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Form and Function: how Estrogen and Progesterone Regulate the Mammary Epithelial Hierarchy

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Abstract The mammary gland undergoes dramatic post-natal growth beginning at puberty, followed by full development occurring during pregnancy and lactation. Following lactation, the alveoli undergo apoptosis, and the mammary gland reverses back to resemble the nonparous gland. This process of growth and regression occurs for multiple pregnancies, suggesting the presence of a hierarchy of stem and progenitor cells that are able to regenerate specialized populations of mammary epithelial cells. Expansion of epithelial cell populations in the mammary gland is regulated by ovarian steroids, in particular estrogen acting through its receptor estrogen receptor alpha (ERα) and progesterone signaling through progesterone receptor (PR). A diverse number of stem and progenitor cells have been identified based on expression of cell surface markers and functional assays. Here we review the current understanding of how estrogen and progesterone act together and separately to regulate stem and progenitor cells within the human and mouse mammary tissues. Better understanding of the hierarchal organization of epithelial cell populations in the mammary gland and how the hormonal milieu affects its regulation may provide important insights into the origins of different subtypes of breast cancer.

Keywords Mammary epithelial cells · Breast · Progenitor cells · Estrogen · Progesterone

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Abbreviations

ALDH	Aldehyde dehydrogenase
CK	Cytokeratin
EGF	Epidermal growth factor
Elf5	E74-like factor
ERα	Estrogen receptor alpha
FACS	Fluorescence-activated cell sorting
GH	Growth hormone
H2BGFP	Histone H2B fused to eGFP
ME	Myoepithelial
MMTV	Mouse mammary tumor virus
MRU	Mammary repopulating units
PI-MEC	Pregnancy-induced mammary epithelial cells
PR	Progesterone receptor
sca-1	Stem cell antigen-1
SMA	Smooth muscle actin
TDLU	Terminal ductal lobule unit
TGFα	Transforming growth factor alpha

Introduction

The mammary gland undergoes dynamic changes over the lifetime of a woman. Although initial development takes place

during embryogenesis, the majority of expansion and differentiation of the epithelium starts during puberty. In humans, the mammary ductal system and immature terminal ductal lobule units (TDLU) lengthen and mature as they grow into the stroma of the breast. This process is under the tight regulation of circulating hormones and localized growth factors. During puberty, epithelial cells of the breast, in particular those in the TDLU, proliferate in response to ovarian steroids, and the mature gland undergoes cyclic proliferation and apoptosis over the course of each subsequent menstrual cycle [1–4]. Similarly, in mice, the mammary gland undergoes ductal elongation during puberty with the development of ductal branches and alveolar budding in the mature gland in response to hormonal changes during the estrus cycle. In both mice and humans, full mammary epithelial maturation occurs during pregnancy, in order to generate complex lobules and specialized epithelial cells in alveoli, which have the ability to synthesize and secrete milk for lactation. Finally, after lactation, evidence suggests that the most mature lobules and differentiated mammary epithelial cells collapse, and the mammary network becomes more similar to the resting state prior to pregnancy [5]. This process of expansion and regression can occur across multiple pregnancies during the reproductive phase of a woman's lifetime, demonstrating that the epithelial cells of the breast have considerable regenerative abilities.

Epithelial cell hierarchies, where undifferentiated stem cells give rise to more differentiated progenitor cell populations, have been well-characterized in epithelia that are rapidly replaced, such as in the intestines and skin (for review, [6, 7]). Given the extensive proliferation and specialization of the epithelial cells in the breast, a linear hierarchy, with stem cells at its apex, is presumed to exist. Stem cells expand through symmetric and asymmetric divisions in order to prevent exhaustion of linage-restricted progenitors. The mammary gland is exquisitely sensitive to the effects of ovarian steroids, demonstrating well-characterized histological changes over the course of the menstrual cycle [8, 9]. In particular, epithelial cells respond to estrogen acting through its receptor, estrogen receptor alpha (ERα), and progesterone signaling through progesterone receptor (PR). Despite this well-accepted notion, determining the specific effects of each hormone on progenitor and more mature cells has been challenging. This is because in adult mammary tissues of both mice and humans, most ERα$^+$ cells also express PR [10–12], and estrogen has been shown to act through ERα to increase expression of PR [13, 14]. In addition, the full repertoire of progenitor cells is not fully understood, nor is their receptor status well defined. Moreover, the effects of estrogen are modulated by interactions at the PR promoter, which may be cell-type specific [15]. Recent evidence suggests that ERα and PR are also expressed independently within separate cell populations in the breast, and these cell populations may have divergent regulation and growth potential [16–19]. Thus, being able to isolate and study a pure population of cells

that are only sensitive to one hormone or the other while only expressing one receptor is difficult.

Identification of progenitor cells and their regulation within the stem cell niche may provide critical insight into the origins of different pathological conditions. For example, transcriptional profiling of breast cancers has revealed intrinsic subtypes, which share similarities to normal epithelial cells of the breast, suggesting that the biology of the normal precursors may play an important role in the underlying phenotype and behavior of the tumor [20–22]. In particular, 70 % of breast cancers express ERα [23–25], and more than half of ERα$^+$ breast cancers also express PR [26, 27]. Therefore, understanding the role of ERα and PR in the epithelial cell hierarchy and its regulation by hormones may lead to a greater understanding about the biology and sensitivity of ERα$^+$ breast cancers. In this review, we attempt to summarize what is currently known in both mice and humans about the identity of progenitor cells in the mammary epithelial hierarchy and focus on those that are regulated by hormones.

Mammary Gland Development

Mammary development begins during embryogenesis with specification of cells from the surface ectoderm that go on to form the nipple and the rudimentary ductal tree. The mammary epithelium is bilayered, composed of luminal cells that line the hollow lumens of the ducts and surrounded by myoepithelial cells that have contractile function. During post-natal development in both mice and humans, mammary ducts grow allometrically into the mammary fat pad until puberty [28–30]. Interestingly during a portion of this developmental period, ERα is readily detectable, while PR expression is low to absent [28, 31]. In humans, ERα is detected in the breast epithelium of the fetus beginning in the third trimester of pregnancy, whereas PR is not detected until 2–3 months after birth [31]. Following birth, 80–90 % of infants continue to produce milk proteins [32, 33], suggesting that the developmental changes in response to the complex maternal hormonal environment of pregnancy exhibit long-lasting effects. Although in mice this lactational response is not seen, the imbalance favoring of ERα expression over PR expression in the early post-natal period is observed [18, 28].

At the start of puberty, estrogen, binding to its receptor ERα, is necessary for rapid growth and expansion of the ducts into the mammary fat pad. In both humans and mice, cells that express ERα however, do not co-localize with markers for proliferation [10–12]. Thus, estrogen's action through ERα is mediated by a paracrine mechanism to promote the proliferation of surrounding cells [34–36]. In fact, transplanted murine ERα$^{-/-}$ cells are unable to undergo ductal elongation when inoculated alone, but when co-mixed with wild type cells, ERα$^{-/-}$ cells can take part in ductal elongation [34, 35]. These

elegant studies illustrated the notion that paracrine factors produced by ERα^+ cells are necessary for the proliferation of ER$\alpha^{-/-}$ cells. One paracrine mediator of estrogen is amphiregulin, which is also necessary for ductal elongation [37–39]. However, growth factors of the EGF protein family can also rescue the ER$\alpha^{-/-}$ phenotype [40]. Epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and heregulin are all necessary for pubertal ductal elongation suggesting that activation of EGF family receptors by estrogen is critical for ductal elonagation.

