, subtending ducts to eventually become sporadically dispersed through the ductal tree. It is envisaged that advanced intravital imaging will be required to define the precise roles of unipotent and rare multipotent progenitors in puberty by tracking the dispersion of labeled progeny through the developing ducts.

Neutral lineage tracing based on low-efficiency labeling has enabled the quantification of unipotent progenitor cells in ductal morphogenesis (134, 191). One study estimated that there are ~260 unipotent progenitor cells per TEB, inferring that most cells in a TEB function as lineage-restricted progenitor cells (191), while the other report estimated 35 lineage-restricted progenitors per TEB that actively contribute to ductal development (134). While the precise numbers of precursors need to be resolved, it is apparent that lineage-restricted progenitor cells function as equipotent pools and colonize the growing ducts via neutral drift dynamics (191). That is, the random segregation of progenitors during bifurcation from the TEB leads to an unequal distribution of progeny between the emanating branches. The observation that ducts generated later in development were either devoid of color or unicolored after induction of the "confetti" locus implies that unipotent progenitors rather than bipotent stem cells drive this process. Interestingly, cells at the border of the TEB but not the tip were found to primarily contribute to growth of the subtending duct (191).

C. Stem/Progenitor Cells in Alveologenesis During Pregnancy

Lineage tracing studies have provided unequivocal evidence that unipotent luminal progenitors in the resting adult gland give rise to mature alveolar cells in pregnancy. In contrast to models in which the labeled population remains relatively stable over the reproductive cycle (117, 183, 226, 239), a new pool of Elf5$^+$ progenitor cells appears to be recruited for each round of alveologenesis. Moreover, multicolor lineage tracing of the *Elf5*-driven model indicated that multiple luminal progenitor cells can contribute to each alveolus. Interestingly, *K8*-marked luminal cells were recently reported to dedifferentiate to basal-like cells during pregnancy but not in the virgin mammary gland (205). These cells exhibited hybrid lineage features and a suprabasal localization but were not observed upon saturation lineage tracing of K8$^+$ luminal cells (239) nor of *Elf5*-expressing cells in pregnancy, in which >80% of alveolar cells were labeled (181). These basal-like cells may represent "transit" cells in the basal compartment that are primed for the alveolar luminal lineage, consistent with the unique molecular profile of basal cells in pregnancy (6).

The role of basal stem/progenitor cells in pregnancy remains obscure given the disparate data obtained from trans-

plantation and lineage tracing studies. Whereas the majority of basal cell lineage tracing models read-out unipotent cells in pregnancy, certain lineage tracing models (*Lgr5, Procr, Axin2*, some *K5*) have labeled both basal and alveolar luminal progeny. For example, large numbers of labeled alveolar luminal cells were observed in pregnancy for the *Axin2*-driven reporter model, which is exclusive to basal cells in the nonpregnant gland (223). Moreover, quiescent MaSCs marked by *Lgr5* in the proximal region were activated by the hormonal milieu of pregnancy and generated mixed-lineage clones (75). Thus, under the influence of pregnancy hormones, it is envisaged that bipotent MaSCs can generate large numbers of alveolar luminal progenitor cells to fuel the production of milk-producing alveolar cells. Indeed, MaSCs may show lineage bias according to the physiological requirements of the mammary gland. FACS analysis of the basal population from mid-pregnant mammary glands revealed a striking but transient expansion in the number of mammary repopulating cells (6). These cells also exhibited a unique gene expression signature enriched for alveolar lineage commitment genes, indicating that they are primed for the alveolar sublineage. This subset appears to be analogous to $H2B^{high}$ cells that lie between the basal and luminal compartments in pregnant mammary glands (108). The increased breast cancer risk following pregnancy suggests the presence of a stem cell or long-lived progenitor that is expanded in pregnancy and susceptible to oncogenesis (119).

VIII. DECONVOLUTION OF LINEAGE RELATIONSHIPS AT THE SINGLE-CELL LEVEL

To unravel cell heterogeneity and potential cell dynamics during mammary gland development, several groups have recently utilized single-cell (sc)RNA-seq technology. This enables a molecular interrogation in an unbiased manner and the reconstruction of developmental trajectories when linked to functional single-cell data (88). Global analyses of gene expression at the single-cell level (mainly using the 10× Chromium platform) have been performed on large numbers of mouse mammary epithelial cells from embryonic and different postnatal developmental stages. Primitive mammary $EpCAM^+$ cells in the embryo (E16 and E18) formed a single population (78). Of note, while it has been reported that 10–30% of embryonic mammary epithelial cells displayed stem cell activity (208) and that the luminal- and basal-restricted lineages are established around E15.5 (125, 240), distinct subsets of cells could not be identified based on their transcriptomes (78). In the adult mammary gland, three major clusters corresponding to luminal progenitors (alveolar-like), mature luminal cells (hormone-sensing), and basal cells were detected on comparison with the gene expression signatures of the definitive cell types (7, 78, 159). Single-cell profiling of thousands of epithelial cells also revealed a new luminal subset that displayed properties intermediate between the two luminal subsets, as well as rare cell clusters with mixed-lineage features (159). These mixed-lineage clusters were more prominent upon deeper sequencing using the C1 Fluidigm platform, which yielded 8,000–10,000 genes/cell. These cells first emerged during puberty and expanded during pregnancy, consistent with the presence of a transient but "lineage-primed" subset that is poised for alveologenesis. However, not all the smaller epithelial clusters were identified in the different studies, likely reflecting the depth of sequencing and the precise algorithms employed. The limited number of epithelial cells captured in the recent data sets that profiled total mammary gland cells (89, 214) precluded further insights into the dynamics of the mammary epithelium, but the identification of several nonepithelial cell populations underscored a complex signaling network in pregnancy (89).

Prepubertal epithelial cells exhibit a unique gene expression program. Analysis of day 4 postnatal cells showed two clusters forming a continuum between primitive and adult mammary epithelial cells, with the dominant cluster representing a mixed-lineage mammary precursor cell (78). Another study of 2-wk versus 5- and 10-wk mammary epithelia revealed that prepubertal cells were more homogeneous in their expression state than pubertal or adult cells (159). Although 14-day postnatal cells expressed luminal genes, they were biased towards a unique "basal-like" signature. Despite the ducts comprising both luminal and myoepithelial layers, it appears that cells lie in a precursor-like state prior to committing to definitive lineage expression near the onset of puberty. It will be of interest to determine the molecular relationship between prepubertal cells and the multipotent progenitors identified in embryogenesis given their hybrid lineage signature (240). Single-cell RNA-seq of 91 cells isolated from TEBs during puberty showed two main clusters but suggested that the identity of progenitor cells within TEBs is not linked directly to a specific gene signature. Analysis of thousands more cells, however, will be required to understand the molecular and cellular relationships within the TEB structure (191).

Exploration of the molecular dynamics of the mammary cell lineages during the pregnancy-lactation-involution cycle uncovered 15 potential cell clusters, 4 with basal cell/myoepithelial features and 11 with luminal features (7). Cells within the luminal lineage showed the most change during postnatal ontogeny, although myoepithelial cells in lactation were also molecularly distinct (7). In particular, the luminal compartment appears to form a continuum of differentiation states across the different developmental stages, but it remains unclear whether a common luminal progenitor exists upstream of ER^+ and ER^- cells. Curiously, the $Procr^+$ MaSC population was only detected in the lactating gland and exhibited features of myoepithelial rather than stem cells, but this may reflect sequencing depth.

Parallel to the mouse, single-cell RNA-seq of >25,000 human breast epithelial cells derived from four premenopausal women revealed a single basal population and two distinct luminal cellular subsets (153). All three subsets contained proliferative cells. Notably, pseudotemporal analysis indicated bifurcation from a common Zeb1$^+$ basal progenitor cell into the two distinct mammary lineages. These data support a continuous differentiation trajectory from basal cells to the myoepithelial, secretory (alveolar-like progenitor) and hormone-responsive luminal populations, in favor of a bipotent MaSC model (153).

It is important to note that no unique molecular fingerprint could be ascribed to MaSCs based on scRNA-sequencing. While this is a powerful technique for exploring heterogeneity among thousands of individual cells, the depth of sequence data remains low and inevitably leads to loss of transcriptome information compared with bulk RNA-sequencing, which yields >25,000 unique transcripts per cell. It is noteworthy that the functional heterogeneity observed in the basal compartment through cell fractionation studies is not apparent at the single-cell transcriptome level, as the basal cell compartment resolves as a single large cluster (7, 78, 153, 159). Indeed, the basal compartment can be fractionated into four molecularly and functionally distinct subsets (75). The finding that the dormant subpopulation exhibits distinct histone methylation profiles for active and repressive marks argues against a random continuum of stem cell states in the basal compartment. Thus cell surface markers continue to play an important role in deciphering heterogeneity at a functional level in the MaSC compartment. Parallel findings have emerged for the more complex hematopoietic system, in which the hematopoietic stem cell (HSC) cannot be defined by a definitive molecular profile and must be defined functionally. Recent single-cell analyses of the blood system have indicated a "continuous model" in which unipotent cells emerge from a pool of "low" lineage-primed HSCs and lineage-restricted progenitors in the stem cell compartment (88).

