Mammary gland development: cell fate specification, stem cells and the microenvironment

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ABSTRACT
The development of the mammary gland is unique: the final stages of development occur postnatally at puberty under the influence of hormonal cues. Furthermore, during the life of the female, the mammary gland can undergo many rounds of expansion and proliferation. The mammary gland thus provides an excellent model for studying the ‘stem/progenitor’ cells that allow this repeated expansion and renewal. In this Review, we provide an overview of the different cell types that constitute the mammary gland, and discuss how these cell types arise and differentiate. As cellular differentiation cannot occur without proper signals, we also describe how the tissue microenvironment influences mammary gland development.

KEY WORDS: MMPs, Mammary gland, Microenvironment, Progenitor cells, Stem cells

Introduction
The mammary gland, which distinguishes mammals from all other animals, functions to produce and secrete milk in order to nourish offspring. It is also a unique glandular organ in that it reaches full development only after birth. As such, the mammary gland provides a unique model for biologists to study development and organ specificity. The embryonic rudiment of the gland, the anlage, is present at birth and, in response to hormonal cues, begins branching into the fat pad as the female reaches puberty. During the lifetime of the female, the mammary gland undergoes many changes in structure and function, including cyclic expansions corresponding to the hormonal changes induced by the estrous/menstrual cycle, as well as the dramatic changes that occur during pregnancy, lactation and involution. During these different stages, the cells of the mammary gland proliferate, differentiate or apoptose in response to stimuli, giving rise to significant remodeling of the glandular tissue architecture.

Indeed, studies of mammary gland development have offered unique insights into the mechanisms regulating cell fate specification, cell and tissue polarity, branching morphogenesis and the involution of a functional organ. Moreover, many dysregulated pathways and processes observed in breast cancer progression mimic those observed during normal mammary gland development and tissue remodeling; these developmental programs are thus of interest also to cancer biologists. Here, we provide an overview of mammary gland development, highlighting the different cell types that make up the murine mammary gland, with a particular focus on mammary gland ‘stem/progenitor cells’. We also discuss how external factors in the mammary gland microenvironment, such as extracellular matrix (ECM) and cell-cell interactions, influence cell fate and function.

An overview of embryonic mammary gland development
In mice, embryonic mammary gland development occurs between embryonic day (E) 10.5 and E18.5 (Hens and Wysolmerski, 2005; Sakakura, 1987; Veltmaat et al., 2003). It begins when the single-layered ectoderm enlarges to form the mammary lines on E10.5. These lines of cells extend from the anterior limb bud to the posterior limb bud. It is thought that mammary line cells then migrate to the location of the future mammary buds (five pairs in mice) (Hens and Wysolmerski, 2005; Propper, 1978; Robinson, 2007). At E11.5, lens-shaped multilayered ectodermal structures called placodes are observed, rising slightly above the surrounding ectoderm. The mammary placodes then become bulps of epithelial cells that are distinct from the surrounding epidermis. These buds are elevated knob-like structures at E12-E13.5 but sink into the underlying dermis at around E13.5 (Sakakura, 1987; Watson and Khaled, 2008). Mesenchymal cells around the sunken bud condense and become the mammary mesenchyme. Androgen receptor activation in the mesenchyme of male embryos between E13.5 and E15.5 signals for the degradation of the mammary buds (Sakakura, 1987). A second mesenchyme, the fat pad precursor, differentiates beyond the mammary mesenchyme at E14.5 (Sakakura, 1987). Female mammary gland development continues at E15.5, with epithelial cell proliferation and elongation in the bud leading to the formation of a sprout that invades the fat pad precursor. The nipple is formed from epidermal cells overlying the bud, and a lumen is formed in the sprout at E16.5 (Hogg et al., 1983). The sprout then branches into the fat pad, giving rise to the rudimentary ductal tree by E18.5 (Sakakura, 1987).

An overview of postnatal mammary gland development
From birth to puberty, the mammary epithelium originating at the nipple remains quiescent (Fig. 1A). During puberty, and under the control of hormones and other factors, the ductal epithelium of the mammary anlage invades into the mammary fat pad (Fig. 1B) in a process referred to as branching morphogenesis (Lyons, 1958; Nandi, 1958). Highly proliferative terminal end buds, which contain an outer layer of cap epithelial cells surrounding multilayered body epithelial cells located at the invading front of the branch, lead the way (Silberstein and Daniel, 1982; Williams and Daniel, 1983). The invading epithelial cells display some characteristics of mesenchymal cells, suggesting that some degree of epithelial-to-mesenchymal transition (EMT) occurs at the end bud (Kouros-Mehr and Werb, 2006; Nelson et al., 2006). However, unlike tumor cells, EMT genes employed during branching morphogenesis are highly regulated. Indeed, recent work has shown that the transcription factor Ovol2, a master negative regulator of EMT, is required during mammary gland morphogenesis to regulate the expression of EMT genes (Watanabe et al., 2014). The mechanism by which the branching process stops...
The mammary gland development is multistage and occurs after birth. (A) The mammary anlage is present at birth and remains quiescent until puberty. (B) Due to puberty hormones, the epithelial ductal cells expand into the mammary fat pad, led by highly proliferative multilayered terminal end buds (TEBs; inset). The TEBs have an outer layer of epithelial cap cells that surround multilayered epithelial body cells in rodents. (C) The mammary gland of adult virgin mice is filled with epithelial branching structures. The ducts of this structure (inset) contain an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells. (D) Pregnancy is accompanied by different hormonal changes that signal a large expansion of alveolar cells that mature to milk-secreting acini/alveoli during lactation. The alveoli (inset) expand out from the ducts filling the majority of the fat pad. (E) Upon weaning, involution proceeds through cell death and ECM remodeling, giving rise to a state that resembles the resting adult mammary gland. Schematic reproduced with permission from Chuong et al. (2014) and Hennighausen and Robinson (2005).

Cell types of the mammary gland and their specification
As with most glandular tissues, the adult mammary gland is composed of multiple cell types, including epithelial, adipose, fibroblasts, immune, lymphatic and vascular cells, that work together to sculpt and maintain a functional organ. These different cell types have been demonstrated to be of importance at specific stages of mammary gland development.

Epithelial cells
Multiple epithelial cell types can be found in the mammary gland. The mammary bilayer found throughout the adult gland of virgin mice is traditionally described as being composed of apically oriented luminal epithelial cells that line the ducts and of basally oriented myoepithelial cells in contact with the basement membrane (BM) (Fig. 1C, inset). Luminal cells express keratins 8 and 18, whereas myoepithelial cells express keratins 5 and 14 as well as smooth muscle actin that mediates their contractile function. In addition to myoepithelial cells, cell-sorting experiments have identified several putative stem and progenitor cell types in the basal cell population which we will discuss in later sections (Shackleton et al., 2006; Stingl et al., 2006; Visvader and Stingl, 2014).