Following puberty, the mouse mammary gland undergoes lateral branching in response to ovarian steroids produced during the estrous cycle. This process is driven primarily by the actions of progesterone. Thus, unlike ER$\alpha^{-/-}$ epithelial cells, PR$^{-/-}$ cells do undergo normal ductal elongation, but the resulting growths lack secondary branches and a complete lack of alveolar development in response to pregnancy [41, 42]. Similar to ER$\alpha^{-/-}$ cells, transplant of PR$^{-/-}$ cells co-mixed with wild type cells also results in normal ductal branching and alveolar development, suggesting that PR also acts through a paracrine mechanism of action on surrounding cells [41].

One paracrine mediator of progesterone activity is RANKL, a member of the tumor necrosis factor superfamily. Ectopic expression of Rankl in PR$^{-/-}$ mice rescues the PR$^{-/-}$ phenotype [43]. Further, transgenic overexpression of Rankl leads to precocious ductal side branching and alveologenesis, similar to the effects of progesterone stimulation [44]. Another downstream target and paracrine mediator of PR is WNT-4, which is upregulated in primary mammary epithelial cells in response to progesterone both *in vitro* and *in vivo* [45]. Cyclin D1, which is a critical component of the cell cycle, is also required for progesterone-induced proliferation during mammary gland development. Deletion of PR results in a significant reduction in cyclin D1 expression, and similar to PR$^{-/-}$ mice, mice lacking cyclin D1 do not properly develop alveoli and are unable to nurse their pups [46, 47]. These results suggest that cyclin D1 is important for progesterone-induced proliferation. However, it is not clear whether Rankl, Wnt-4, and cyclin D1 promote proliferation of the same or different cell types in the mammary gland.

Since the human breast exhibits greater anatomical and lobule complexity compared to the mouse mammary gland, it is not surprising that its development is also more complex. Unlike the murine mammary gland, terminal end buds do not emerge and grow into the breast stroma. Rather lobules, separated by connective tissue, develop and are joined to central ducts that range in number from 11 to 48 [48]. Given this difference in early development, it is not clear whether ERα alone mediates this growth. Lobules range in size and have been categorized with regard to their degree of development. Type I lobules are the least developed and have been characterized as having the highest expression of ERα and PR expression [10]. Lobules mature through increasing their size and complexity through pregnancy, with Type IV lobules only

present in lactating women [10, 49–51]. Although breast tissue of nulliparous women primarily contains Type I lobules, Type II and Type III lobules are also present [32, 33, 52].

Humans also exhibit important differences in their cycling hormones which also likely affects breast development. In humans, as well as mice, proliferation of mammary epithelial cells is not at its peak during the follicular phase, when circulating estrogens are at their maximum, but rather during the luteal phase, when the ratio of circulating progesterone to estrogen is increased [10, 12]. However, unlike mice, the human corpus luteum secretes estrogen in addition to progesterone [53]. As such, tamoxifen use in women can inhibit breast epithelial proliferation during the luteal phase of the menstrual cycle [54]. This suggests that both estrogen and progesterone regulate proliferation in the human breast. The changes in hormonal activity over the menstrual cycle may also impact the types of lobules observed within the breast, as Type I lobules have been shown to be more abundant during the follicular phase of the menstrual cycle, whereas Type II lobules are more common during the luteal phase [55]. Interestingly, ERα and PR are expressed in different subsets of cells over the course of the menstrual cycle [18], although this response is variable among patient samples, possibly due to differences in parity or history of hormone-based contraceptive use. However, studies to dissect the changes in the specific breast lobules types have met with technical challenges. Lobules have been primarily characterized in human tissues that were fixed and stained as whole mounts or on histological sections, which limited the types of analyses that were performed. Further work to isolate breast lobules for the delineation of the cell populations responsive to estrogen and progesterone may improve our understanding of the complex anatomical development of the human breast.

Transcription Factors Regulate Cellular Differentiation

Transcription factors play a central, cell-specific role in lineage selection and cell fate decisions. Some transcription factors have been shown to regulate steroid receptor expression, which in turn can alter the behavior of the surrounding cells through paracrine signaling. For example, Gata-3 is expressed in luminal epithelial cells and has been shown to play a central role in regulation of mammary gland morphogenesis and luminal differentiation during development [56] and in the mature gland [57]. Loss of Gata-3 specifically during lactation results in significantly decreased numbers of differentiated alveolar cells [56]. One mechanism by which Gata-3 regulates luminal differentiation is through the activation of the transcription factor FoxA1 [57]. FoxA1 is important for expression of ERα; FoxA1-deficiency results in significantly reduced ERα expression levels, a block in terminal end bud formation, and an

inability of the ducts to properly invade the mammary fat pad [58]. The majority of FoxA1^{+} cells also express ERα [58]. Therefore, one mechanism through which luminal differentiation is regulated by Gata-3 is likely due to its indirect effects on steroid receptor expression, which in turn alters the behavior of the surrounding cells through paracrine signaling.

The transcription factor C/EBPβ also plays an important role in luminal cell differentiation and the correct patterning of steroid receptors [59, 60]. C/EBPβ^{-/-} mice demonstrate elevated PR expression and increased epithelial proliferation [60]. This results in an increase in differentiated luminal cells but a decrease in cells that secrete milk proteins during lactation [61]. Thus, C/EBPβ may regulate the expansion of luminal cells as well as their ability to differentiate into a population that is able to give rise to alveoli.

While one set of transcription factors regulates cell populations that are responsive to ovarian steroids, another group of transcription factors appears to act downstream of steroid receptors to expand populations of cells that are able to mature into alveoli. One such factor that regulates alveolar cellular proliferation is Stat5a [62]. During pregnancy, Stat5a is required for lobuloalveolar outgrowth and lactogenesis [63, 64], while in nulliparous glands, its active form (p-Stat5a) is necessary for ductal branching and proliferation [65]. Stat5 is regulated by both estrogen and progesterone [66], and p-Stat5a localizes with both ERα and PR expression [66]. Another transcription factor, E74-like factor 5 (Elf5, also known as ESE-2), regulates alveolar differentiation. Elf5 expression is induced by progesterone, although ERα[+]/PR^{+} cells do not express Elf5 [67, 68]. Progesterone may regulate Elf5 expression levels through Rankl, since blockade of Rankl signaling prevents progesterone-induced side branching and the expansion of Elf5^{+} mature luminal cells [68]. During pregnancy, Elf5 is critical for the differentiation of secretory cells and is regulated by both Stat5-mediated and independent mechanisms [62]. Although ERα and PR have been shown to regulate Stat5 and Elf5 in nulliparous glands, delineating the regulation of Stat5 and Elf5 specifically by ERα and PR signaling during pregnancy is complex. This is because both transcription factors are also regulated by the pituitary hormone prolactin, which is critical for alveologenesis during pregnancy and differentiation during lactation [69, 70].