IX. MODELING THE MAMMARY DIFFERENTIATION HIERARCHY

In this section, we summarize the salient features of two models that have emerged for the mammary differentiation hierarchy in the adult gland. In model A, predicated on combined transplantation and lineage tracing data, multipotent MaSCs occupy the apex of the differentiation hierarchy and give rise to heterogeneous populations of progenitors. In contrast, model B focuses on lineage tracing data and posits that multipotent stem cells only exist in the mid-embryonic period and are supplanted by lineage-restricted progenitors around E15.5. In the postnatal period, the two models essentially converge: different populations of unipotent progenitors in the postnatal gland are self-sustained and largely drive morphogenesis during puberty and pregnancy and day-to-day homeostasis **(FIGURE 7)**. The progenitor compartment appears to be particularly diverse, with the identification of both short- and long-lived cells: cell-fate tracking in vivo has revealed long-lived progenitors that survive multiple rounds of pregnancy but also shorter-lived luminal progenitors that are replenished with each round of pregnancy. There is cumulative evidence that ER$^+$ and ER$^-$ luminal cells are independently maintained in the adult gland, inferring the existence of distinct progenitors for these luminal branches. The majority of luminal progenitors in the steady-state gland are ER$^-$, but a small subset of cycling ER$^+$ progenitors may produce large numbers of mature ER$^+$ cells to line the ducts. Whether there exists a common upstream precursor cell that coordinates the generation of ER$^+$ and ER$^-$ cells remains to be determined. Of note, single-cell RNA-seq analyses have not provided insight into the number of unipotent progenitor types in the resting gland nor defined a specific stem cell population (see above). More sophisticated lineage tracing systems based on dual recombinases driven by two distinct lineage-specific promoters (91) may be required to address these questions in the future.

The question of the relative contributions of the different precursor cells to the distinct stages of postnatal development is particularly relevant to model A. Evidence from certain reporter models suggests that a small pool of long-lived multipotent cells plays a role in long-term ductal homeostasis over the lifespan of the mammal. These adult stem cells may also help coordinate alveolar expansion during pregnancy through the production of large numbers of lineage-restricted progeny, which then serve as the "workhorses." Multipotent MaSCs may also participate in tissue remodeling during involution based on the extensive labeling of both lineages (181). Finally, it is plausible that the activity of MaSCs and basal-restricted cells is uncoupled during development according to contextual cues and the specific requirements.

Emerging evidence from other cellular systems (231) suggests that stem cell hierarchies are not rigid during tissue regeneration. Moreover, facultative stem cells, i.e., progenitor cells that acquire stem-cell characteristics under specific conditions, have been demonstrated in certain organs that are frequently subject to damage such as the intestine or skin. For example, enterocyte-restricted progenitors in the intestine can regain stemness upon acute ablation of Lgr5$^+$ stem cells (218). Facultative cells may exist in adult mammary tissue (202), but it is noteworthy that the mammary gland is not prone to injury. The degree of flexibility built into the mammary differentiation hierarchy under physiological conditions is yet to be determined but is likely to be significant in pathological contexts.

Although lineage tracing is not possible for human breast, earlier studies of X chromosome inactivation in human breast tissue have demonstrated the presence of identical

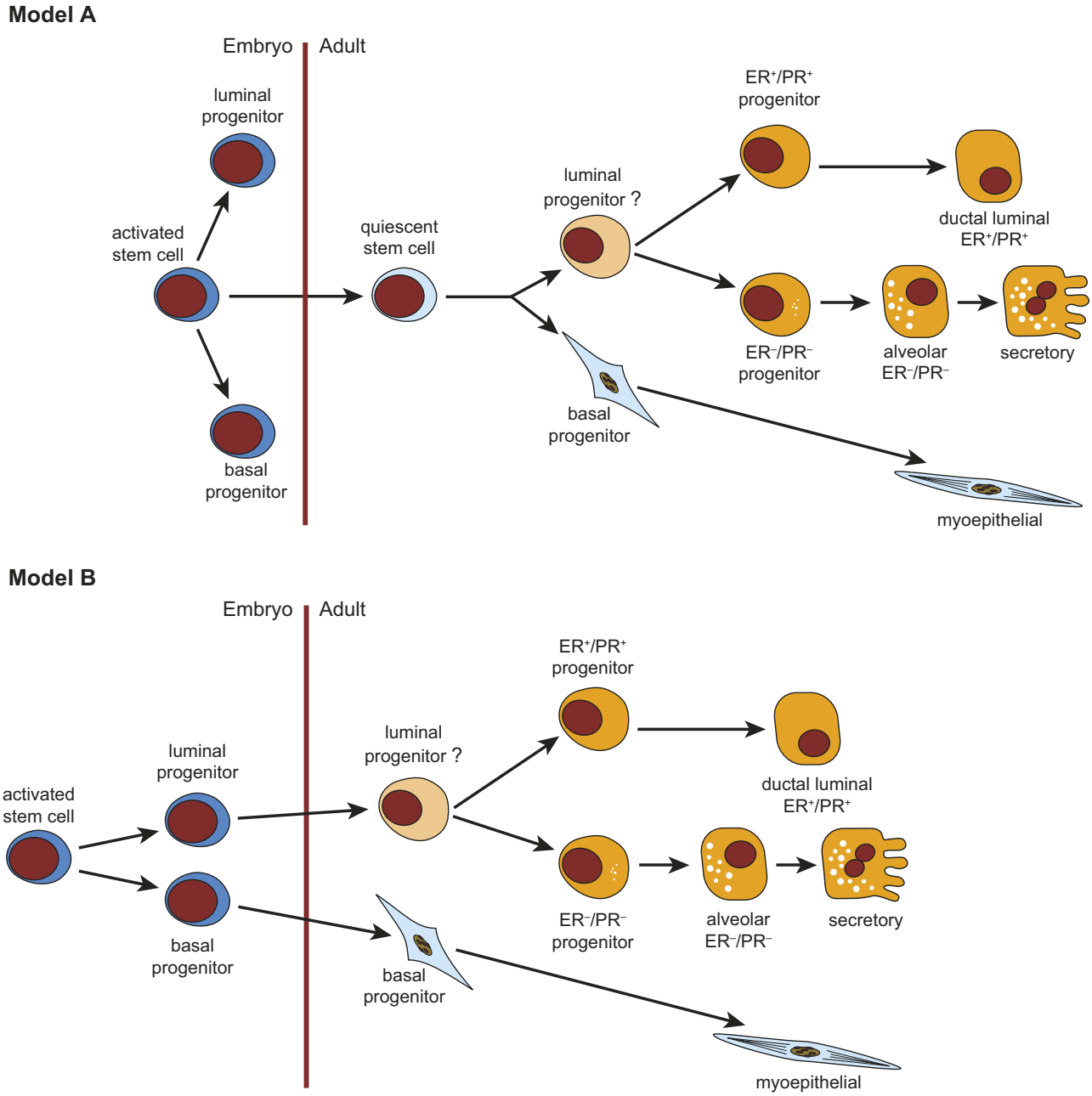


FIGURE 7. Two opposing models of the mammary epithelial differentiation hierarchy. In model A, the stem cell compartment in the adult gland is heterogeneous and comprises quiescent mammary stem cells (MaSCs). These give rise to committed progenitors for the myoepithelial and luminal epithelial lineages. In model B, there are no adult MaSCs but rather embryonic multipotent stem cells that become restricted in their potential around E15.5. Thereafter, unipotent progenitors drive most aspects of postnatal development. In both models A and B, there are independent ductal and alveolar luminal sublineages, which produce ER^+ and ER^- cells, respectively. There may be a common luminal progenitor upstream of these sublineages.

chromosomal aberrations in contiguous patches (221), suggesting their derivation from long-lived cells. More recent tracking of cells deficient in the mitochondrial enzyme cytochrome *c* oxidase (CCO) has revealed multilineage differentiation within the CCO-deficient areas, implying the presence of multipotent precursor cells. Interestingly, CCO-deficient domains were present in some areas of ductal carcinoma in situ, consistent with the clonal expansion of luminal lineage-restricted cells (37).

X. HORMONAL SIGNALING AND PARACRINE REGULATION OF STEM CELLS

The ovarian steroid hormones estrogen and progesterone are integral to the major periods of morphogenesis that occur in puberty and pregnancy, and to the smaller changes that accompany each estrus cycle (27). Estrogen and progesterone (Pg) exert their mitogenic influence primarily

through paracrine signaling to adjacent cells. Basal MaSCs lack expression of ER and PR yet are exquisitely sensitive to ovarian hormonal signals (6, 105). Hormone deprivation drives the MaSC subset into a quiescent state, whereas ovarian hormone stimulation during the estrus cycle or pregnancy results in the expansion of MaSCs (6, 105). The hormone receptor-negative (HR^-) status of MaSCs emphasizes the importance of paracrine interactions to mammary gland development. HR^+ cells are widely distributed throughout the epithelial network in the adult, suggesting that HR^- stem and progenitor cells are in close proximity to receive signals transmitted from HR^+ cells.