Throughout puberty and pregnancy, there appear to be unique cell types undoubtedly relating to the mammary functions at these developmental phases. During puberty, for example, cap cells and body cells, which are both specialized epithelial cells, arise in the end bud. Cap cells, so named because they line the end bud forming the cap of the structure (Fig. 1B, inset), contact the surrounding stroma through a thin basal lamina and appear to differentiate into myoepithelial cells that generate a thicker basal lamina (Daniel and Silverstein, 2000; Williams and Daniel, 1983). The body cells, by contrast, fill the interior of the end bud. The central body cells then apoptose to form the lumen, and the remaining body cells differentiate into luminal epithelial cells, giving rise to the ductal epithelium of the adult breast (Fig. 1C, inset) (Hennighausen and Robinson, 2005). During pregnancy, luminal epithelial cells rapidly expand, forming alveoli that are lined with cells primed to secrete milk at parturition (Fig. 1D, inset).

Adipocytes
Fat-filled adipocytes comprise a large proportion of the stromal fat pad in the adult and non-lactating gland. The dense fat pad precursor, which is observable at E14, develops throughout embryogenesis, yet conversion to typical white fat tissue is not observed until two to three days after birth (Sakakura, 1987). During pregnancy and lactation, adipocytes with reduced lipid content are observed, suggesting that this reservoir of fat is necessary for the metabolically demanding process of milk production (Gregor et al., 2013; Hovey and Aimo, 2010). Adipocytes also serve an endocrine function in the gland: they are thought to regulate epithelial growth and mammary epithelium function, as well as to communicate with other cell types in the mammary gland (Bartley et al., 1981; Hovey and Aimo, 2010). As an example, adipocytes secrete vascular endothelial growth factor (VEGF) and probably regulate angiogenesis in the mammary gland (Hovey et al., 2001).
Fibroblasts
Stromal fibroblasts are embedded within the fat pad and are often found in close proximity to the basal side of the epithelial branching tree (Muschler and Streuli, 2010; Sakakura, 1987). Fibroblasts serve many functions, one of which is bi-directional communication with the epithelium during branching morphogenesis, providing instruction in the form of growth factors, proteases and other elements (Howard and Lu, 2014). In vivo and in vitro studies suggest that fibroblasts play an important role in supporting both epithelial cell survival and morphogenesis in the fat pad (Liu et al., 2012; Makarem et al., 2013; Wang and Kaplan, 2012). Furthermore, fibroblasts are believed to be the chief stewards of the mammary ECM: they synthesize a number of ECM components, such as collagens, proteoglycans and fibronectin. Additionally, fibroblasts synthesize many enzymes, such as matrix metalloproteinases, that are capable of not only degrading the ECM but also of releasing the growth factors and cytokines harbored or embedded within the ECM that influence cellular and tissue function (Simian et al., 2001; Wiseman and Werb, 2002). As such, these cells can regulate epithelial cell features and the epithelial cancer phenotype by altering ECM composition or density (Lühr et al., 2012). It is important to note, however, that myoepithelial cells produce copious amounts of laminin-111, which influences many aspects of mammary gland development and function, including tissue polarity (Gudjonsson et al., 2002) and survival (Boudreau et al., 1995) of luminal cells.

Vascular and immune cells
The mammary gland is intercalated with extensive vascular and lymphatic networks present throughout the fat pad. During pubertal mammary gland morphogenesis, the lymphatic network develops in close association with the mammary epithelial tree and blood vasculature (Betterman et al., 2012). Lymphangiogenesis in the mammary gland is probably driven by myoepithelial-derived VEGF-C (Vegfc – Mouse Genome Informatics Database) and/or VEGF-D (Vegfd – Mouse Genome Informatics Database) (Betterman et al., 2012). Immune cells, such as macrophages and eosinophils, are also required for branching morphogenesis, and they are recruited to the branching tips of the epithelium to mediate invasion into the fat pad (Gouon-Evans et al., 2000). Macrophages are also required for epithelial cell death and adipocyte repopulation during involution (O’Brien et al., 2012). Through activation of their serine proteases and degranulation, mast cells are involved in normal mammary branching during puberty, and they accumulate, and possibly activate, plasma kallikrein, thus activating the plasminogen cascade in involution (Lilla et al., 2009; Lilla and Werb, 2010).

Although it is evident that the many cell types of the mammary gland contribute to its structure, development and ultimate function in a dynamic and reciprocal fashion, the vast majority of research has focused on the epithelium alone. This perhaps can be explained because it is the epithelial cells that show dramatic alterations in function and structure in pregnancy and lactation, and because mammatory tumors predominantly arise in the epithelial compartment. In an effort to better understand epithelial behavior, recent research efforts have focused on elucidating the hierarchy of epithelial cell differentiation, as well as identifying factors that can influence mammary gland function.

Mammary gland stem and progenitor cells: dramatic regenerative potential
The mammary epithelium displays dramatic regenerative potential and the ability to undergo many cycles of growth and involution, suggesting that the mammary gland epithelial compartment contains mammary epithelial stem cells, that is, single epithelial cells capable of generating the entire epithelial architecture. The remarkable regenerative capacity of mammary epithelium was first demonstrated in the late 1950s and early 1960s by transplanting small numbers of epithelial cells into mammary fat pads that had been cleared of their epithelial rudiments (Daniel, 1975; Faulkin and Deome, 1960). These cells were able to generate branching epithelia that filled the whole fat pad (i.e. reconstitute the glandular epithelium), and cell populations from these epithelia were able to reconstitute the gland again in subsequent cleared fat pads, demonstrating strong stem cell-like behavior. More recently, it was shown that an entire functional mammary gland can be derived from the progeny of a single cell (Kordon and Smith, 1998; Shackleton et al., 2006; Stingl et al., 2006), supporting the notion that the mammary epithelium contains a stem cell population.

Since these demonstrations, multiple studies have focused on identifying and isolating mammary stem cell (MaSC) populations and defining the differentiation potential of different mammary epithelial populations (Plaks et al., 2013; Shackleton et al., 2006; Spike et al., 2012; Stingl et al., 2006). Many of these studies have used flow cytometry, or fluorescence-activated cell sorting (FACS), to select different epithelial cell populations, which are then tested for reconstitution efficiency by injecting them into cleared mammary fat pads at limiting dilutions (Plaks et al., 2013; Shackleton et al., 2006; Spike et al., 2012; Stingl et al., 2006; Zeng and Nusse, 2010). However, confusion plagues this field of study, as several different epithelial cell populations with stem cell properties have been identified, some of which might act as stem cells during the non-physiological process of cell transplantation but not during normal development and differentiation. To ensure clarity, we will hereafter refer to cells with the ability to reconstitute the glandular epithelium on transplant as mammary gland-reconstituting units (MRUs). These are distinct from cells that, during normal development, give rise to both luminal and myoepithelial cells, which we will refer to as bipotent MaSCs, and cells that give rise to just a single lineage during normal development, which we will refer to as unipotent mammary epithelial progenitors.