Although gene expression profiling has led to the identification of transcription factors that are expressed in various stem and progenitor cells, identifying their role during lineage commitment and differentiation is an area of great interest [71–73]. It is likely however that transcription factors activated in progenitor cells that drive cell fate decisions may also have continued functions in daughter cells to maintain lineage differentiation [74]. Thus, identification of unique combinations of transcription factors in early progenitor cells compared to more mature progeny may provide insights into how steroid receptors regulate lineage specification and how cell fate decisions in the mammary epithelial cell populations are achieved in response to hormones.

Stem Cell Activity in the Mammary Gland

The discovery that mammary epithelial cells and tissue fragments could regenerate entire mammary tissues led to the notion that stem cells must be present in mammary tissues. Pioneering work by DeOme and colleagues demonstrated the existence of mammary stem cells through transplant experiments. This technique relies on the removal of endogenous mammary tissue fragments prior to puberty and allografting donor epithelium into the cleared fat pads; the epithelium can repopulate and recreate a fully functional mammary ductal tree [75, 76]. These observations were extended using insertional marking of mammary epithelial cells with mouse mammary tumor virus (MMTV) integration, which showed that one putative stem cell could contribute to and account for all cellular renewal of the mammary epithelium over several transplant generations [77]. Similarly, in humans, genetic tracking of cells through inactivation of the same X-chromosome suggested that lobules with contiguous patches of epithelium were derived from the same stem/progenitor cell [78, 79].

Prospective identification of stem cells was subsequently achieved with the identification of cell surface markers that could separate populations of epithelial cells based on their functional abilities to form colonies *in vitro* or demonstrate outgrowth potential *in vivo*. When epithelial cells are dissociated, stained with antibodies to detect CD24 and CD29/β1-integrin, and sorted using fluorescence-activated cell sorting (FACS), a single lacZ^{+} cell from the CD29^{hi}CD24^{+} population could generate an entirely lacZ^{+}, functional mammary gland [80]. These data demonstrated that cells from the basal/myoepithelial (ME) lineage are enriched for mammary repopulating units (MRU). Similar results have been observed when epithelial cells were sorted using CD24^{+}CD49f/α6-integrin^{hi} [81].

Although cells in the basal/ME lineage are able to reconstitute an entire mammary tree when transplanted, physiologic lineage tracing models have been less clear as to whether bipotent stem cells exist and are able to reconstitute both lineages *in situ*. In lineage tracing experiments marking luminal or basal/ME cells, some studies have shown that lineage restricted progenitor cells maintain the separate epithelial lineages after birth [82–84]. Luminal progenitor cells gave rise to mature luminal cells without contribution from a putative stem cell in the basal lineage (Fig. 1a). However, other studies have shown that during pregnancy, the lineage restriction of luminal and basal cells is not maintained, and that cells from the basal lineage can also contribute to the luminal lineage during alveologenesis [85–87] (Fig. 1b). Yet another study has suggested significant contribution of basal/ME stem cell populations to the continued development of the luminal epithelium.
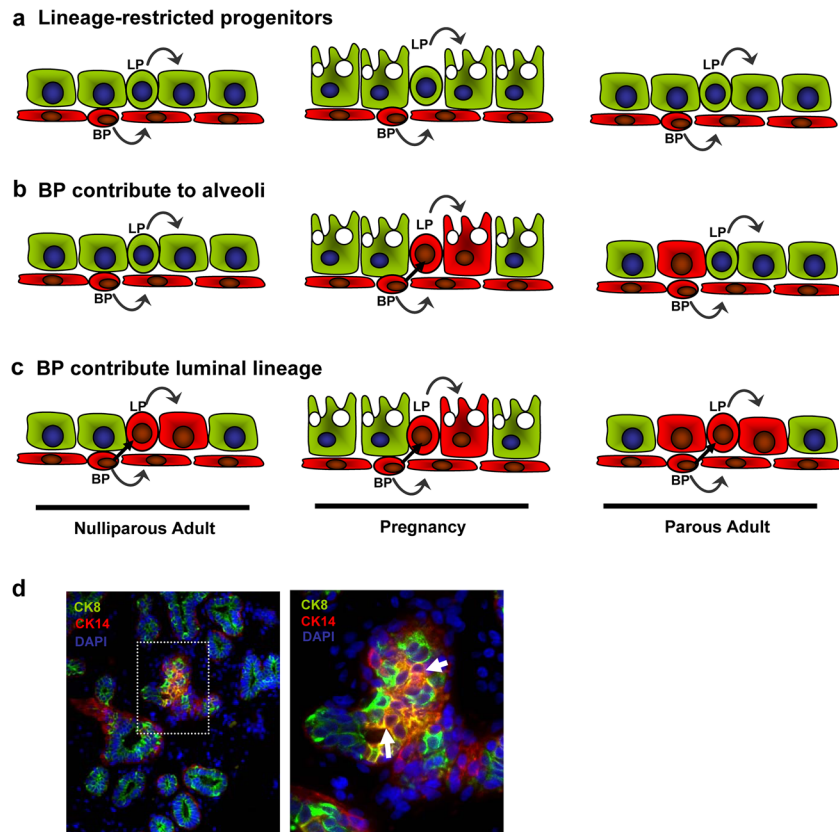
Fig. 1 Models for the contribution of stem cells of basal origin to the luminal lineage in the mammary glands of adult mice. Epithelial cells of basal origin are depicted in red; BP denotes basal progenitor cells. Cells of luminal origin are depicted in green; LP denotes luminal progenitor cells. **a** In the adult gland, cells of each lineage are restricted. LPs divide in order to expand the luminal cell population, while BPs proliferate in order to generate basal epithelial cells. **b** In adults, the lineages are restricted until pregnancy, when progenitor cells of the basal lineage contribute to the formation of the luminal alveolar cells. A portion of these cells remain in the gland following involution to expand the alveolar cell population during subsequent pregnancies. **c** BPs proliferate over the course of the estrus cycle to contribute to the LP population. These cells contribute to formation of the luminal alveolar cells. A portion of these cells remain in the gland following involution to expand the alveolar cell population during subsequent pregnancies. **d** In humans, cytokeratin (CK) 8, a marker for luminal cells, and CK14, a marker for basal cells, are co-expressed in a subset of cells. Sections of tissue obtained from reduction mammoplasty surgeries were labeled with CK8 (*green*), CK14 (*red*), or both (*yellow*) and counterstained with the nuclear stain, DAPI (*blue*) using immunofluorescence. White arrows highlight cells that are co-labeled with CK8 and CK14. Image magnification: 200x, 400x

Using a stochastic multicolor *cre* reporter specific to mark either basal or luminal epithelial lineages, cells from the basal lineage significantly contributed to the luminal lineage, even in nulliparous female mice [88]. This contribution of the basal lineage to the luminal lineage has also been observed in other tracing studies, although the occurrence is rare [86, 89] (Fig. 1c). The reasons for the differences among these studies are not clear, but may due to differences in mouse strains or the use of the estrogen antagonist, tamoxifen, to induce lineage tracing. The dosage of tamoxifen has also been shown to have direct effects on mammary gland development, as well as long-lasting effects on progenitor cell activity in the mammary gland and other organs, thus potentially confounding the results of some of the findings [88, 90, 91]. Hence, to reconcile these disparate observations, additional studies are necessary to fully define the contribution of basal/ME cells to progenitor populations in the luminal lineage under physiologic conditions *in vivo*. A recent review on mammary lineage tracing provides an in-depth discussion of these challenges for determining progenitor contributions to each lineage in the mammary epithelial hierarchy [92].