A. Estrogen Signaling

The mouse knockout of *ER* has established its critical role in pubertal morphogenesis, attributed in part due to diminished prolactin levels (18). Complete deletion of ERα demonstrated the absence of TEBs and failure of development during puberty (139), while conditional deletion of this receptor revealed it was necessary for ductal elongation during puberty and alveologenesis in late pregnancy (69). The EGFR ligand amphiregulin (Areg) is a key paracrine mediator of ER signaling to adjacent ER^- cells **(FIGURE 8)** and mice lacking *Areg* phenocopy ERα-null mice. Moreover, Areg can rescue the ER-null phenotype in transplantation assays (44). Pertinently, Areg must be activated by MMP17 to mediate estrogen signaling (211). Given that MaSCs in the basal compartment are $ER^-PR^-EGFR^+$ (3, 176), it is likely that mature ER^+ luminal cells secrete Areg (127), which then activates $EGFR^+$ MaSCs in a paracrine manner. Although ER^+ "sensor" luminal cells produce Areg, paracrine signaling from ER^+ luminal progenitor cells may also be important. Recent work has described a gradient of ERα-expressing luminal cells, with ~30% expressing low levels (33). These cells appear essential for ductal expansion during puberty but are growth inhibitory during pregnancy and are dependent on the AF-2 estrogen ligand-binding domain that controls transcript levels linked to cell motility, cell adhesion, and the epithelial-to-mesenchymal transition (EMT). In keeping with this, single-cell profiling has suggested that prepubertal ER^{low} cells are more likely to have progenitor properties (159).

B. Progesterone Signaling

Progesterone induces side-branching in the adult virgin mammary gland and alveologenesis in pregnancy. Together with prolactin, Pg stimulates the differentiation of alveoli to milk-secreting cells in late pregnancy (28). *Rankl* and *Wnt4*

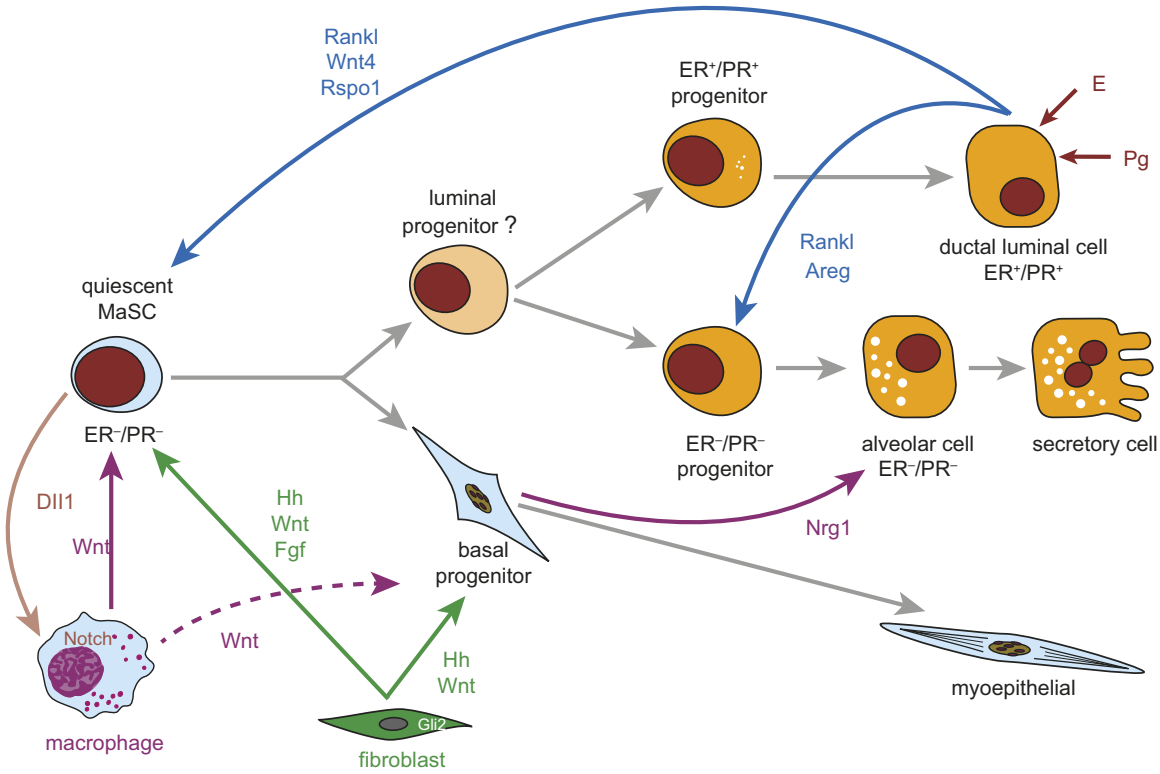


FIGURE 8. Schematic depiction of cross-talk between different ductal mammary cells and the stromal environment. Estrogen or progesterone activate ER^+/PR^+ epithelial cells, which secrete paracrine factors that then act on hormone receptor-negative stem cells or luminal progenitor cells. A further layer of interaction occurs between stromal cells (fibroblasts and macrophages are depicted) and cells within the basal layer of the mammary ducts. MaSC, mammary stem cells.

are direct targets of PR and serve as key paracrine effectors of Pg-induced mitogenic signals **(FIGURE 8)**. Rankl (receptor activator of nuclear factor-κB ligand), a cytokine member of the TNF superfamily, and its receptor Rank are essential for the formation of functional alveolar structures during lactation (67). Evidence that the Rank-Rankl signaling axis is a major mediator of Pg signaling has stemmed from findings that ectopic expression of *Rankl* in $PR^{-/-}$ epithelial cells rescues branching morphogenesis and alveolar development (13, 150).

In the context of the mouse mammary differentiation hierarchy, Rankl expression is strongly induced by Pg in mature PR^{+} luminal cells (70, 80, 151, 158) and mediates mitogenic signaling to both neighboring MaSCs and a subset of luminal progenitor cells that express Rank in diestrus and pregnancy (6, 105, 158). Following Pg-induced Rankl-mediated amplification of the MaSC/progenitor compartment in pregnancy, commitment to the secretory lineage during pregnancy may be achieved in part through *Elf5*. Induction of *Elf5* in luminal progenitors in response to Pg treatment in vivo or upon forced *Rankl* expression in transgenic mouse models indicates that Rankl-induced *Elf5* is important for the differentiation of progenitors towards a secretory cell fate (121).

Wnt4 has emerged as a paracrine mediator in both human and mouse mammary tissue (26, 27, 105, 215). Although the expression of Wnt4 and PR colocalize in the mouse mammary epithelium (26), there appear to be distinctions between the actions of Wnt4 and Rankl. *Wnt4*-deficient mammary glands show reduced side-branching and alveologenesis, and this phenotype is rescued as pregnancy progresses, suggesting compensation by other Wnt ligands (26). Furthermore, *Wnt4* overexpression in the mouse mammary gland failed to elicit a morphogenic response (111). Hence, Pg-induced *Wnt4*, unlike Rankl, does not appear to be sufficient for alveolar development in the mouse. In the resting adult gland, however, a functional interaction between the Rankl and Wnt pathways has been proposed (106): Pg-induced Rankl signaling amplified Wnt pathway activity in mammary progenitor populations by triggering the upregulation of *R-spondin1* and stabilization of the Wnt receptor complex (53). Of note, the Wnt receptor agonist R-spondin1 was recently invoked as a novel mediator of ovarian hormone signaling to MaSCs (34). The production of R-spondin1 and Wnt4 by ER/PR^{-} and ER/PR^{+} luminal cells, respectively, appears to synergistically control the function of MaSCs.

XI. MOLECULAR CONTROL OF THE DIFFERENTIATION HIERARCHY

Over recent years, targeted mouse models have highlighted multiple genes and pathways that govern specific cell types along the hierarchy. A number of these will be discussed, with emphasis on transcriptional regulators that are determinants of the stem cell state, "basalness" or luminal identity **(FIGURE 9)**. It should be noted that in many cases it is difficult to designate where basal-specific regulators act along the hierarchy since this requires dissecting the effects of gene deletion on MaSCs versus committed progenitors, ablation of which can result in a similar outcome in transplantation assays.