Markers and features of mammary gland-reconstituting cells
Using cell surface marker sorting (see Table 1) and the gland reconstitution assay, several different markers for MRUs have been identified. The murine mammary epithelium is typically first isolated by selecting against markers of immune/hematopoietic (CD45), erythrocyte (Ter119) and endothelial (CD31) cells (commonly referred to as the lineage-marker negative, or Linpopulation). These epithelial cells are then sorted for expression of moderate-to-high levels of CD24 (heat-stable antigen), high levels of CD29 (αv-integrin) and/or high levels CD49f (α6-integrin) (Badders et al., 2009; dos Santos et al., 2013; Plaks et al., 2013; Shackleton et al., 2006; Stingl et al., 2006; Zeng and Nusse, 2010). The expression pattern of these markers appears to vary between studies, making quantitative comparisons of the prevalence of different populations difficult (Fig. 2). Nonetheless, recent work suggests that the cells isolated via the sorting approach described above are all myoepithelial: myoepithelial cells expressing smooth muscle actin were the only cells able to repopulate the gland in one recent study (Prater et al., 2014).
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<td>&lt;1/230 CD24&lt;sup&gt;hi&lt;/sup&gt;CD49&lt;sup&gt;low&lt;/sup&gt; 1/3400 CD24&lt;sup&gt;low&lt;/sup&gt; CD49&lt;sup&gt;low&lt;/sup&gt; 1/1400 unsorted cells</td>
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<td>Shackleton et al., 2006</td>
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<td>1/14 Lrp5&lt;sup&gt;+&lt;/sup&gt; Lrp&lt;sup&gt;6+&lt;/sup&gt;</td>
<td>1/485 Lrp5&lt;sup&gt;-&lt;/sup&gt; 1/142 CD24&lt;sup&gt;-&lt;/sup&gt; CD49&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>1/7760 in unsorted population 1/100,000 Lrp5&lt;sup&gt;-&lt;/sup&gt;</td>
<td>All Lrp5&lt;sup&gt;+&lt;/sup&gt; and Lrp6&lt;sup&gt;+&lt;/sup&gt; are K5&lt;sup&gt;+&lt;/sup&gt; or SMA&lt;sup&gt;+&lt;/sup&gt; basal cells but not all are CD24&lt;sup&gt;+&lt;/sup&gt;CD49&lt;sup&gt;hi&lt;/sup&gt;. 40% of Lrp5&lt;sup&gt;+&lt;/sup&gt; are Vwf&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Badders et al., 2009</td>
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<td>11/16 glands Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; Axin2&lt;sup&gt;+&lt;/sup&gt; (50 cells)</td>
<td>5/16 glands Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;-&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; Axin2&lt;sup&gt;-&lt;/sup&gt; (50 cells)</td>
<td>5% of Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; are Axin2&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>1/1400 fetal CD24&lt;sup&gt;med&lt;/sup&gt;CD49&lt;sup&gt;hi&lt;/sup&gt;–matrigel</td>
<td>0/8 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; CD1d&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5% of Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; are Axin2&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>1/44 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt;CD1d&lt;sup&gt;+&lt;/sup&gt;CD1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1/149 unlabeled DNA</td>
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<td>dos Santos et al., 2013</td>
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<td>1/8 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt; CD1d&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1/44 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;-&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt;CD49&lt;sup&gt;hi&lt;/sup&gt;</td>
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<td>1/66 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt;large</td>
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<td>1/132 Lin&lt;sup&gt;-&lt;/sup&gt;CD24&lt;sup&gt;-&lt;/sup&gt;CD29&lt;sup&gt;hi&lt;/sup&gt;</td>
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The reported likelihood of a single cell from this MRU-enriched population in a limiting dilution giving rise to a full glandular epithelium is \( \sim 1/40-1/800 \), depending on the study (Badders et al., 2009; dos Santos et al., 2013; Plaks et al., 2013; Shackleton et al., 2006; Stingl et al., 2006; Zeng and Nusse, 2010). This can be compared with 1/1400 to 1/7600 in the unsorted Lin\(^-\) epithelial population (Badders et al., 2009; Shackleton et al., 2006) or less than 1/3000 in the Lin\(^-\)CD29\(^-\) or Lin\(^-\)CD24\(^-\) MRU-depleted epithelial populations (Shackleton et al., 2006). However, Lin\(^-\)CD24\(^{med-high}\)CD29\(^{high}\)CD49f\(^{high}\) populations are not pure MRU populations; rather, they are MRU-enriched populations, and the complement populations are mostly, but not completely, MRU-depleted (Shackleton et al., 2006).

### Table 1. Continued

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| Plaks et al., 2013 | FACS and gland reconstitution on cells from 7-9-week-old mice (50% matrigel+FGF2) | CD31\(^+\)CD45\(^{Ter119^{-}}\)
CD24\(^{CD49f^{hi}}\)
Ck14\(^{Lgr5^{*}}\) | 5/16 glands Lin\(^-\)
CD24\(^{CD49f^{hi}}\)
Ck14\(^{Lgr5^{*}}\) (10 cells) | 0/16 glands Lin\(^-\)
CD24\(^{CD49f^{hi}}\)
Ck14\(^{Lgr5^{*}}\) (10 cells) | Lgr5\(^+\) cells are 6\% of K14\(^+\) basal cells |
| Prater et al., 2014 | FACS, in vitro culture and gland reconstitution on cells from 10-14-week-old mice (25% matrigel) | CD31\(^+\)CD45\(^{Ter119^{-}}\)
CD49f\(^{hi}\)EpCAM\(^{hi}\) or SMA\(^+\) | 1/57 EpCAM\(^{hi}\)
1/79 cultured CD49f\(^{hi}\) cells
15/18 cultured EpCAM\(^{hi}\) colonies
1/93 SMA\(^+\) | 1/300 EpCAM\(^{hi}\)
1/205 non-cultured CD49f\(^{hi}\)
11/18 cultured EpCAM\(^{hi}\) colonies >1/300 SMA\(^+\) |
| Wang et al., 2015 | FACS and gland reconstitution on cells from 8-12-week-old mice (in 50% matrigel, 20% FBS) | Lin\(^-\)CD24\(^{CD29^{hi}}\)
ProcR\(^{+}\) | 1/12 CD24\(^{CD29^{hi}}\)
ProcR\(^{+}\)
1/15 CD24\(^{CD29^{hi}}\)
ProcR\(^{+}\)
Lgr5\(^{+}\) | 1/70 CD24\(^{+}\)
CD29\(^{hi}\)
1/2000 CD24\(^{+}\)
CD29\(^{hi}\)ProcR\(^{+}\)
Lgr5\(^{+}\) | 0/16 glands CD24\(^{CD29^{hi}}\) ProcR\(^{+}\) Lgr5\(^{+}\) |