The cellular hierarchy and identity of stem/progenitor cells in the human breast is not as clearly defined as in rodents. This is in part because the expression of luminal and basal markers are not as lineage restricted as they are in mice. In adult mice, mammary luminal cells exclusively express cytokeratins 8 and 18 (CK8, CK18), while basal/ME cells strictly express basal cytokeratins 5 and 14 (CK5, CK14). However in human breast tissue, CK14 and CK8 double-labeled cells are readily observed in both the luminal and basal/ME compartments [52, 93] (Fig. 1d). Type I lobules contain the greatest number of dual-labeled cells, while Type II and Type III lobules exhibit increased lineage restriction of these markers to the luminal and basal epithelial cells [52]. It is possible that these dual-labeled cells reflect a more primitive bipotent state with the ability to differentiate into different lineages. Indeed, microdissection of

ducts and lobules in the human breast to isolate cells for functional assays *in vitro* has shown the presence of enriched progenitor activity in cells that exhibit both luminal CK19 and basal CK14 expression [94]. Further, a population of luminal cells that is enriched for progenitor activity [94, 95] is also enriched for cells that co-express CK8 and CK14 [52, 96, 97]. However, a recent study examining progenitor activity from dissociated primary breast epithelial cells found no significant correlation between reduction mammoplasty samples that exhibit high CK8/14 double labeling and the ability to form colonies in culture [73]. Therefore, whether cells that express CK8/14 are bonafide progenitor cells still remains unclear.
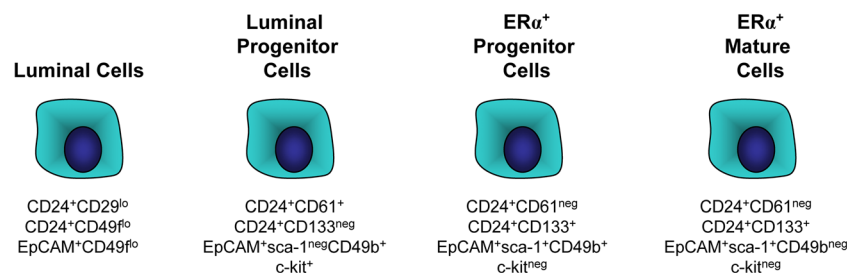
Diversity of Luminal Progenitor Populations

While all reports support the notion that basal/ME epithelial cells are enriched for cells with the most stem/progenitor cell activity, there are conflicting reports regarding stem/progenitor potential of luminal epithelial cells in the same assays [80, 81]. In humans, cells expressing the luminal cell surface marker EpCAM/ESA$^+$ are able to form branching structures reminiscent of TDLUs as well as acinar restricted colonies on Matrigel [75]. On a plastic substrate, EpCAM$^+$ luminal cell populations also generate colonies that express both CK8 and CK14 as well as CK8 only colonies, suggesting that luminal cells have the ability to lose lineage restriction and give rise to basal cells [98–100].

Cells within the luminal lineage can be fractionated into two major populations: an EpCAM$^+$CD49f^{neg} mature luminal population in which 55 % of cells express ERα and 71 % express PR or an EpCAM$^+$CD49f$^+$ luminal progenitor population in which 28 % cells express ERα in the absence of PR [95]. When such cells are sorted and transplanted into humanized mammary fat pads of immunocompromised mice, both luminal progenitor and mature luminal cell populations give rise to bi-layered structures that contain both luminal (CK8$^+$) and basal/ME (CK14$^+$ and smooth muscle actin positive (SMA$^+$)) cells. [96]. Thus, luminal cells do have the capacity to exhibit bipotent progenitor activity. *In vitro*, luminal cells isolated from both populations also form both branching and acinar structures on a collagen substrate [19, 52, 96]. Additionally, it has been shown that aldehyde dehydrogenase (ALDH) activity,

which enriches for progenitor activity in multiple cell types [101], is enriched in luminal progenitor cells [102]. These luminal progenitor cells exhibit elevated expression of the ALDH1A3 isoform, suggesting that this isoform of the ALDH family may regulate progenitor activity in the breast [102, 103]. Further, the addition of ErbB3 expression in conjunction with ALDH activity segregates luminal cells into 3 separate populations [104]. However the size and incidence of these populations are not consistent among all reduction mammoplasty samples examined [104]. Improved delineation of luminal cells may be strengthened by clinical information about patients donating breast tissue samples, including parity, history of hormonal contraceptive use, and stage of the menstrual cycle. However, this information is often challenging to obtain.

In mice, multiple luminal epithelial cell populations have also been identified, although some of the populations are overlapping. Fractionation of luminal cells with the surface markers CD61/β_3-integrin enriches for progenitor activity in the CD24$^+$ population [56]. With the addition of this marker, the CD24$^+$CD61$^+$ cells have greater progenitor activity with significantly reduced numbers of ERα^+ cells, while the CD24$^+$CD61^{neg} cells are enriched for ERα^+ cells [56]. Similar to CD61, the use of CD133/prominin-1 separates an ERα/PR enriched population from the CD24$^+$ cells [105]. Stem cell antigen-1 (sca-1) has been associated with stem/progenitor activity in a number of murine cell types (for review, [106]), and initial studies in the mammary gland suggested that sca-1$^+$ epithelial cells exhibit increased progenitor activity compared with sca-1^{neg} cells [107]. In combination with CD49b/α_2-integrin, sca-1 separates EpCAM$^+$ luminal cells into three populations: sca-1^{neg}CD49b$^+$, sca-1$^+$CD49b$^+$, and sca-1$^+$CD49b^{neg} populations. Sca-1^{neg}CD49b$^+$ cells have significant progenitor activity and express detectable CK18/CK5 double labeling, suggesting that they maybe an intermediate cell type [104]. Both sca-1$^+$CD49b$^+$ and sca-1$^+$CD49b^{neg} populations are enriched for ERα, but only the sca-1$^+$CD49b$^+$ population contains functional progenitor activity [104]. Similar to the sca-1^{neg} progenitors, cells isolated using c-kit as a marker exhibit progenitor activity [108]. Together, these studies suggest that many of the cell surface markers used to isolate presumably different luminal progenitor populations identify cells with similar functional activities (Fig. 2). Thus,

	Luminal Cells	Luminal Progenitor Cells	ERα^+ Progenitor Cells	ERα^+ Mature Cells

CD24$^+$CD29^{lo} CD24$^+$CD61$^+$ CD24$^+$CD61^{neg} CD24$^+$CD61^{neg}
CD24$^+$CD49f^{lo} CD24$^+$CD133^{neg} CD24$^+$CD133$^+$ CD24$^+$CD133$^+$
EpCAM$^+$CD49f^{lo} EpCAM$^+$sca-1^{neg}CD49b$^+$ EpCAM$^+$sca-1$^+$CD49b$^+$ EpCAM$^+$sca-1$^+$CD49b^{neg}
c-kit$^+$ c-kit^{neg} c-kit^{neg}

Fig. 2 Cell surface markers to identify luminal epithelial cell populations in mouse mammary glands. In mouse mammary glands, multiple cell surface markers have been identified that detect luminal, luminal progenitor, estrogen receptor alpha (ERα) positive progenitor, and mature luminal cells

additional work is necessary to determine whether combining these markers will further refine the cellular populations and identify more pure populations of progenitor cells.