A. Regulation of Basal Stem/Progenitor Cells

1. The Wnt pathway

The Wnt pathway executes major roles in regulating the renewal and quiescence of stem cells in multiple cellular systems. The role of canonical Wnt receptors of the Frizzled family in the regulation of MaSCs is likely to be complex since 8 out of the 10 receptors are expressed in the mammary gland. Fzd7 has been identified as a direct transcriptional target of ΔNp63, and the impaired MaSC function caused by loss of one *p63* allele could be rescued by restoration of Fzd7 expression (39). In the context of Wnt pathway coreceptors, Lrp5 and Lrp6 are coexpressed in the majority of basal cells (9, 129), and cells expressing the highest levels of Lrp5/Lrp6 showed >200-fold enrichment of mammary repopulating activity. Nonetheless, mammary gland development in $Lrp5^{-/-}$ females was found to be relatively normal, suggesting functional redundancy (9). Knock-down of the alternative Wnt receptor Ror2 demonstrated its role in ductal development (182). Interestingly, there appears to be a gradient of Wnt activity in the vicinity of the TEB with canonical signaling in the cap cell region and noncanonical activity in the neck region (182).

The Lgr4/5/6 family of receptors amplifies the intensity of Wnt signaling through their binding to R-spondin ligands. Lgr4 is expressed in both the cap and body cells of TEBs and in the basal cells of mature ducts (235), while *Lgr5* appears to be exclusively expressed in the basal compartment of adult glands (55, 75, 167). *Lgr5* does not enrich substantially for repopulating activity in limiting dilution assays when used as a sole marker (55, 220, 223), although single $Lgr5^{+}$ cells can give rise to multipotent ER^{+} organoid structures in vitro (247). Transplantation studies on *Lgr4*-null mammary glands revealed diminished regenerative capacity but only a minor delay in pubertal development (235). Similarly, conditional targeting of a floxed *Lgr5* allele did not reveal any discernible mammary phenotype in the adult (220; N. Y. Fu, unpublished data). Conversely, acute deletion of *Lgr5*-expressing cells using diphtheria toxin-mediated ablation was shown to impair ductal development (168), highlighting differences between gene ablation in early development versus puberty. Like *Lgr5*, the Wnt target gene *Axin2* is specifically expressed in basal cells but alone does not define MaSCs (223). Nonetheless, stem cells

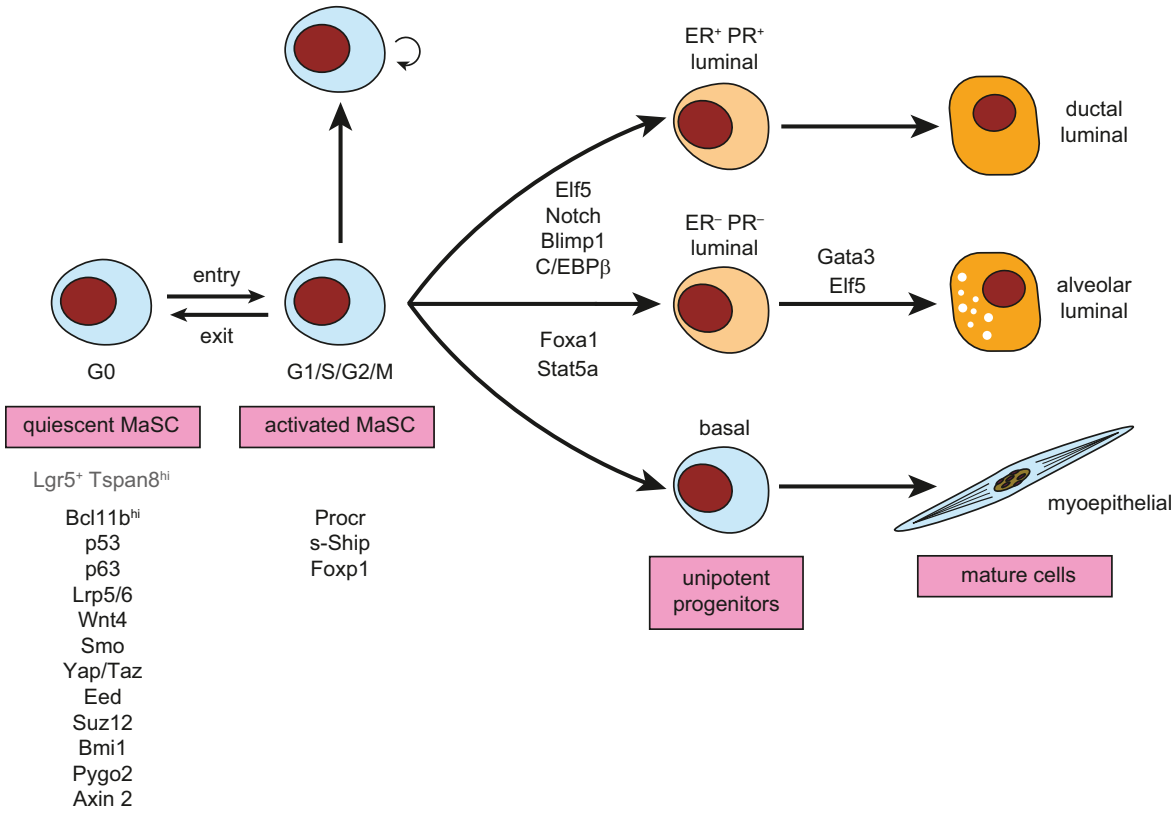


FIGURE 9. Molecular regulators that have been shown to act along the mammary epithelial hierarchy. These have been largely implicated in influencing mammary stem cell (MaSC) quiescence, MaSC activation, lineage commitment, or luminal differentiation. In some cases, marker genes have yet to be demonstrated to play a functional role.

mutant for Axin2, a negative feedback regulator of Wnt signaling, outcompeted wild-type cells in in vivo reconstitution assays due to the cells being sensitized to Wnt signals (246). The Wnt target *Procr*, which is expressed in a small subset of basal cells and a larger proportion of mammary stromal cells, has been shown to mark a distinct subset of cycling MaSCs in both transplantation and lineage tracing assays (233).

Wnt4 is the best studied of the Wnt ligands in the context of MaSCs. Wnt4 acts as an effector of canonical Wnt signaling in basal cells and a mediator of MaSC expansion in response to ovarian hormone cues (175). Interestingly, Wnt4 is activated in the mammary gland as early as P5 (175), suggesting that it regulates prepubertal development in a hormone-independent manner. Although *Wnt4*$^{-/-}$ mice die at birth, serial transplantation assays using embryonic *Wnt4*$^{-/-}$ mammary tissue revealed that Wnt4 is critical for long-term regenerative capacity (175). Conditional targeting of the *Wnt4* locus led to impaired ductal expansion during puberty, underscoring a role for Wnt4 in the postnatal gland (175). Other members of the Wnt family involved in the spatiotemporal control and fine tuning of MaSC activity are yet to be determined. Along these lines, it is noteworthy that Wnt3a exerts potent effects on stem cell activity ex vivo although this ligand is not expressed in the mammary gland (246). Studies on downstream target genes of the Wnt pathway provide further evidence that the Wnt pathway governs MaSC function. For example, targeting of the limb-bud and heart (LBH) transcriptional cofactor regulated by the Wnt pathway led to a severe delay in ductal morphogenesis (128). LBH appears important for the renewal of MaSCs through mediating the expression of ΔNp63. Upstream of this pathway, SLIT-Robo1 has been implicated as a negative regulator of Wnt/β catenin signaling and suppresses basal cell proliferation (136). Together these data underscore an important yet not fully understood role for the Wnt pathway in the regulation of MaSCs.

2. *Foxp1*

Quiescent cells must be tightly governed during adult life to enable tissue expansion in response to stimuli and then to reenter quiescence when no longer required. Although Foxp1 is not restricted to basal cells in the mouse mammary gland, this winged-helix transcriptional repressor executes a specific role in regulating the exit of MaSCs from quiescence for ductal morphogenesis (74). Foxp1 is not required for embryonic or early postnatal mammary development. Rather, it is critical for expansion of the ductal tree during puberty, and its absence results in a severely stunted tree throughout life and the accumulation of dormant Tspan8^{+}

MaSCs. Notably, Foxp1 binds the *Tspan8* promoter in basal/stem but not luminal cells, and the profound defect in *Foxp1*-deficient mice can be rescued through loss of *Tspan8*. At a molecular level, Tspan8 operates within a plasma membrane microdomain that comprises a network of signaling proteins (251), and Lgr5 and Tspan8 can physically interact to enhance Wnt signaling (74). Together these data invoke a potential scenario in which Wnt and Tspan8 participate in maintaining stem cell quiescence while Foxp1 is crucial for stem cell activation to allow the production of progenitors for ductal morphogenesis.

3. p63 and p53

ΔNp63, the major isoform of the p63 gene expressed in the mammary gland, is restricted to the basal cell compartment. ΔNp63 is considered to be a master regulator of MaSCs based on knockout mice and transplantation studies. Homozygous loss of p63 leads to embryonic lethality, and mice lack even a rudimentary mammary gland indicating an indispensable role in embryonic mammogenesis (147). Loss of one allele led to a modest decrease in repopulating activity (39) while a different *p63* knockout strain showed that a single wild-type allele was sufficient to maintain postnatal mammary gland function through the different stages (242). In an unexpected twist, loss of Δ*Np63* in the basal compartment led to a complete failure of lactation owing to impaired differentiation and accumulation of luminal progenitor cells (71). Mechanistically, paracrine signaling from basal to luminal cells was uncovered to be essential for luminal cell maturation during lactogenesis via p63-induced expression of Nrg1, a ligand for the ErbB family of tyrosine kinase receptors. The role of ΔNp63 as a master regulator has been confirmed by p63 overexpression studies in basal cells, which blocked the generation of luminal cells (241), or its overexpression in $K8^+$ luminal cells that induced a switch to a basal-like fate (240).