Abbreviations: MRU, mammary reconstituting unit; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting; Vwf, Von Willebrand factor; SMA, smooth muscle actin; Ck, cytokeratin; FBS, fetal bovine serum; CD31, CD1d, a glycoprotein typically found on antigen-presenting cells, was identified in the DNA label-retaining population (dos Santos et al., 2013). Adding this marker to the current sorting technique led to a fivefold enrichment of MRUs in the Lin\(^-\)CD29\(^{high}\)CD49f\(^{high}\)CD24\(^{+}\) population to 1/8. However, CD1d\(^-\) cells might not represent the entire stem cell population, given the rarity of the CD1d\(^-\) population (1\% of CD29\(^{high}\)CD49f\(^{high}\)CD24\(^+\)) compared with the frequency of MRUs observed in this study (1/44 Lin\(^-\)CD24\(^{CD29^{hi}}\) versus 1/8 Lin\(^-\)CD24\(^{CD29^{hi}}\)CD1d\(^+\)), suggesting that CD1d\(^-\) cells are a subset of MRUs. This study also did not determine whether the Lin\(^-\)CD24\(^{CD29^{hi}}\)CD1d\(^+\) population was depleted of stem cell activity. Refining MRU surface markers by interrogating the Wnt signaling pathway

Another fruitful approach for isolating MRUs has been to identify elements of the Wnt signaling pathway that are active in mammary development, given the importance of Wnt signaling in stem cell behavior (Reya and Clevers, 2005) and the high activation of the Wnt/β-catenin pathway in DNA label-retaining cells (dos Santos et al., 2013). Indeed, a downstream target of Wnt, the G-protein-
coupled receptor Lgr5, appears to act as a marker of stem cells in several other organ systems (Barker et al., 2010, 2007; Jaks et al., 2008). In the mammary gland, Lgr5+ cells are a subset of the keratin 14+ (K14) basal Lin−CD24−CD49f+ MRU-enriched population and are superior to their parent population in regenerating functional mammary glands (de Visser et al., 2012; Plaks et al., 2013). Supporting a role for Lgr5 in not just gland reconstitution but normal development, loss-of-function and deletion experiments show that Lgr5 and its principal ligand, R-spondin, are necessary for normal postnatal mammary gland organogenesis (Chadi et al., 2009; de Visser et al., 2012; Plaks et al., 2013). The Lin−CD24−CD49f+ Lgr5− depleted population showed no gland-reconstituting ability in one study (Plaks et al., 2013), whereas Lgr5− cells showed rare repopulating activity in another (Rios et al., 2014). However, a third study found the opposite, such that Lgr5− cells that also expressed protein C receptor (ProcR) showed stronger MRU behavior than Lgr5+ cells (Wang et al., 2015).

Other Wnt pathway elements, including the canonical Wnt pathway receptors Lrp5 and Lrp6 (Goel et al., 2012), also appear to mark MaSC-enriched populations (Badders et al., 2009; Lindvall et al., 2009) compared with epithelial cells as a whole. Accordingly, selection for Lrp5, an LDL receptor-related protein, increases gland reconstitution efficiency compared with unsorted epithelial cells (Badders et al., 2009), and Lrp6 is necessary for normal mammary branching invasion in vivo (Lindvall et al., 2009). A very recent study reported that ProcR, which is a Wnt3A target, is also a marker of MRUs: CD24−CD29hiProcR− cells showed much higher gland reconstitution potential than their parent or ProcR-depleted populations (Wang et al., 2015). Furthermore, anthrax toxin receptor 1 (Antrx1), which also acts in the Wnt pathway, might act as a stem cell marker based on in vitro colony-forming assays (Chen et al., 2013). It will be of interest to investigate whether the cell populations characterized by these various Wnt signaling components overlap with the CD1d+ MaSC-enriched populations.

Issues with MRU studies

Although flow cytometry-based studies have been useful for identifying various populations of MRUs and the markers that they express, variations in FACS results and the frequency of MRU detection are observed from study to study (Fig. 2). These might partially be explained by methodological variations, including those in donor age and transplant conditions. As mice undergo puberty and massive changes in epithelial architecture around week 6-8, followed by estrous cycles every 4 days, 6-week-old murine mammary glands might show a very different cellular composition to that from 12-week-old mice. The estrus cycle can also dramatically alter gland reconstitution efficiency, resulting in tenfold higher MRU numbers during progesterone-high luteal phases (Joshi et al., 2010). Likewise, we recently demonstrated that very subtle differences in cell isolation can result in discordance in FACS data (Hines et al., 2014). Furthermore, transplant conditions may dramatically affect reconstitution efficiency, leading to a tenfold increase in gland reconstitution in one study (Spike et al., 2012). For example, the use of matrigel to prevent anoikis, and the injection of epithelia in their native configuration with attached stroma, both strongly increased gland reconstitution efficiency (Spike et al., 2012). Likewise, the culture of myoepithelial cells for one week in vitro was sufficient to massively increase MRU activity (Prater et al., 2014). Given these many potential variables, a consensus technical paper, comparing leading separation methods and providing guidance for future studies, has been published (Smalley et al., 2012).

It should also be noted that, although gland reconstitution studies have demonstrated the remarkable regenerative capacity of MRUs, it is unclear whether normal mammary development relies on the same cellular populations. Gland reconstitution likewise involves injection site wounding, which might induce different fate decisions than those occurring during normal gland development (Plaks et al., 2013; Shackleton et al., 2006; Van Keymeulen et al., 2011).

Mammary gland stem and progenitor cells: insights from lineage tracing

Given some of the issues with FACS-based approaches, lineage tracing of cell populations in transgenic animals is increasingly being employed to define and better understand the stem and progenitor cell populations in the mammary gland. However, such studies have also yielded some conflicting results on the nature and potency of mammary stem and progenitor cells during pubertal development, pregnancy and involution (summarized in Figs 3 and 4).

Pubertal development might rely on bipotent MaSCs

Two single-color lineage-tracing studies concluded that mammary development during puberty predominantly occurs through unipotent progenitor cells, such that all basal cells arise from basal progenitor cells alone, and all luminal cells from luminal progenitors (Fig. 4C) (Van Keymeulen et al., 2011; van Amerongen et al., 2012). By contrast, recent, extensive lineage-tracing studies demonstrated that the pubertal gland contains several different stem and progenitor cell populations, including bipotent stem cells that give rise to both the luminal and myoepithelial cells of the duct (Rios et al., 2014) (Figs 3 and 4). This work used multicolor lineage tracing to mark single cells and their clonal progeny, and confocal microscopy on thick sections to analyze large regions of the ductal tree, thereby allowing for more sensitive detection of rare cell types.