Less clear but equally important are bipotent progenitor cells that appear to exhibit structurally limited outgrowths. Transplantation of murine cells by limiting dilution can produce ductal-limited and secretory alveolar-limited outgrowths [77, 109, 110]. Progenitors that yield these specific outgrowths can be found in CD24^{hi}CD49f^{lo} cells when transplanted into pregnant hosts [110]. In humans, breast epithelial cells also have the ability to form ductal-limited or acinar-limited structures when grown on a collagen substrate. Enrichment for ductal-only progenitors can be found in EpCAM^{neg}CD10$^+$ basal cells while acinar-only progenitors are present within the EpCAM$^+$ luminal populations [52, 96]. It is currently unclear if these progenitors within the human breast have similar patterns of growth *in vivo*. It is also unclear whether these duct-limited or alveolar-limited progenitor cells represent distinct progenitor population or are an intermediate progenitor state that has yet to be described.

Although luminal populations enriched for steroid receptors can exhibit progenitor activity, whether the steroid receptor positive cells themselves have progenitor activity is not clear. This is because cells isolated with the current complement of cell surface markers do not isolate pure steroid receptor positive cells. This leads to the possibility that cells closely associated with steroid receptor positive cells are the source of progenitor activity. Technical challenges of cell sorting may also contribute to this issue; contaminating cells isolated during the sorting process may have measurable progenitor activity, leading to erroneous conclusions. Steroid receptor expression may be measured using immunofluorescence or immunohistochemistry on sorted fractions, which can also lead to challenges for interpretation of the data among different studies. Further, marker differences exist among mouse strains, with some cell surface markers such as c-kit or CD61 having different ranges of expression on progenitor cells in different models [104, 111]. In addition, culture conditions affect lineage restriction as well as progenitor activity *in vitro* [112]. ERα expression is dependent upon basement membrane components [113], and expression of ERα is rapidly lost in 2D culture making it challenging to directly test ERα progenitor activity *in vitro* [113, 114]. Similarly, responsiveness to progesterone may depend on other cell-cell interactions in addition to matrix. Cultures of primary cells in matrix do not increase the expression of downstream mediators of progesterone signaling, WNT-4 and RANKL in response to stimulation with progesterone [115], whereas culture of primary epithelial cells in microstructures which retain endogenous cellular interactions do [114]. Even utilizing transplantation assays, which are the gold standard for assessing progenitor activity, has proven challenging. Early studies indicated that luminal cell populations had little ability to generate structures within cleared mammary fat pads [80, 81, 105], however, luminal progenitor populations have the ability to grow in mammary fat pad in the presence of Matrigel [104, 110, 116] or under conditions of pregnancy, suggesting that some populations of progenitor cells may have specific growth requirements.

Together, these observations suggest that the cells in the luminal epithelial lineage have greater progenitor activity than previously appreciated. The progenitor cells in this population may require different stimuli to proliferate, including the hormonal environment of pregnancy. Through the use of multiple assays to detect progenitor activity, such as growth in matrix and treatment with combinations of hormones or injection into pregnant recipients, specific progenitor populations within the luminal lineage may be identified. These assays will also clarify the roles that they play in the mammary hierarchy.

Progesterone Effects on Progenitor Populations

In mice, progesterone signaling through PR has been shown to be necessary during puberty and over the course of subsequent estrous cycles for ductal branching and for full alveolar development during pregnancy. In both mice and humans, proliferation of mammary epithelial cells is at its highest during the luteal phase of the menstrual cycle in women or diestrus in mice, when progesterone peaks. The effects of progesterone on cell proliferation in the mammary gland has been examined both in the physiological period of diestrus as well as following exogenous progesterone administration. During diestrus, luminal cells with the cell surface markers CD24$^+$CD49f^{lo} undergo a 3-fold expansion, however the specific luminal progenitor populations that may be affected have not been examined [117]. In addition, stem/basal progenitor cells also undergo a 14-fold increase during diestrus, compared to estrus when estrogen levels are comparatively elevated [117]. To investigate the effects of progesterone on epithelial cell proliferation, mice were treated with exogenous progesterone and proliferating cells were identified using BrdU incorporation. Mammary epithelial cell proliferation occurred in two waves related to PR expression. In the first wave, PR$^+$ cells co-labeled with BrdU, suggesting that PR$^+$ cells themselves proliferate, while in the second wave, PR^{neg} cells incorporated BrdU [43]. Although PR$^+$ progenitor cells have not been identified, progesterone has been shown to regulate the proliferation of neighboring PR^{neg} cells through the secretion of paracrine mediators.

Although PR is expressed only in luminal cells in mice, progesterone coordinates the expansion of both luminal and basal progenitor populations. In luminal cells, progesterone has been shown to increase Rankl, which in a paracrine manner promotes proliferation of neighboring epithelial cells [43, 68, 118]. A paracrine mechanism for PR activity also appears to be conserved in humans. Using a "tissue microstructure"
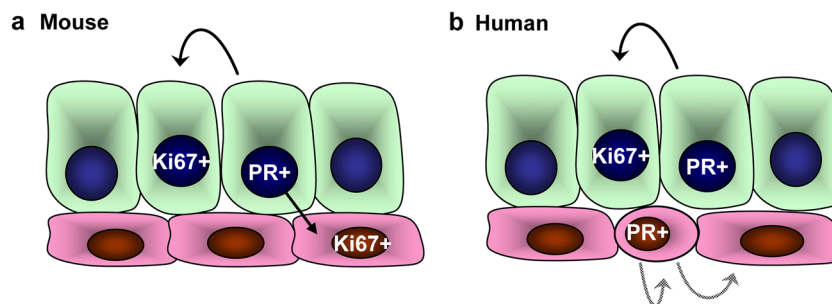
a Mouse

b Human

Fig. 3 Model for progesterone receptor (PR) activity in human and mouse epithelial cell populations. **a** In murine mammary glands, PR acts through a paracrine mechanism to promote the proliferation of cells in both the luminal and basal lineages. PR expression in basal epithelial cells has not been detected. **b** In the human breast, PR is expressed in both luminal and basal epithelial cells. Luminal PR acts through a paracrine

mechanism to simulate the proliferation of neighboring cells. PR is also expressed in basal epithelial cell populations and promotes the proliferation of basal epithelial cells, however it is currently unclear whether PR activity is mediated through a paracrine or autocrine mechanism

approach to study progesterone action on human epithelial cells, treatment with R5020, a PR agonist, led to a significant increase in RANKL mRNA and its cognate receptor RANK [114]. In addition to RANKL, progesterone induces the secretion of another paracrine acting factor, growth hormone (GH). In mammosphere cultures, primary human epithelial cells treated with progesterone significantly upregulated the expression of GH, and treatment of mammary cells with GH stimulated mammosphere formation in a dose-dependent manner [119]. Staining human breast tissue revealed that growth hormone receptor positive cells were found in the luminal epithelial cell layer and were $ER\alpha^{neg}$ and PR^{neg} [119]. This suggests that GH may also function as a paracrine mediator of progesterone action in luminal epithelial cells. Collectively, these results suggest that luminal PR acts in a paracrine manner to promote cellular proliferation of adjacent luminal PR^{neg} cells (Fig. 3a, b).