The family member p53 has emerged as a pivotal regulator of MaSC renewal but in contrast to p63 exerts a negative influence. Tissue fragments of *p53*-null glands can be transplanted in an unlimited manner compared with the decline in regenerative capacity apparent for wild-type tissue (143). Compatible with a physiological role for p53 in restricting the renewal of MaSCs, *p53*-null basal cells exhibited a higher frequency of symmetric cell division (42). This transcriptional regulator appears to control asymmetric cell division through the partitioning of Numb protein into the daughter cell that retains stem cell identity (45). In concert, loss of *Numb* in the mammary gland led to the expansion of MaSCs (219). It remains to be determined why high levels of p53 may be required for one daughter cell to retain a stem cell fate during asymmetric division while loss of p53 leads to MaSC expansion. In addition to basal cells, p53 has also been found to play a critical role in restraining the expansion of luminal progenitor cells without affecting their lineage identity (42, 217).

4. Mesenchymal-associated transcription factors

A number of transcriptional regulators initially identified as mesenchymal genes are highly expressed in mammary basal epithelial cells albeit at higher levels in mammary gland fibroblasts (e.g., Snail, Twist, and Zeb1) (152, 244). The expression of *Slug* is restricted to a subset of basal cells in the mammary epithelium (86, 152, 244); however, germline deletion of this gene results in a mild phenotype that recovers by 8 wk of age (152). Thus Slug is dispensable for MaSC function. Nevertheless, the misexpression of luminal genes observed in $Slug^{-/-}$ MECs implies a role in maintaining the basal state (152). Similarly, *Snai1* knockout mice did not display a mammary gland phenotype, suggesting compensation by other EMT factors. Therefore, neither Snail1 nor Slug/Snai2 is an essential determinant of the stem cell state in the mammary gland, inferring that activation of the EMT program in mammary epithelial cells is not a prerequisite for stem cell function. Snai1, however, may influence the symmetric division of MaSCs via inscrutable mINSC, a member of the spindle orientation machinery (12).

Although loss of *Slug* alone does not impact on MaSC function, overexpression of Slug dramatically enhanced the self-renewal capacity of stem/progenitor cells in mammosphere and transplantation assays (86). More interestingly, ectopic expression of Slug alone could convert luminal progenitors into repopulating stemlike cells. Indeed, mature luminal cells could be reprogrammed to bona fide stem cells by transient expression of Slug together with the transcriptional cofactor Sox9. The dedifferentiation of mature cells to luminal progenitors seems to be induced by Sox9 and was a prerequisite for conversion to a stem cell state (86). Given that a small basal pool coexpresses Slug and Sox9, it would be of interest to characterize the features of this subpopulation. Sox9, a member of the wider Sox family of transcription factors that governs cell-fate decisions and stem cell states in different tissues (188), is expressed in luminal as well as basal cells. Paradoxically, *Sox9*-expressing cells primarily contribute to the development of ER^- but not ER^+ luminal or basal cells in cell-fate mapping experiments in vivo (232). Mouse knockout models with compound deletion of multiple EMT transcription factors may provide further insight into their role in regulating MaSCs.

The mesenchymal gene Sox10 was recently identified as a target gene of fibroblast growth factor (FGF) signaling in the mammary primordia (62). Sox10 is highly expressed by most cells in the mammary rudiment and resides in almost all basal cells as well as 50% of the luminal population in the adult. *Sox10*-deficient basal cells exhibit a similar reconstituting frequency to wild-type cells but did not yield full outgrowths, suggesting that Sox10 may be required for proper progenitor function. The physiological role of the Sox10-FGF signaling axis requires further investigation

since *FGFR2*-deficient (248) but not *FGFR1*-deficient basal cells show dramatically reduced repopulating capacity (170).

5. The Hedgehog pathway

The Hedgehog (Hh) pathway has been explored in MaSC/progenitors using a variety of mouse models, yet definitive conclusions await on the physiological role of this pathway in the mammary gland. Germline deletion of the key Hh transcriptional effectors Gli2 and Gli3 profoundly impaired embryonic mammary development (90, 123) and resulted in perinatal lethality. Furthermore, the perinatal lethality associated with either germline or conditional deletion of the Smoothened Receptor (Smo) or Sonic Hh (Shh) (82) precluded analysis of this pathway in postnatal development. Long-term label retention studies in mice heterozygous for *Ptch1*, a negative regulator of the Hh pathway, have suggested that Hh signaling may be important for regulating MaSC quiescence via intraepithelial interactions between p63 and Hh (124). Mice overexpressing constitutively active Smo displayed lower regenerative capacity, concomitant with precocious differentiation of MaSCs to progenitors, suggesting a role for Hh signaling in promoting stem cell restriction (149, 229). Notably, the Hh pathway appears to be activated in quiescent MaSCs (75). In addition, the EMT program may enhance stemness through activating the Hh pathway (85).

6. The Hippo pathway

Increasing evidence suggests that the Hippo pathway positively regulates the expansion of stem cells in different tissues. Taz was identified as a novel regulator of MaSC fate in a functional screen aimed at identifying transcription factors that could convert human mammary luminal cells into stemlike cells (199). Analysis of Yap/Taz target genes revealed enrichment of the Yap/Taz signature in basal but not luminal cells. Moreover, transient expression of Yap/Taz could reprogram primary differentiated mouse cells to tissue-specific somatic stem cells in a range of different tissues, including a switch in the fate of mammary luminal cells to stemlike cells that harbor in vivo regenerative capacity (160). Interestingly, transient expression of *Yap/Taz* led to the activation of endogenous *Yap/Taz*, as occurs in the case of *Slug*- and *Sox9*-induced reprogramming (86). In contrast, a RNAi screen identified Lats1/2 as regulators of MaSC maintenance and suppressors of their differentiation (29). The opposite outcomes observed upon knockdown of Lats1/2 versus overexpression of Yap/Taz on mammary cell fate may reflect a Yap/Taz-independent function for Lats1/2. Functional redundancy between Yap and Taz most likely contributes to the lack of phenotype in mammary glands lacking either *Yap* or *Taz*. Indeed, adenovirus-cre-mediated deletion of both *Taz* and *Yap* in basal cells markedly impaired self-renewal capacity during passaging (160).

7. The HGF/Met pathway

Signal transduction by c-Met requires a coreceptor such as intercellular adhesion molecule (ICAM) 1 (CD54). In the mammary gland, ICAM is expressed in the basal, stromal and luminal progenitor populations, with much higher levels occurring in luminal progenitors (56). The addition of hepatocyte growth factor (HGF) to mammosphere cultures of $ICAM^+$ progenitor cells led to the acquisition of in vivo reconstituting capacity and therefore stemlike properties. Moreover, HGF induced the expression of basal genes, including *Snai1*, *Slug*, *p63,* and *Sox9*, and repressed the expression of luminal-specific genes such as *Hey1*, *Elf5,* and *Gata3*. Mammary gland development, however, was found to be relatively normal in conditionally targeted *c-Met* mice, suggesting functional compensation downstream of this receptor (77).

8. C/EBPβ

Deletion of this CCATT/enhancer binding protein in the mammary gland appears to have multiple outcomes. Severe impairment of reconstituting activity was observed together with premature MaSC senescence based on serial transplantation assays. Moreover, the luminal lineage was affected, with a paucity of luminal progenitor cells and the misexpression of basal markers in this compartment, suggesting a role in stem cell fate determination (118).

9. ID4

Id4 represents an example of a largely basal-restricted gene that may not serve a specific function in MaSCs but rather appears essential for maintaining "basalness." Gene deletion revealed that Id4 has an important role in restraining the expression of key factors involved in luminal differentiation such as ERα and its associated network of transcription factors (11, 16). Although deletion of *Id4* impaired ductal elongation and branching in puberty (16, 58), transplantation of *Id4*-deficient basal cells from pubertal females showed no difference in the frequency of repopulation. Thus stem cell activity is not dependent on Id4, and the phenotype most likely reflects a perturbation in lineage commitment (11, 16, 107).

B. Regulation of the Luminal Lineage

1. The Notch pathway

The exquisite luminal specificity of the Notch signaling pathway has been exemplified through lineage tracing models (117, 183). Furthermore, constitutive Notch signaling in

adult basal epithelial cells or early embryonic progenitor cells dictates luminal cell fate specification (24, 125). Notch pathway activation in human cells led to decreased p63 expression before their restriction to the luminal lineage, implying a reciprocal relationship between these key determinants of cell-fate (241), and consistent with recent overexpression studies (125, 240). In the context of cancer, constitutive activation of the Notch pathway in luminal progenitor cells conferred self-renewal and culminated in hyperplasia, inferring that they serve as "cells of origin" of cancer (24). Conditional targeting of the key Notch effector molecule Cbf-1/Rbpj or the fucosyltransferase Pofut1, which is critical for activity of all Notch receptors, revealed that luminal cells in *Rbpj*- and *Pofut1*-deficient glands failed to differentiate into mature alveolar cells during pregnancy and acquired basal-like cell characteristics, despite the glands displaying normal morphology (31). Together, these findings confirm the importance of the Notch pathway to luminal cell-fate specification.