In this study, keratin 5 (K5)-expressing cells labeled at puberty were all found in the basal compartment at the time of labeling, but gave rise to contiguous patches of both luminal and basal cells, suggesting bipotency (Rios et al., 2014). At the initial labeling time point, all K5-labeled cells were in the basal compartment, as detected both by FACS analysis (91% CD29hi, CD24− and less than 1% luminal CD29hi, CD24+) and by histopathology. At 1 week after K5 induction, no single-colored luminal patches were observed, and 65% of clonal patches were solely myoepithelial. After an 8-week chase, 61% of contiguous, single-color patches had both luminal and myoepithelial cells, a result unlikely to be due to adjacent, commonly colored stem or progenitor cells giving rise to single-color patches (Fig. 4A'). Indeed, cells labeled for either K5, K14 or Lgr5 in the prepubertal gland all appeared to give rise to clonal patches of cells from both lineages when assessed either by confocal microscopy or by FACS (Rios et al., 2014). A small subset of dividing K5− cells at puberty were also observed to express markers of multiple lineages, including the basal marker K14, the luminal marker Elf5 and the putative stem cell marker Lgr5, suggesting that this cell population represents a progenitor population.

In addition to these bipotent stem cell populations, Elf5+ luminal cells labeled before puberty gave rise to luminal and alveolar cells alone, suggesting that Elf5+ cells contain a luminal progenitor pool (Fig. 4D) (Rios et al., 2014). This result is mirrored by the earlier finding that keratin 8+ (K8) and keratin 18+ (K18) luminal cells in the prepubertal gland give rise to luminal and alveolar cells alone (Van Keymeulen et al., 2011).
Remodeling in the adult mammary gland of virgin mice
When using lineage tracing in a postpubertal mammary gland of virgin adult mice, contiguous patches of labeled cells were observed in multiple studies (Rios et al., 2014; Van Keymeulen et al., 2011), suggesting that, even in the quiescent gland, extensive cell turnover and remodeling occurs. Using lineage tracing of Elf5⁺ luminal cells to assess adult animals, large patches of luminal cells derived from the same progenitor were observed at 8 weeks, but fewer clonal patches were observed at 20 weeks, consistent with the expansion and slow depletion of luminal progenitors over time (Rios et al., 2014). When using lineage tracing of K5⁺ basal cells in the adult gland, patches of both luminal and myoepithelial cells were observed, including some physically coupled cell pairs, suggesting recent asymmetric division (Rios et al., 2014). Over long periods of time, fewer, larger patches, containing both luminal and basal labeled cells were observed, suggesting massive clonal expansion of these K5⁺ bipotent progenitors during glandular remodeling (Rios et al., 2014). Additionally, over long chases, a shift from mixed or myoepithelial-only clonal patches to predominantly luminal clonal patches was observed with this labeling scheme, suggesting that bipotent progenitors give rise to a population of luminal progenitors that then expands for luminal ductal maintenance (Fig. 4B) (Rios et al., 2014).

Conflicting evidence for stem/progenitor cells in pregnancy and involution
During pregnancy, the glands of virgin adult mice develop an extensive network of secretory alveoli lined by specialized luminal cells. These alveolar luminal cells are believed to be derived from the ductal luminal cells seen in the adult virgin mice, based on lineage-tracing experiments using luminal lineage markers, such as Elf5, K8 or K18 (Rios et al., 2014; Van Keymeulen et al., 2011). In
multicolor labeling approaches, most alveoli were shown to express one to four distinct colors, suggesting that, on average, two luminal progenitors give rise to each alveolus (Fig. 4D) (Rios et al., 2014). When tracing the bipotent K5+, K14+ or Lgr5+ populations, both labeled luminal alveolar and myoepithelial cells are observed. However, multicolor experiments suggest that contiguous alveolar and myoepithelial cells do not arise from the same progenitor. This suggests that, although bipotent progenitors in the adult gland can give rise to alveolar cells, these pass through a luminal progenitor state first (Rios et al., 2014). However, a separate study using lineage tracing of Axin2, which is expressed exclusively in basal cells in the gland of virgin mice, showed that the induction of pregnancy resulted in both basal cells and luminal alveolar cells within the same alveoli being labeled, suggesting that alveolar cells arise from a bipotent stem cell instead of a luminal progenitor (van Amerongen et al., 2012). Additionally, a separate, rare, population of Notch2-expressing luminal cells appears necessary for tertiary branching and formation of alveoli during pregnancy, although the lineage and properties of this cell type have not been fully explored (Šale et al., 2013).

The fate of alveolar cells during involution remains unclear. Using the luminal progenitor cell marker Elf5, one study showed that a new pool of Elf5-labeled luminal progenitors gave rise to alveolar luminal cells in pregnancy, but that this labeled population then died off during involution, to be replaced with a new pool of progenitors for subsequent rounds of pregnancy (Rios et al., 2014). However, a different study showed that descendants of K8- or K18-labeled luminal progenitor cells persist through multiple cycles of pregnancy (Van Keymeulen et al., 2011). A different study found that progeny of secretory alveolar cells, marked according to whey acidic protein promoter (Wap) expression, persist through multiple cycles of pregnancy and involution. These parity-induced epithelial cells are found in the lumens between pregnancy and in the secretory alveoli during pregnancy; they express markers of luminal cells, including Elf5, and appear to not express hormone receptors (Chang et al., 2014).

Potential causes of discrepancies in lineage-tracing studies

The discrepancies between these various lineage-tracing studies can be explained partially by differences in gene-labeling techniques, labeling times and induction agents (Rios et al., 2014). Some studies choose to label fewer total cells by inducing with lower drug concentrations (Van Keymeulen et al., 2011), which aids in the identification of clonal patches, whereas others use stronger induction techniques to label all potential cells of a given lineage (Rios et al., 2014), which compromises some ability to identify clones. In addition, tamoxifen, which is commonly used for cre-lox recombination, is in fact an estrogen blocker, and the doses of this drug used in floxing appear to alter mammary epithelial cell fate decisions (Rios et al., 2014; Visvader and Stingl, 2014).

When a lineage is traced with a single-color label, it is very difficult to determine whether adjacent, labeled cells are derived from a single precursor or from two different ones (Van Keymeulen et al., 2011). The more recent use of multi-color labeling schemes makes this less likely, as the probability of two cells being labeled with the same color is related to the probability of labeling squared times the probability of the two having the same color (1/16 for four-color labeling), but it is still possible that some single-color patches
were not clonal. Although lineage tracing studies appear to be superior for identifying the normal developmental potential of various mammary gland stem and progenitor cell populations, further studies are needed to reconcile the relationship between the K5−, K8−, K14−, Lgr5−, Axin2−, CD1d− and parity-induced mammary epithelial cell populations (Chang et al., 2014; dos Santos et al., 2013; Plaks et al., 2013; Sale et al., 2013; Van Keymeulen et al., 2011; van Amerongen et al., 2012; Wagner et al., 2002).