Basal/ME progenitor activity also appears to be regulated by luminal expression of PR paracrine mediators in mice. Luminal epithelial cells express Wnt-4, while axin2 activity, an indicator for canonical Wnt signaling, is restricted to the basal/ME layer [120]. Axin2 expression increased in response to progesterone and is highest during diestrus [120]. Wnt ligand receptors Lrp5 and Lrp6 are also preferentially localized to the basal epithelium [121, 122], suggesting that upregulation of Wnt-4 in response to PR activity acts in a paracrine manner to promote the proliferation of basal epithelial cells (Fig. 3a). Recently, Cxcl12/Sdf-1α was identified using microarray analysis as a novel paracrine mediator of progesterone action on progenitor populations in the mammary gland [123]. Cxcr4, the cognate receptor for Cxcl12, is expressed by both basal and luminal epithelial cells, and inhibition of this signaling pathway led to reduced mammary repopulating ability [124]. This suggests that this pathway also regulates progenitor activity. Together, these observations show that progesterone coordinates the expansion of the luminal and basal epithelial compartments through the secretion of paracrine mediators. The specificity of the signals induced by the

paracrine mediators may result from the restricted expression of their receptors on either luminal or basal epithelial cells.

While mouse mammary basal epithelial cells do not express PR and rely on paracrine signals from PR^{+} luminal cells to be responsive to progesterone, recent studies have revealed that progesterone directly affects basal progenitor epithelial cells in humans. In breast tissue, cells residing in the basal epithelial layer do indeed express PR [17, 52], and colabeling with basal epithelial cell markers p63 and CK14 revealed that some, but not all, basal/ME cells express PR [17]. However, these PR^{+} basal/ME cells do not co-express $ER\alpha$ [17]. Interestingly, PR^{+} basal/ME cells are found primarily within immature Type I lobules, suggesting that these might be primitive basal progenitor cells [52]. Consistent with this, basal cells which are enriched for bipotent colony-forming activity express high PR and low levels of $ER\alpha$ transcripts [16]. Evaluation of cells sorted by FACS has demonstrated that both PR mRNA and protein is robustly detected in the basal $EpCAM^{lo}CD49f^{+}$ fraction of cells compared with the bulk unsorted cells [17, 52]. This suggests that in the human breast, basal/ME progenitor cells do express PR and are likely responsive to progesterone. Indeed, progesterone treatment of primary human epithelial cells in culture increases mammosphere formation [19, 115]. On a collagen substrate, basal/ME $EpCAM^{neg}CD10^{+}$ cells exhibit enhanced ductal colony growth in response to treatment with progesterone, further indicating that progesterone affects basal progenitor activity [19]. These observations suggest that humans differ from mice in regulation of the basal/ME compartment in response to progesterone. While mice regulate basal progenitors indirectly through luminal-derived paracrine mediators, human basal epithelial cells express PR and respond to progesterone signaling.

Although PR has been detected in human basal epithelial cells, how progesterone actually signals to regulate basal cells is not clear. One possibility is that like luminal epithelial cells, PR acts in a paracrine mechanism to induce the proliferation of neighboring PR-negative basal cells. Consistent with this

notion, WNT-11 expression is enriched within basal epithelial cells compared to unsorted cells and is upregulated in mammospheres following progesterone stimulation [19]. This suggests that WNT-11 could act as a paracrine mediator of PR activity in the basal compartment. However, other studies have suggested that basal PR$^+$ cells may themselves be induced to proliferate in response to progesterone. This is supported by the expression of both PR and BrdU in rare basal cells in 3D cultures [115]. Isolation and characterization of PR$^+$ cells from the basal epithelium is necessary to determine the mechanism of PR action in regulating this cell population. In total, these observations suggest that in humans, PR acts through a paracrine mechanism in luminal cells to expand luminal populations, while PR localized to basal progenitor cells may coordinate basal cell proliferation in either an autocrine or paracrine manner (Fig. 3b). More experiments are necessary to determine whether PR$^+$ cells directly proliferate in response to progesterone in the basal epithelial cell layer in human tissues.

The underlying biological reason for the difference in PR expression patterns between mice and humans is not clear. In humans, PR signaling within the basal epithelium may have evolved with the formation of complex lobules in the breast. Further analysis of luminal and basal PR signaling and regulation of the human breast epithelial hierarchy may clarify these species differences.

Estrogen and Luminal Progenitor Cells

Estrogen is critical for ductal elongation in mice during puberty, but a specific role for estrogen in the regulation of progenitor activity in the adult mammary gland is still evolving. Treatment of mice with the aromatase inhibitor letrozole significantly diminishes repopulating cell numbers [125]. Letrozole inhibits the conversion of androgen to estrogen leading to a decrease in levels of circulating estrogen. Since loss of estrogen signaling leads to reduced PR expression, letrozole also inhibits PR signaling pathways. Although a role for progesterone in stem cell maintenance in the adult gland has been identified [117], the direct effect of estrogen on adult stem cells has not been characterized. This has led to the suggestion that estrogen functionally enhances stem cell activity indirectly through the upregulation of PR, and progesterone primarily maintains adult stem cells.

However, multiple luminal progenitor populations in mice do express ERα leading to the possibility that estrogen could directly influence progenitor cell activity. For example, in both humans and mice, rare ERα^+ cells have been identified that co-label with markers of proliferation, [10–12, 126]. Long-term label-retaining studies, which identify slower cycling stem cells or those that undergo asymmetric division, have also identified ERα^+ cells that retain label [60, 84, 127,

128], suggesting the possibility of an ERα^+ progenitor cell. Human ERα^+ long-term label-retaining cells are also enriched for p21^{cip}, msi-1, and CK19, which have been shown to be putative stem cell markers [128, 129]. Utilizing the cell surface marker c-kit to isolate progenitor cells led to the identification of a subset of c-kit$^+$ cells that are enriched for ERα^+ expression and show high proliferative potential [108]. In addition, 6 % of progenitor-enriched CD24$^+$/CD61$^+$ cells express ERα [56], while sca-1$^+$CD49b^{neg} progenitors are also enriched for ERα expression. Although these latter progenitor cells show limited mammary repopulating ability, they do still form small ductal/lobular structures at low frequencies *in vivo* [104] and exhibit multilineage potential. Interestingly, despite expressing ERα, sca-1$^+$CD49b^{neg} luminal progenitor cells are not affected following ovariectomy, suggesting that these cells are able to survive in a low estrogen and progesterone environment [104]. Together, these observations suggest the existence of a distinct luminal ERα^+ progenitor cell type.