2. Gata3 and Foxa1

The zinc finger transcription factor Gata3 is essential for the differentiation of luminal progenitor cells. Loss of *Gata3* impairs development of the mammary gland during puberty, accompanied by an accumulation of $CD61^+$ luminal progenitor cells owing to a block in their differentiation along the secretory alveolar sublineage (4, 114). Notably, while similar levels of *Gata3* mRNA are found in luminal and basal cells at different developmental stages, Gata3 protein is restricted to luminal cells. *Foxa1*, a target of Gata3 initially thought to contribute to the defect in alveologenesis in *Gata3*-deficient mammary glands (114), is not expressed in the vast majority of alveolar cells. In accordance, gene knockout studies have revealed that *Foxa1* is dispensable for alveolar differentiation during pregnancy but instead plays an obligate role in ductal expansion in puberty and is a critical regulator of ERα but not Gata3 expression (15, 133).

3. Stat5 and Zpf157

Stat5a deficiency also culminates in failure of alveolar development but, in contrast to Gata3, the reduced pool of luminal progenitors evident upon loss of *Stat5a* indicates a role in the generation or maintenance of these cells rather than their maturation (243). In another layer of complexity, the KRAB domain zinc finger protein Zfp157 governs the balance of *Stat5a*- and *Gata3*-expressing luminal subtypes in pregnancy (156). In mice deficient in Zfp157, alveologenesis was accelerated and the $Gata3^+$ lineage was suppressed in favor of pStat5-expressing cells. Curiously, *Gata3* was dispensable for lactation in the absence of *Zfp157* in knockout mice, indicating an unexpected degree of complexity in the alveolar compartment (156).

4. ELF5

The ETS transcription factor Elf5 plays a dual role in the mammary gland where it serves as a key determinant of luminal lineage restriction and the differentiation of progenitors into milk-secretory alveolar cells (155). Elf5 is exclusively expressed by luminal progenitor cells in the resting adult gland and by mature alveolar cells in pregnancy. Even loss of a single allele results in a severe lactational defect that phenocopies that seen in Prolactin Receptor $(PrlR)^{+/-}$ mammary glands (250). Conversely, overexpression of Elf5 stimulated alveolar differentiation and milk secretion in virgin mice (155). Analysis of transplanted $Elf5^{-/-}$ cells revealed an accumulation of $CD61^+$ luminal progenitor cells, which failed to differentiate into mature alveolar cells (155). Moreover, conditional ablation of *Elf5* in the mammary gland confirmed the lactational defect but did not reveal abnormalities in the virgin state (40, 43). Indeed, the acquisition of an EMT-like molecular phenotype suggested that Elf5 normally suppresses a basal gene expression program in luminal cells. It is not clear why Elf5 only promotes the differentiation of luminal cells in pregnancy, given that the expression of *Elf5* in reporter mice is comparable between luminal progenitor and mature alveolar cells (N. Y. Fu, unpublished data). It is likely that ovarian hormone-induced factors such as Rankl collaborate with Elf5 to promote alveolar differentiation (121). Compatible with the restriction of this ETS protein to ER^- cells, Elf5 has been shown to suppress estrogen sensitivity and underpin the acquisition of anti-estrogen resistance in luminal breast cancer (109).

C. Regulation of Cell Fate by Epigenetic Regulators

The fundamental role of epigenetic regulation in cell fate decisions in the mammary gland is underscored by the genome-wide histone methylation profiles of basal/MaSC, luminal progenitor, and mature luminal cells. Epigenome data sets for mouse and human mammary epithelial subsets in adult tissue show marked cell-type specificity in chromatin states (141, 158, 164). In particular, H3K27me3-mediated gene silencing increased with lineage restriction along the hierarchy, and luminal progenitors showed a higher number of bivalent promoters, pointing to a role for H3K27me3 marks in establishing luminal identity. Analysis of chromatin accessibility in embryonic MaSCs and adult epithelial populations further revealed that fetal and adult MaSC-enriched cells share similar epigenetic patterns that are associated with multipotency and are thus consistent with the existence of multipotent MaSCs (61).

The PRC1 gene *Bmi1* was first implicated in regulating the self-renewal of MaSCs in human breast epithelial cells (131). In the mouse, $Bmi1^{-/-}$ mammary epithelial cells failed to generate outgrowths upon transplantation, and *Bmi1*

was further implicated in the maintenance of faithful committed progenitor cells (166). The canonical PRC2 complex comprises the histone methyltransferases Ezh1 and Ezh2, Suz12, Eed, and the accessory protein RBBP4/7. Ezh2 appears to be the dominant methyltransferase in PRC2 during early development, and *Ezh2* levels are also markedly higher than that of *Bmi1* in embryonic MaSCs (208). In the adult gland, Ezh2 plays an essential role in regulating the proliferation of all progenitor populations, and its loss resulted in delayed pubertal development and defective alveologenesis (146, 158). Strikingly, pregnancy hormones triggered genome-wide changes in chromatin modifications, raising the possibility that sustained exposure to hormones initiates breast cancer through altering the chromatin landscape in progenitor cells (158). Similar to the combined loss of *Ezh1* and *Ezh2* (245), disruption of the essential PRC2 component *Suz12* completely blocked mammary gland development and the deposition of H3K27me2/me3 chromatin marks (145). Another epigenetic regulator, the histone methylation reader Pygo2, is essential for suppression of luminal differentiation in the MaSC population. These effects appear to be mediated through Pygo2 facilitating the binding of β-catenin to *Notch3* in MaSC/basal cells to suppress its expression (83).

The role of DNA methylation in mammary cell fate decisions is still emerging. Both basal and luminal cells show unique methylation patterns that correlate with gene expression (59, 101, 122). Intriguingly, comparison of genome-wide DNA methylation patterning in cells from nulliparous and parous females revealed an epigenetic memory associated with pregnancy (59). Integration of ATAC-seq, DNA methylation, gene expression, and proteomic data for adult basal and luminal cells also indicated that DNA methylation is regulated by the balance of activity between DNA methyltransferase enzymes (Dnmt1, Dnmt3a/3b) and translocation (Tet) methylcytosine dioxygenases (36). *Dnmt1* loss led to marked defects in ductal growth and appears indispensable for the maintenance of stem or progenitor cells (162).

XII. ROLE OF THE MICROENVIRONMENT IN INSTRUCTING MAMMARY STEM CELLS

A. Reprogramming of Epithelial Cells from Other Tissues

Early seminal tissue recombination experiments demonstrated the influence of the mesenchymal stromal compartment on epithelial cell fate. When embryonic salivary epithelium was combined with the mammary mesenchyme under the kidney capsule, outgrowths were generated that resembled a functional mammary gland capable of milk production (47, 186). In the reverse experiment, tissue recombinants between salivary mesenchyme and mammary epithelium (E12 or E16) were found to generate epithelial outgrowths reminiscent of the salivary gland, demonstrating that the mesenchyme provides crucial inductive signals. These findings also highlight the inherent adaptability of the primordial epithelium to respond to instructive signals.

In the adult setting, the mammary stroma can be instructive and contributes to mammary epithelial specification via indirect mechanisms. Nonmammary cells such as testicular cells, neural stem cells, or bone marrow cells could be reprogrammed by the mammary stroma, only when coinjected with mammary epithelial cells, to yield progeny that contributed to ductal epithelial outgrowths (20, 22, 23, 30). These data imply that reprogramming by the putative stem cell niche requires mammary epithelial cells in addition to the stromal microenvironment.

B. The Stem Cell Niche

The ductal epithelium is surrounded by distinct stromal cell types, including fibroblasts, adipocytes, macrophages, T cells, and other immune cells. In the presumptive stem cell niche, reciprocal interactions between specific cell types are envisaged between basal MaSCs and luminal cells, and between MaSCs and the basement membrane or neighboring stromal cells. Even a single stem cell in the absence of supporting mammary epithelial cells can reconstitute an entire mammary ductal tree, indicating the importance of the stromal compartment. Although the biochemical signaling networks that support proper tissue architecture have yet to be fully deciphered, it is notable that myoepithelial and stromal cells synthesize extracellular matrix (ECM) components such as laminins, fibronectin, collagens, and proteoglycans, which also constitute the basement membrane and may contribute to a stem cell niche (207). In fact, a recent study indicates that laminins are important constituents of the MaSC niche since basal cells depleted of laminin receptors comprising α3- and α6-integrin subunits have increased myosin II and reduced repopulating activity (184).