The differentiation of mammary gland cell types
Lineage tracing-based studies have thus revealed that, during mammary gland development and homeostasis, bipotent stem cells in the prepubertal gland give rise to both luminal and myoepithelial cells, possibly by passing through a unipotent progenitor state. The mechanisms governing the differentiation of mammary progenitors and/or their progeny into luminal, alveolar or myoepithelial cells are not well understood, but recent transcriptional profiling studies coupled with mouse knockout models are very useful in providing new insights into these cellular differentiation pathways. Here, we summarize the factors known to act on epithelial cell differentiation in the murine mammary gland.

Luminal progenitor cell differentiation
During pregnancy, luminal cells or luminal progenitors give rise to alveolar epithelial cells, and this differentiation is driven by a number of factors. The transcription factor Gata-3, for example, is known to be a crucial regulator necessary for luminal epithelial differentiation from luminal progenitors (Asselin-Labat et al., 2007; Kouredes et al., 2006). It has been demonstrated that β3-integrin/CD61 is a marker of luminal progenitor cells (Asselin-Labat et al., 2007, 2011); using transgenic models it was shown that Gata-3 expression is necessary for the differentiation of the CD61+ population into luminal cells and for alveolar development in pregnancy (Asselin-Labat et al., 2007). Furthermore, induction of Gata-3 in a MaSC-enriched population drove differentiation to an alveolar luminal phenotype, defined by Wap and β-casein (Csn2 − Mouse Genome Informatics Database) expression (Asselin-Labat et al., 2007). These studies indicate that Gata-3 is a crucial regulator of commitment and maturation of the luminal epithelial lineage.

The massive hormonal changes in puberty, menstruation and pregnancy profoundly alter breast structure, yet luminal progenitor cells themselves are believed to be hormone insensitive (Asselin-Labat et al., 2010; Yoshi et al., 2010). The effects of hormones on MaSCs and progenitors are thus believed to occur via paracrine signaling from adjacent cells. Indeed, recent studies have shown that, in pregnancy, progesterone induces mature luminal cells to signal to MaSCs via RANK Ligand (Tnfsf11) in a Stat5a-dependent manner (Obr et al., 2013). In line with this, using a floxed Stat5a/b allele and mammary-specific Cre expression, Yamaji et al. (2009) showed that Stat5a/b was required for alveolar differentiation but not for ductal branching. It was shown also that RANK Ligand induces expression of the transcription factor Elf5 in CD61+ luminal progenitors (Lee et al., 2013). When Elf5 expression is blocked, the population of K5−K14−p63+ MaSCs expands and overaccumulates in the mammary gland of pregnant mice, and the mammary glands of these animals fail to lactate (Chakrabarti et al., 2012; Lee et al., 2013; Oakes et al., 2008). Accordingly, Elf5 conditional knockouts exhibit a complete block in alveolar differentiation (Choi et al., 2009).

Stat5a signaling induced by myoepithelial cell-derived neuregulin 1 (Nrg1) is also necessary for MaSC activity (Vafaizadeh et al., 2010; Forster et al., 2014). Nrg1 is expressed in myoepithelial cells in a p63-dependent manner and is detected by luminal cells via the Erbb4 receptor. This signaling induces the expression of the Stat5a targets Elf5 and cyclin D1, necessary for luminal and luminal progenitor cell function (Forster et al., 2014). The expansion of progenitor cells also requires Myc, suggesting that this proto-oncogene has a physiological role in mammary development (Moumen et al., 2012). Finally, Lgr5 appears to act as a receptor for an alveologenic signal in pregnancy (de Visser et al., 2012; Plaks et al., 2013; Rios et al., 2014), and when either Lgr5 or its ligand, R-spondin (de Lau et al., 2011), are absent, secretory alveoli fail to develop (Chahi et al., 2009; Chakrabarti et al., 2012).

Functional mammary differentiation is also mediated by contact of mammary epithelial cells with the ECM via laminin-111-ligation of integrins. Luminal cells express β-casein in response to laminin-111 signaling, independent of cell-cell contact (Streuli et al., 1991, 1995). It was further demonstrated that there is a transcriptional enhancer (BCE1) in the bovine β-casein promoter that is responsive to prolactin and ECM signaling (Schmidhauser et al., 1992), and laminin-111 was shown to be the specific protein mediating the ECM signaling (Streuli et al., 1995). Molecular characterization of the BCE1 enhancer revealed two essential regions that bind C/EBP-β (Cebp − Mouse Genome Informatics Database) and Stat5 (Myers et al., 1998). Importantly, the enhancer had to be integrated into the genome in order to be activated by laminin-111 and prolactin, suggesting that chromatin structure probably plays an essential role in activation of this element (Myers et al., 1998). In line with this finding, later studies determined that sustained activation of Stat5 mediated by laminin-111 is essential for chromatin remodeling and β-casein transcription (Xu et al., 2009).

Myoepithelial cell differentiation
Mammary myoepithelial cells are identified by the expression of specific proteins, including keratin isoforms and contractile proteins [reviewed by Moumen et al. (2011)]. However, the transcription factors that mediate the differentiation of a basal progenitor into a myoepithelial cell in vivo are not well understood. In the human breast, p63 has been identified as a potential mediator of the basal phenotype (Yalcin-Ozuysal et al., 2010). The differentiation of myoepithelial cells is dependent on serum response factor (Srf), as demonstrated by studies of the knockout mouse model of the Srf coactivator myocardin-related transcription factor A (MRTF-A; Mk1 − Mouse Genome Informatics Database), which reveal that MRTF-A is required for myoepithelial differentiation upon lactation (Li et al., 2006; Sun et al., 2006). Additional transcription factors, such as Slug and Smad3, as well as Notch signaling, have been implicated in the basal phenotype [reviewed by Moumen et al. (2011)]; however, it is clear that still more studies are required to understand the mechanisms underlying myoepithelial differentiation.