The progeny of proliferating ERα^+ cells have not been identified, however, a population of dividing ERα^+ cells is detected at a 10-fold higher frequency in murine mammary glands during early pregnancy [130, 131]. This suggests that this population is expanding specifically during this time and may be contributing to alveologenesis. Mammary glands from mice expressing a mammary inducible form of Histone H2B fused to eGFP (H2BGFP) have also been used to identify a population of proliferating ERα^+ cells [132, 133]. Following label, H2BGFP was co-expressed with ERα, but these cells did not express PR [133]. Gene expression analysis suggested that the H2BGFP$^+$CD24$^+$CD29^{lo} cells show an intermediate transcription profile between mammary stem cells and luminal progenitor cells [133]. Functionally, H2BGFP$^+$CD24$^+$CD29^{lo} cells are able to generate mammary structures capable of differentiation and lactation when transplanted into pregnant recipients, but cannot be serially transplanted [133]. This is consistent with the notion that these cells are likely limited multipotent progenitors rather than true stem cells. Collectively, these studies suggest that ERα^+ cells may have bipotent progenitor activity that is regulated by the hormonal environment of pregnancy.

Little is known about the activity of human ERα^+ progenitor cells. Using a novel ERE-reporter system, human ERα^+ primary mammary epithelial cells were isolated by FACS and characterized [103]. *In vitro*, these ERα^+ cells could form adherent luminal colonies but were unable to form mammospheres [103]. Further, transplant of isolated ERα^+ cells into humanized glands of immunocompromised mice resulted in only single layer outgrowths that expressed luminal markers [103], suggesting that in humans, ERα^+ cells are luminal restricted progenitor cells. In breast tissues, a population of ERα^+ luminal cells has also been identified that expresses p27$^+$ [134]; p27 was shown to regulate stem and progenitor cells in mice [135, 136]. Low but detectable numbers

of p27^{+} cells that also express the proliferation marker Ki67 are observed during early pregnancy [134], although their functional significance is not clear. Further work is necessary to determine whether ERα[+]p27[+] have progenitor activity during pregnancy in the human breast.

Currently, little is known about the regulation of ERα^{+} progenitor cells. In transgenic mice, Wnt-1 overexpression enhances the proliferation of ERα^{+} cells [130], suggesting that Wnt family members may play a role in expansion of these progenitors. *In vitro*, estrogen-treatment of human mammospheres also leads to the upregulation of Wnt ligands (WNT-3A, WNT-4, and WNT-8A), suggesting that estrogen stimulates progenitor activity through the WNT pathway [19]. Human luminal progenitor cells treated with estrogen show increased acinar colony formation, which is dependent on WNT ligand receptor, LRP6 expression [19]. Identification of the specific regulation of ERα^{+} progenitor cells may provide insight into ERα[+] tumors, particularly those that are resistant to treatment with anti-estrogen therapies.

Collectively, these various lines of evidence suggests that 2 pools of ERα-expressing cells reside within mammary tissues: ERα-expressing PR^{+} cells that regulate progesterone responsiveness, and ERα^{+} cells that have progenitor activity, which may be enhanced during pregnancy or through WNT signaling (Fig. 4).

Parity and Progenitor Cell Populations

During pregnancy, the expanding lobule-alveoli that become terminally differentiated to produce milk are essentially entirely derived from the progeny of progenitor cells. Pregnancy-induced mammary epithelial cells (PI-MECs) were first shown to be the stem/progenitor cells that contribute to the formation of alveoli. Following involution, these cells remain in the gland to be called upon for alveolar expansion during subsequent pregnancies. Isolated PI-MECs have been shown to contain the potential for both ductal- and lobule- limited
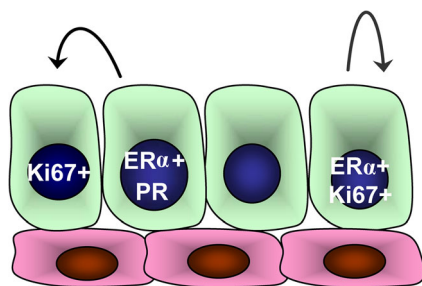
Fig. 4 Model for estrogen receptor alpha (ERα) activity in human and mouse epithelial cell populations. In luminal epithelial cells, ERα and PR are expressed together and act through a paracrine mechanism to stimulate the proliferation of surrounding cells. A population of ERα^{+} cells has also been detected that acts through an autocrine mechanism to expand cells of the luminal lineage

outgrowths [137], and the expansion of PI-MECs during pregnancy suggests that these cells may be hormonally regulated. Indeed, ERα may be important for the maintenance or expansion of PI-MECs since a lactation defect was uncovered when exon 3 of ERα was excised during pregnancy [35].

Although PI-MECs indeed serve as the precursor to alveoli during pregnancy, there have been conflicting reports regarding whether they contribute to both luminal and basal/ME lineages. Using a genetic tracking approach in which WAP-cre mice were crossed with Rosa26-lox-Stop-lox-YFP mice, the lineage contribution of PI-MECs during pregnancy was examined. Following involution, YFP-labeled cells were shown to be restricted to the luminal epithelial layer; the majority displayed an ERα^{neg} sca-1^{lo}CD49b^{hi} luminal progenitor immunophenotype, although ~6 % of the YFP labeled cells displayed the hormone-sensing ERα-enriched sca-1^{hi}CD49b^{lo} phenotype [138]. In contrast, another study also using WAP-cre to genetically label PI-MECs during the second half of pregnancy observed that PI-MECs were primarily localized in the CD24^{+}CD49f^{hi} basal population following involution [139]. The reason for this difference in lineage restriction between the two studies is not clear. One possibility is that WAP is expressed at slightly different time points during pregnancy in each model. If PI-MECs are labeled with YFP late during pregnancy, it is possible that basal progenitor cells have already contributed to the luminal progenitor population earlier in pregnancy and therefore would not have been detected as precursors to alveoli. In fact, contribution of the basal progenitor population to the luminal epithelium during pregnancy is consistent with other lineage tracing studies that demonstrated a basal epithelial cell contribution to alveologenesis [85–87].

The importance of PI-MECs extends beyond serving as the precursors for alveoli during pregnancy. PI-MECs may also serve as the cellular precursors to MMTV-ErbB2 driven tumorigenesis [140–143]. This notion was uncovered by the finding that PI-MECs require cyclin D1 expression for their proliferation. Cyclin D1^{-/-} mice are able to undergo alveologenesis during pregnancy and are particularly resistant to tumor formation in response to overexpression ErbB2 [124, 144]. In addition, transgenic mice that carry a point mutation in cyclin D1, which renders the interactions with cyclin-dependent kinases 4 and 6 catalytically inactive (cyclin D1[KE/KE]), also exhibit similar phenotypes. Whole mounts of glands from cyclin D1[KE/KE] mice show a ductal system that is devoid of side branching [110], reminiscent of the phenotype seen in PR^{-/-} mice. In addition, although normal lobuloalveoli develop in cyclin D1[KE/KE] mice following the first pregnancy, a decline in lobule-alveologenesis is observed in these mice such that by the third pregnancy, lobuloalveoli are absent [145]. Moreover, cyclin D1[KE/KE] mice are also resistant to MMTV-ErbB2 tumorigenesis [110]. These results suggest that the kinase activity of cyclin D1 is necessary for the development and maintenance of luminal progenitor cell

populations, in particular those that are necessary for ductal branching and alveologenesis. Loss of these luminal progenitor cell populations results in the protection from ErbB2-induced mammary tumorigenesis.