The localization of dormant MaSCs to the proximal region of the fat pad may be indicative of a specific microenvironment. In the distal portion, there is no evidence that MaSCs specifically reside at branch points, but a suprabasal location has been described for some basal cells (35). Thus the different stem cell subsets in the proximal and distal parts of the ductal tree likely exist in distinct niches. If there is a fixed configuration of cells in the mammary niche, it is possible that progenitors or premalignant cells may enter the niche under perturbed conditions and become reprogrammed towards a basal stem cell fate.

C. Signals from Fibroblasts to Epithelial Cells

Stromal fibroblasts appear to be a major determinant of development in the mammary gland. A number of fibroblast-derived factors have been implicated in transmitting signals to the epithelium including Hh ligands **(FIGURE 8)**. In the context of Hh signaling, *Gli2* expression is restricted to stromal fibroblasts, and increased numbers of these cells occur in the vicinity of the TEBs (90, 123). While *Gli2*-null epithelial cells generate morphologically normal outgrowths upon transplantation, engraftment of whole tissue fragments revealed abnormalities suggesting that Gli2 plays an important role in the stroma (90, 123). Targeted deletion of *Gli2* in fibroblasts severely impaired MaSC activity despite only 20% of stromal fibroblasts expressing *Gli2*, suggesting that a specific fibroblast subset forms a stem cell niche. Hh ligands expressed by MaSCs may also reciprocally activate the Hh pathway in fibroblasts. Interestingly, *Gli2*-expressing fibroblasts responded to ovarian hormones via the secretion of multiple growth factors, including insulin-like growth factor, Wnt, FGF, and HGF family members (249), all of which have been implicated in MaSC function and different aspects of mammary gland development. FGFs are potent mammary morphogenic factors and likely impact on MaSCs, which express FGFRs. The importance of stromal fibroblasts in the regulation of MaSCs is also underscored by the genetic loss of *Pten* in fibroblasts resulting in stem cell expansion through Jagged-1-mediated activation of Notch signaling (198).

D. Communication Between Immune Cells and Mammary Stem Cells

Macrophages surround the TEBs in the developing mammary gland and are associated with mature ducts in the established gland. Early studies, based on depletion of macrophages by genetic or biochemical strategies, suggested that macrophages are essential for postnatal mammary gland development (81, 227). Recent insights into the mechanisms by which macrophages govern mammary epithelial cells have implicated the Notch and Wnt pathways in an integrated paracrine signaling network **(FIGURE 8)**. Notch ligand Dll1 is expressed by a subset of basal epithelial cells and binds Notch receptors on the macrophage cell surface. Activation of the Notch signaling pathway appears to be important for the maintenance of mammary macrophages as well as the induction of Wnt ligands, which are then secreted to promote the renewal of juxtaposed MaSCs (38). Other immune cells such as T cells can also influence ductal development. For example, $CD4^+$ Th1 cells secrete interferon-γ, which binds to its cognate receptor in a subset of luminal progenitor cells and inhibits their differentiation and mammary organogenesis (167).

XIII. IMPLICATIONS OF THE CELLULAR HIERARCHY FOR BREAST CANCER

A. Cells of Origin of Breast Cancer

Breast cancer is the most common cancer affecting women worldwide, accounting for ~30% of new cancer cases diagnosed each year and 15% of all deaths (196). It is not a single disease but a heterogeneous collection of tumor subtypes with diverse pathological features, molecular signatures, and clinical outcomes. Breast tumors are largely classified on the basis of expression of ER, PR, and human epidermal growth factor receptor 2 (HER2) by immunohistochemistry. At a transcriptome level, breast cancers have been stratified into five main intrinsic subtypes: luminal A, luminal B, HER2-positive, claudin-low, and basal-like (95, 165, 206). Classification of newly diagnosed tumors into a breast cancer subtype has important implications for tailoring treatments and predicting patient outcomes; however, patient response to targeted therapy or chemotherapy remains highly variable.

Intertumoral heterogeneity is hypothesized to reflect distinct breast epithelial cells that serve as the cell of origin for malignant transformation, as well as the repertoire of oncogenic driver mutations (66, 230). In addition, the microenvironment encompassing immune cells and fibroblasts plays a pivotal role in influencing tumor histopathology and behavior (169). Comparison of the established molecular signatures of the normal breast epithelial subpopulations with those of the different breast cancer subtypes has provided an important framework to understand the cellular origins of breast cancer, at both the sporadic and hereditary cancer levels **(FIGURE 10)**. The cancer subtypes appear to segregate along the normal differentiation hierarchy commencing with undifferentiated claudin-low tumors, followed by basal-like tumors, $HER2^+$ tumors, and finally the luminal A and B tumor subtypes (126, 171). The MaSC-enriched subset, in particular dormant MaSCs (75), is remarkably similar to the claudin-low cancer subtype at the molecular level. The luminal progenitor subset harbors a molecular profile that is very closely aligned with basal-like tumors (126), which also aligns closely with $ALDH^+ER^-$ luminal progenitors (194). The $HER2^+$, luminal A, and luminal B subtypes almost certainly reflect different cell types within the luminal lineage, and not surprisingly, the molecular profile of luminal A tumors is closest to that of mature luminal cells. Importantly, these molecular relationships remain correlative until functionally proven in tumor-initiating studies. Determination of the cells of origin of these subtypes and novel biomarkers could enable earlier detection of breast cancer and the development of effective prevention therapies.

In terms of familial breast cancer, early cellular changes arising in premalignant tissue from *BRCA1*-mutant carriers

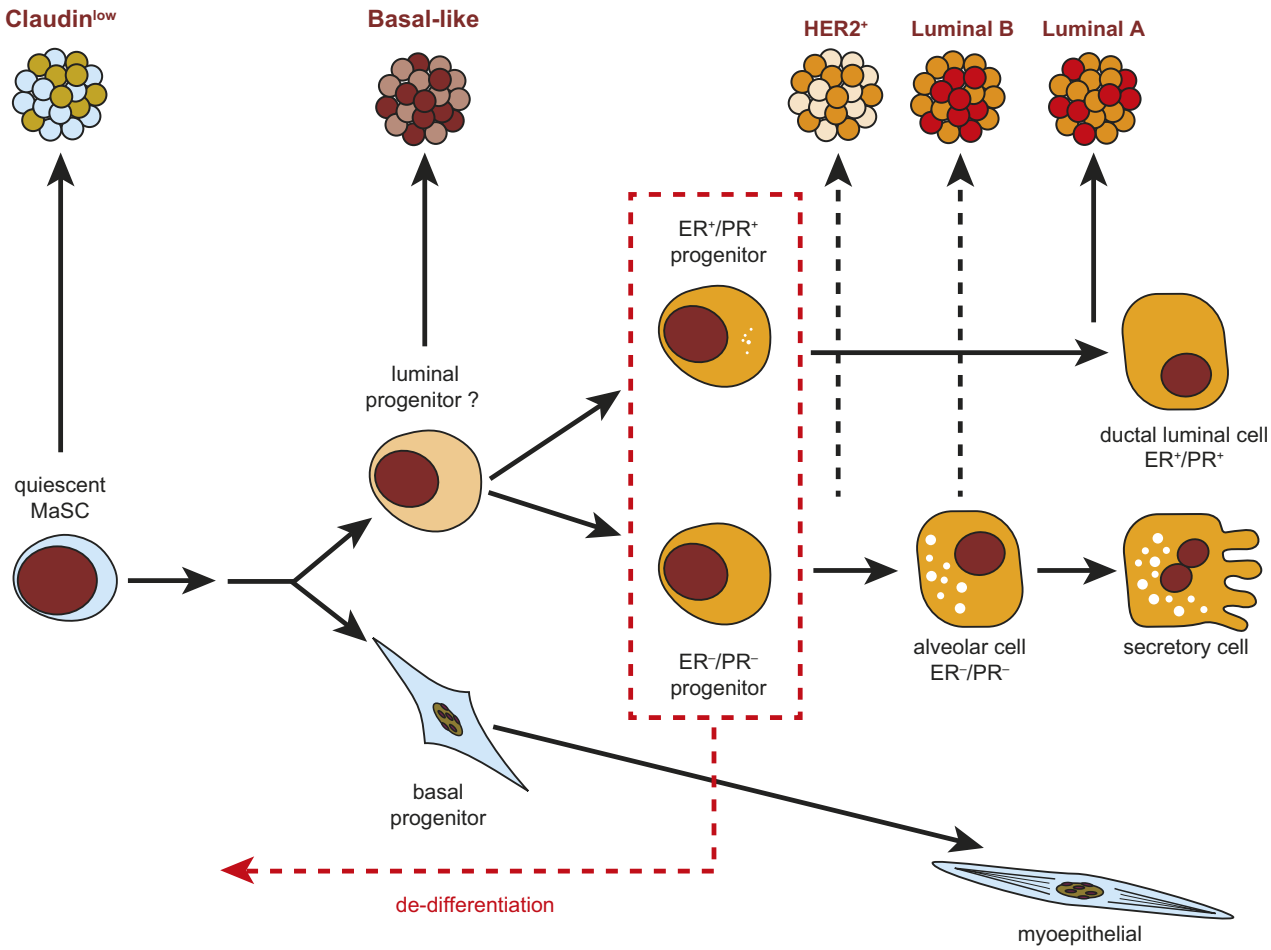


FIGURE 10. Schematic model of potential relationships between cells in the human breast epithelial hierarchy and breast tumor subtypes. The five intrinsic breast tumor subtypes are shown together with their closest normal epithelial counterpart based on gene expression profiling. The luminal B and HER2$^+$ subtypes likely originate in target cells restricted to the luminal cell lineage. Luminal progenitors also exhibit marked "plasticity" or dedifferentiation during oncogenesis.