The microenvironment as a regulator of mammary gland development and homeostasis
The maintenance and differentiation of the various mammary gland cell types is also dependent on the features and properties of the local tissue microenvironment, in particular those of the surrounding ECM. The importance of the ECM and stroma in mammary gland development and function were proposed several decades ago ([Williams and Daniel, 1983], reviewed by Varner and Nelson (2014)). Chimeric recombination models [reviewed by Nelson and Bissell (2006)] demonstrated the power of the stroma
to influence the developing epithelium. When mammary epithelium was recombined with mammary mesenchyme, not surprisingly, the outcome was that of a typical mammary ductal tree. However, mammary epithelium recombined with salivary gland mesenchyme resulted in structures that resembled salivary gland epithelium (Sakakura et al., 1976). On the other hand, outgrowths of salivary epithelium in contact with mammary mesenchyme resembled a mammary gland ductal tree and could even become competent for lactation and respond to hormonal stimuli (Cunha et al., 1995). These and other studies revealed that the epithelial component is highly malleable and that cell fate and tissue function are strongly influenced by the stromal component of the gland.

More recent studies (Bruno and Smith, 2012; Bussard and Smith, 2012) demonstrated, amazingly, that non-mammary cells and even human cancer cells can be reprogrammed and incorporated into mammary outgrowths that are capable of self-renewal upon further transplantation into a murine cleared mammary fat pad (Boulanger et al., 2013, 2012, 2007). In these studies, co-injection with mammary epithelial cells was required, indicating that a ‘stem cell niche’, which includes the microenvironment and ECM components, is also necessary for signaling from bona fide mammary epithelial cells. How does the tissue microenvironment support growth, cellular differentiation and development of the mammary gland? As we discuss below, an intricate signaling network exists between the epithelium and its microenvironment, and includes signaling in response to mechanical forces and cell-cell contact, signaling from the ECM molecules, stromal-derived growth factors and cytokines, and the activities of proteolytic enzymes in the microenvironment.

The role of cell contacts and mechanical forces
Mammary cells are tightly connected both to each other and to their surrounding environment, and they require this connection for normal function. Myoepithelial cells, for example, are anchored to other myoepithelial cells and luminal epithelial cells via desmosomes, and to the BM via hemidesmosomes (Adriance et al., 2005; Pandey et al., 2010). Similar properties, mediated by the integrin α6β4 complex, are exhibited by mammary luminal cells when cultured in laminin-rich, three-dimensional (3D) ECM gels (Weaver et al., 2002). In vivo, the two layers of the duct express different cell-cell contact mediators (Chanson et al., 2011; Huebner et al., 2014; Mroue et al., 2014) and cell-ECM connections (Brizzi et al., 2012); as a result, the layered structure of the duct self-organizes when luminal and myoepithelial cells are mixed in 3D cultures (Chanson et al., 2011; Gudjonsson et al., 2002; Huebner et al., 2014; Runswick et al., 2001). Furthermore, these connections are necessary for normal cell function: mammary cells in the lactating gland require E-cadherin to survive (Boussadja et al., 2002). Together, these tight connections between mammary cells allow the transmission of mechanical forces and biochemical signals throughout the growing epithelium and allow collective cell motion (Gjorevska and Nelson, 2010), in which mechanically active cells push or pull more passive cells along with them (Rørth, 2012).

ECM-mediated control of mammary gland development
The ECM is a major regulator of epithelial architecture and function. Within the mammary gland, myoepithelial cells reside on a specialized layer of ECM, termed the basement membrane (BM). Myoepithelial and stromal cells synthesize ECM components, such as laminins, collagens, fibronectin and proteoglycans, which are incorporated into the ECM as well as the BM. These matrices provide not only physical support for correct tissue architecture but are also essential biochemical signaling networks that guide cellular fate and function of the gland. The BM also anchors the myoepithelial cells by ligating cell surface molecules into position and by organizing the basal side of the myoepithelium, through myoepithelial-secreted laminin-111 and hemidesmosomes (Inman et al., 2011).

Studies using microenvironmental protein microarrays have started to uncover the ECM molecules that influence cell fate decisions in mammary progenitor cells (LaBarge et al., 2009; Lin et al., 2012). These studies revealed that laminin-111 regulates mammary progenitor self-renewal, whereas other combinations of ECM proteins result in growth, differentiation and apoptosis (LaBarge et al., 2009). As discussed above, myoepithelial cells are a major source of laminin-111 of the BM, and it is this laminin that is essentially responsible for establishing luminal epithelial cell apical-basal polarity (Gudjonsson et al., 2002; Weir et al., 2006). Furthermore, because of their position and function within the mammary tissue, myoepithelial cells are thought to act as tumor suppressors in the adult gland (Adriance et al., 2005; Bissell and LaBarge, 2005; Pandey et al., 2010; Sternlicht and Barsky, 1997; Sternlicht et al., 1997).

Other stromal ECM proteins are important for instructing the development of the mammary arboreal structure. For example, recent studies show that collagen I fiber orientation in the mammary fat pad is a patterning cue for mammary branch orientation during development (Brownfield et al., 2013). These collagen I fibers appear to be oriented prior to mammary branching morphogenesis, indicating that epithelial architecture might be pre-patterned in the stroma of the pubertal mammary gland. Such structural components, which support tissue architecture, are often overlooked as regulators of morphogenesis, but we now understand that correct tissue architecture and stiffness of the ECM are essential components of normal development, differentiation and function within the mammary gland (Bissell et al., 1982; Maller et al., 2013; Schedin and Keely, 2011). Future studies of the stroma in mammary gland development would undoubtedly provide additional clues into the role of stroma in breast cancer initiation and progression.

Proteases are essential regulators of mammary gland morphogenesis and differentiation
Proteolytic action remodels the ECM and stroma, and releases sequestered growth factors and cytokines. Not surprisingly, proteases are essential for mammary gland development and function. For the epithelial rudiment to fill the fat pad upon hormonal signals during puberty it is essential for the cells to take on an invasive phenotype and for proteinases, in this case matrix metalloproteinases (MMPs), to pave the way by remodeling the ECM (Talhouk et al., 1991). To understand the role of proteinases in the mammary gland, a number of transgenic mice in which a particular proteinase (or proteinase inhibitor) is silenced or overexpressed have been developed. Although compensation by different MMPs makes it difficult to exclusively define the function of a single proteinase in vivo, the transgenic mouse models have provided insight into the importance of proteinases during mammary gland development and function. Additionally, 3D physiologically relevant organotypic culture models have shed light on the importance of both the catalytic activity and the newly discovered functions of the non-catalytic domains of proteinases in mammary gland development (Correia et al., 2013; Kessenbrock et al., 2013; Mori et al., 2013).

Several different classes of proteases are necessary for proper mammary gland development and function. Proteinases provide...
local environmental signals to promote EMT and invasion. For example, we showed more than two decades ago that Mmp3 has a crucial role in remodeling the mammary gland in involution (Talhouk et al., 1991), and that its aberrant activity in 3D collagen gels leads to EMT and a premalignant phenotype (Locher et al., 1997). Others have shown that cathepsins play important roles during involution and apoptosis of the mammary gland (Sloane, 2012; Watson and Kreuzaler, 2009). Serine proteinases are important as well: mice deficient for the serine protease plasminogen have difficulties supporting lactation, due to a disruption in factors that control involution (Green et al., 2006).