The existence of PI-MECs in the human breast is unknown. Analysis of breast tissue from parous and nulliparous women based on cell surface markers has revealed changes in epithelial cell populations. Not surprisingly, compared with nulliparous glands, parous glands exhibit more complexity within the luminal cell populations following pregnancy [52]. These differences in parity-induced epithelial cell populations may contribute to the transcriptional differences observed between the breasts of parous and nulliparous women [146–149]. Following pregnancy, ERα and PR expression is significantly reduced in breasts of parous women compared to nulliparous women [149]. Similarly, mammary glands from parous mice demonstrate significantly reduced levels of ERα and PR compared with age-matched virgin mice, although circulating progesterone levels are similar [150].

One way to differentiate among cell populations that develop during pregnancy may be to identify signaling pathways that are specific to each population. Notch is a highly conserved signaling system that plays important roles in stem cell niches and differentiation processes (for review, [151, 152]). All four Notch receptors are expressed within the mammary gland, and each appears to regulate separate epithelial progenitor niches. Notch1 signaling regulates progenitor cells in the basal lineage [153, 154]. Consistent with this, transgenic mice that overexpress the activated form of Notch1 under control of the MMTV promoter (MMTV/N1IC) demonstrate an expansion of the CD24$^+$CD29^{hi} basal/ME cell cells, which when isolated and grown *in vitro* exhibit predominantly basal differentiation potential [154]. In addition, transplantation of N1IC epithelial cells into cleared mammary fat pads results in ductal-limited outgrowths, with a loss of secondary and tertiary branches and alveologenesis, which was not observed in transplants of epithelial cells from nontransgenic mice [154].

Other Notch family members appear to only regulate luminal progenitor populations. Notch2 has recently been implicated in patterning of the tertiary branches and formation of alveolar clusters [155]. Given the role of progesterone in tertiary branching and alveologenesis, it would be interesting to determine whether Notch2$^+$ cells are also PR$^+$ or in close proximity to PR$^+$ cells. Notch3 (N3IC) mice that contain an activated intracellular form of Notch exhibit a cyclin D1-dependent expansion of CD24$^+$CD29^{lo} luminal progenitors. Transplants show reduced alveoli in pregnant hosts, with a higher proportion of cells expressing ERα [156], suggesting Notch3 may regulate hormone sensing cell populations. Examination of Notch4 signaling defined yet another type of luminal population. Transgenic mice that overexpress INT3 (Notch4) under control of the WAP promoter fail to undergo

secretory alveolar development and are unable to lactate [157]. Mammary epithelial cells of these mice exhibit normal ERα expression, but have significantly reduced numbers of PR$^+$ and Rankl$^+$ epithelial cells [157]. Interestingly, these mice are prone to mammary tumor formation and notably they develop tumors that are strongly ERα^+/PR^{neg} [157]. Both Notch1 and Notch4 are significantly upregulated in response to knockdown of Elf-5, a transcription factor necessary for alveologenesis [158], and loss of Elf-5 results in increased luminal progenitor populations during pregnancy [67]. This suggests that Elf-5 may regulate the expression of specific Notch receptors to allow for the maturation of alveolar progenitor cells. Collectively, these studies suggest that the Notch family signaling pathway is a central player in mammary progenitor cell biology and may differentially stimulate hormonally responsive progenitor cell populations that expand during pregnancy.

Studies from mice have suggested that multiple populations of cells emerge in the hormonal milieu of pregnancy. As we continue to delineate specific cells types that are responsive to either estrogen or progesterone or the combination of the two hormones in mice, this may provide critical insight into human counterparts that have not yet been identified experimentally, including the potential cells of origin for ErbB2/HER2$^+$ cancers. This may also have implications for pregnancy-associated breast cancer as well as malignancies that develop in parous aging women.

Stem Cell Populations and Aging

After menopause, serum plasma estrogen and progesterone levels decline sharply, whereas androgen levels, largely testosterone, are unchanged. This leads to regression of the breast epithelium with a specific reduction in the number of Type II and Type III lobules and an increase in the number of Type I lobules [51]. In addition to regression of the lobules, the stroma also regresses with a generalized reduction in density and an increase in adipose tissue [159–161]. While systemic estrogen and progesterone levels dramatically decrease after menopause, local levels of estrogen have been reported to actually elevate due to the increased activity of aromatase, the necessary enzyme in the biosynthesis of estrogen [162].

The elevated levels of localized estrogen likely have an effect on epithelial cells that are present within the breast. This is because in the human breast, the number of ERα^+ cells increases after menopause, and in some lobules the expression pattern changes to areas of contiguous expression with over 90 % of epithelial cells expressing ERα [163]. A similar increase in ERα^+ cells is also observed in mouse models of menopause generated through ovariectomy [164–166]. Prior to menopause, ERα expressing cells rarely express markers for proliferation [10–12]. However, ERα^+

proliferating cells significantly increase with age in the human breast [163], although overall proliferation in the breast is reduced [163, 167].

Besides changes in ERα expression, other age-related changes in epithelial cell phenotypes have been reported. In a study examining a large collection of normal mammary epithelial cell strains derived from primary breast tissue of aging women, a decrease in myoepithelial cells with an increase in luminal progenitor cells that expressed CD49f, CK19, and MUC1/CD227, as well as c-kit^{+} progenitor cells was observed [168]. In a different study examining 20-month old mice that ceased ovarian cycling, mammary epithelial cells demonstrated diminished colony forming activity *in vitro* suggesting decreased activity and/or fitness of progenitor cells; however, no other assays were conducted to examine luminal progenitor populations [117].

It is well established that with aging, there is a higher incidence of breast cancer [169]. However, what is frequently not appreciated is that there is also a specific increased incidence of ERα[+]/PR[+] tumors with age which, is different from the incidence of ERα[+]/PR[neg], ERα[neg]/PR[+], or ERα[neg]/PR[neg] tumors [170, 171]. The reason for this subtype-specific difference is not clear. Little is known about the complement of mammary gland progenitor cells that are present in the breast and their activity following menopause. While luminal progenitor cells are though to be the precursors to the majority of breast cancers, the mechanism of how they give rise to the different tumor subtypes is not clear. In addition, it is not fully understood whether different types of luminal progenitor cells are the precursors to different type of ERα[+] tumors. Since more than 80 % of breast cancers occur in women over 50 years of age [169], understanding the effect of menopause on progenitor populations and their regulation in response to locally produced estrogen may be beneficial for reducing the incidence of ERα[+] tumors in aging women.

Conclusions

Diverse types of mammary progenitor cells have been identified, some that are specifically induced during pregnancy, which precluded their identification as progenitor cells in earlier studies. In particular, determining mechanisms for regulation of stem and progenitor cells by estrogen and progesterone has been challenging due to the loss of steroid hormone receptors *in vitro* and the intertwined nature of ERα and PR regulation. However, as further progress is made with the identification of additional cell surface markers and sophisticated lineage tracing models, more epithelial cell populations in the human breast may be delineated, including those that are specific to parous women. Although many similarities exist between mouse mammary glands and human breasts, the regulation of the epithelial hierarchy as well as its response

to estrogen and progesterone in women may be more complex than in mice. Understanding these similarities and differences may lead to improved models to study breast cancer origin and treatment.

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