have been recently dissected and provided significant insights into a key target population. While it was initially proposed that putative breast stem cells residing within the basal layer of the mammary epithelium were the "cell of origin" for *BRCA1*-mutated basal-like tumors (72), this was primarily based on histological studies that noted similarities between basal-like tumors and basal epithelial cells (i.e., expression of basal cytokeratins and lack of HR expression). Early work suggested that *BRCA1* regulates the fate of human progenitor cells and that *BRCA1* mutation carriers show perturbed differentiation (132). Analysis of preneoplastic tissue from *BRCA1*-mutation carriers revealed a target population predisposed to neoplastic transformation. An expanded population of aberrant luminal progenitor cells was identified in *BRCA1* carriers, and preneoplastic *BRCA1*-mutant tissue showed highest molecular similarity to normal luminal progenitor cells (126). Conditional deletion of *Brca1* in the mammary luminal compartment predisposed mice to the development of basal-like tumors that recapitulated the histological, pathological, and molecular features of human basal-like tumors, whereas deletion in the basal compartment did not (148). Furthermore, transduction of human breast epithelial cells with a cocktail of potent oncogenic lentiviruses followed by implantation into humanized mammary fat pads (116) showed that *BRCA1*-mutated luminal cells were more susceptible to transformation compared with basal cells (173). While transduced cells derived from wild-type breast tissue gave rise to both luminal and basal-like tumors, the transformation of *BRCA1*-mutant cells predominantly yielded basal-like tumors. Thus *BRCA1*-deficient breast tissue exhibits an inherent defect that dictates tumor phenotype. Further fractionation of human breast has revealed that the likely cell of origin is a hyperproliferative RANK$^+$ luminal progenitor cell (154) that exhibits augmented NF-κB signaling (190) and aberrant DNA repair properties. This RANK$^+$ progenitor represents a potentially targetable cell for chemoprevention (154, 197).

Not only do luminal progenitor cells appear more sensitive to germline *BRCA1* mutations, but they may be programmed to acquire basal-like features. The predilection

for forming basal-like tumors may be mediated in part by the *BRCA1*-regulated transcription factor *SLUG*. This EMT factor is upregulated in *BRCA1*-deficient tissue and blocks luminal cell differentiation, thus biasing cells towards a basal fate (173). Autocrine transforming growth factor-β was recently shown to restrict stem/progenitor cell expansion through regulation of *BRCA1* via miR182 (140), perhaps also contributing to basal-like features. Collectively, these studies provide valuable insights into target cells prone to tumorigenesis and the potential molecular mechanisms underlying neoplastic transformation.

Potential "cells of origin" of sporadic or spontaneous breast cancers have been investigated in a range of mouse models, primarily using constitutively active promoters to drive the oncogenic lesion(s). For example, deletion or alteration of two key tumor suppressor genes, *Trp53* and *Pten,* using different gene promoters that include *β-lactoglobulin*, *K14, WAP,* and *MMTV,* resulted in diverse tumors that differed according to the promoter (130, 144, 234). Cells of origin of cancer are ideally addressed using inducible lineage tracing models combined with highly specific promoters. Along these lines, induced expression of an activated form of phosphatidylinositol 3-kinase (PIK3CA), a mutation commonly found in the luminal subtypes of breast cancer, was recently implicated in activating a multipotent gene expression program in basal or luminal-restricted cells in vivo. Expression of $PIK3CA^{H1047R}$ in either *Lgr5*- or *K5*-expressing (basal) or *K8*-marked luminal lineage cells of the adult gland elicited differentiation to a multipotent state (113, 225). Transplantation of the purified basal subset isolated from preneoplastic tissue of either model revealed increased repopulating capacity, and enrichment of luminal progenitor signature genes was evident in the basal compartments. Likewise, enrichment of myoepithelial genes was observed among the luminal subsets. Even though the cell of origin was found to influence tumor histopathology, the pathologies evident in the mouse tumors differ considerably from luminal breast cancers seen in patients, perhaps reflecting architectural differences in this tissue across species. For the basal-cell promoters (*Lgr5* or *K5*) utilized in each study (113, 225), the findings that *Lgr5*-expressing cells could generate luminal progeny (albeit a minority) in the adult mammary gland (113) and that *K5* can behave in a similar manner (179) raise the possibility that rare bipotent cells might have been activated by the PIK3CA mutation, which then promoted the expansion of luminal-restricted cells. In any event, promiscuous differentiation rather than dedifferentiation likely applies to the basal compartment expressing the oncogenic lesion. A multipotent lineage expression program may have been aberrantly activated in this lineage (as well as the luminal) by this important driver mutation.

B. Cellular Plasticity Manifest During Carcinogenesis

Cell fate decisions along the mammary epithelial hierarchy may not be strictly unidirectional. Increasing evidence suggests that the process of dedifferentiation may occur under nonphysiological conditions such as an oncogenic insult, inflammation, or exogenous factors. For example, the presence of the ECM substratum Matrigel (194) or Rho kinase inhibitors (172) can induce progenitor cells to acquire stemlike characteristics in repopulation assays. ER^- cells under defined conditions (Nrg1 and Rspo1) can also direct the formation of organoids comprising both luminal and basal cells in vitro (104), whereas other conditions strictly favor maintenance of the luminal lineage (103). Moreover, luminal epithelial cells can convert to basal-like cells upon oncogenic stress in vivo (92), and inducible ablation of *p53* in luminal cells resulted in tumors that had acquired basal-like features (217). These data reflect the inherent plasticity within the mammary luminal compartment upon perturbation.

Reactivation of embryonic developmental programs may contribute to breast cancer. Along these lines, poorly differentiated adenocarcinomas express higher levels of the ES cell-associated genes MYC, NANOG, SOX2, and OCT4, suggesting the acquisition of primitive stemlike features (14). In addition to the potential reprogramming of luminal cells to a basal stemlike state (discussed above), heterogeneity during oncogenesis may result from the transformation of stem cells. Claudin-low cancers are a rare group of tumors that are HR^-, express low levels of luminal genes as well as tight junction and adhesion-associated genes (95), and may arise directly from a MaSC. Conversely, metaplastic breast cancers, noted for their chemoresistance, have a distinct profile that resembles the claudin-low group but may correspond to tumor cells that have undergone an EMT to a true mesenchymal cell (93). It is important to note that basal mammary epithelial cells should not be confused with mesenchymal cells/fibroblasts as these cell types are distinct and bear no hierarchical relationship to one another. More recently, an unexpected degree of plasticity was observed at a clonal level using inducible *Krt5*- and *Elf5*-driven tumor suppressor deletion models. Molecular profiling of individual, labeled tumor clones revealed that most emergent clones comprised both epithelial and mesenchymal subsets, indicating that the EMT was an inherent property of tumor cells in these models (178). Thus dedifferentiation and the EMT represent two forms of plasticity that are manifest during breast carcinogenesis.

XIV. EPILOGUE

Although there is substantial evidence that the normal mammary epithelium is organized in a hierarchical fashion, many questions remain. The spatiotemporal dynamics of

stem cells and their different states are yet to be uncovered. The presence of long-lived progenitors also questions the current definition of MaSCs and raises the possibility that they could be coined unipotent stem cells. Key issues include the number of distinct unipotent progenitor cells in the mammary gland and the relative contribution of these cells (as well as multipotent stem cells) to each stage of ontogeny. What is the network of intrinsic and extrinsic factors that actively regulate quiescence versus activation of MaSCs? Do stem cells play any role in the distal area of the fat pad given that long-term lineage tracing studies suggest their potential existence? What are the components of the MaSC niche, and do they differ for stem cells in the proximal versus distal region? Are there dormant stem cells in human breast? In the context of breast cancer, there are at least three parameters that impinge on tumor pathology: the cell of origin, genetic/epigenetic mutations, and microenvironmental factors, each of which contributes a layer of heterogeneity. Heterogeneity is further confounded by plasticity, especially manifest in the luminal progenitor compartment, which has a propensity to dedifferentiate towards an "ancestral" basal-like stem cell. Targeting of multiple cell types within tumors may be necessary for effective eradication of tumor cells in the future.

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Address for reprint requests and other correspondence: J. E. Visvader, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia (e-mail: visvader@wehi.edu.au).

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