Other studies identified the serine protease plasminogen activator kallikrein as being important for adipocyte differentiation in the mammary gland. Kallikrein is thought to play a role in the plasminogen cascade of remodeling the fibrin-rich pre-adipocyte stromal ECM (Selvarajan et al., 2001).

The most widely studied enzymes in the context of mammary gland development and differentiation are metalloproteinases, which include MMPs and the ‘a disintegrin and metalloproteinases’ (ADAMs). MMPs consist of a family of over 20 zinc-dependent proteinases synthesized as latent enzymes that must be activated post-translationally (Kessenbrock et al., 2010). Their activity is modulated by endogenous inhibitors referred to as tissue inhibitors of metalloproteinases (TIMPs) (Murphy, 2011). Collectively, MMPs can degrade all protein components of the ECM, and we now know of a number of other proteins, including E-cadherin (Locher et al., 1997). Over the past decades, many transgenic mouse models have been developed to investigate MMP function in development and cancer (Gill et al., 2010; Wiseman and Werb, 2002). Interestingly, the Mmp14 knockout is the only single MMP gene knockout model that is lethal. Overexpression of Mmp14 (Ha et al., 2001) or Mmp3 (Sympson et al., 1994) in the mammary gland leads to excessive side branching and advanced alveolar morphogenesis (Fata et al., 2004). Increased levels of the active form of Mmp3 not only causes supernumerary branching in the mammary gland but is responsible for causing production of reactive oxygen species (ROS), changes in splicing of Rac1, EMT and genomic instability (Radisky et al., 2005), which precedes mammary tumor development (Sternlicht et al., 1999). Cross-in Mmp3-overexpressing mouse model with a mouse overexpressing Timp1 decreases the detrimental effects of Mmp3 overexpression/activity. These data support the notion that Mmp3 activity in the mammary gland is important for branching morphogenesis, but indicate that unscheduled activity alters the mammary tissue microenvironment. Furthermore, the fact that Mmp3 activity could be tempered by Timp1 emphasizes the importance of a balance between enzymes and inhibitors for proper tissue development and homeostasis, and might explain why TIMPs are also risk factors in mammary cancer.

Other studies, using a mouse model in which Mmp3 has been genetically suppressed, have revealed a role for Mmp3 in adipocyte differentiation (Alexander et al., 2001). Timp1 overexpression gives rise to a phenotype similar to that seen following Mmp3 depletion, supporting the idea that MMP activity, in particular Mmp3 activity, is important for adipocyte differentiation during mammary gland involution. In studies in which slow-release pellets of Timp1, 2, 3 or 4 were implanted into mouse mammary glands, it was shown that Timp1, 3 and 4 inhibited ductal elongation, most likely via inhibition of MMP activity. However, Timp2 had an elongation promoting effect (Hojilla et al., 2007). On the surface, the Timp2 result seems contradictory, but increased levels of Timp2 most likely raise the levels of Mmp2 activation by increasing the formation of a tertiary complex (Mmp14-Timp2-Mmp2) that is responsible for Mmp2 activation in vivo (Ellerbrock and Stack, 1999; English et al., 2006).

To unravel the mechanisms underlying these observations in vivo, cell culture studies have been most informative. These studies have revealed that active Mmp3 increases ROS levels, leading to genetic instability and EMT (Radisky et al., 2005), which contribute to tumor development in the aging gland. More recently, non-proteolytic functions of Mmp3 (within the hemopexin domain) were found to be crucial for EMT and invasion during branching morphogenesis (Correia et al., 2013). Proteomic analysis of Mmp3 hemopexin domain binding partners revealed, surprisingly, that the chaperone heat shock protein, 90β (Hsp90β) interacts specifically with the hemopexin domain of Mmp3 in the extracellular space and that this interaction is crucial for Mmp3 function. Other work has implicated the hemopexin domain of Mmp3 as a regulator of Wnt signaling and MaSC activity (Kessenbrock et al., 2013). In line with this, mice deficient for Mmp3 exhibit decreased numbers of MaSCs and diminished mammary-reconstituting activity. Conversely, in the same study it was shown that Mmp3 overexpression elevated MaSC function. Together, these observations suggest that Mmp3 activity is necessary for the maintenance of MaSCs.

Three-dimensional cell culture models have also been essential for elucidating the many functions of MMPs in normal and malignant mammary glands (Barcellos-Hoff et al., 1989; Petersen et al., 1992). For example, investigations into the role of Mmp14 in mammary development were hampered due to the lethality of this gene knockout. However, using micropatterned gels of collagen I, it was revealed that the hemopexin domain of Mmp14 is important for sorting mammary epithelial cells to points of branching, thus highlighting that the non-proteolytic domains of Mmp14 also are essential for proper branching morphogenesis (Mori et al., 2009). In addition to the hemopexin domain, recent evidence indicates that the short intracellular domain of Mmp14 is crucial for epithelial cell invasion (Mori et al., 2013). Using engineered Mmp14 constructs in which different domains were deleted, we discovered that only the short intracellular domain of Mmp14 is needed to rescue branching morphogenesis in Mmp14-deficient cells, despite the fact that this sequence does not contain kinase activity. This deficiency is compensated for by integrin β1, which interacts with the short cytoplasmic domain of Mmp14. It is required for interaction with the ECM, and for transducing the extracellular signals needed for epithelial cells to invade. These recent observations provide strong evidence that MMPs are important not only for ECM remodeling but also for the microenvironmental signaling necessary for morphogenic programs within the mammary gland.

Combining mouse models with physiologically relevant 3D models of human cells will allow further investigations into the mechanisms of action of these proteinases. This information is important not only for understanding tissue development and mammary function, but also for identifying new targets for cancer therapy, as researchers focus on the new functions of the non-catalytic domains of MMPs as regulators of tissue morphogenesis and tumor development (Dufour and Overall, 2013; Rodriguez et al., 2010; Strongin, 2010). The non-catalytic domains are targetable using antibodies or blocking peptides directed specifically against those domains (Basu et al., 2012). Such biological pharmaceuticals directed to domains other than the catalytic domains of these proteinases surely will provide more specificity in therapy.

**Conclusions**

Like all other organs, the mammary gland is composed of many specialized cell types that carry out mammary functions, with
interconnected signaling occurring between the different cellular compartments. With its unique developmental mode occurring essentially after birth and its remarkable regenerative properties, the mammary gland provides a superb model for investigating developmental programs, stem and progenitor cell properties, and the stability of the differentiated state.

Early serial transplant experiments and transgenic mouse models have shed light on the identity and role of MaSCs and progenitor cells. More recently, lineage-tracing experiments have identified multiple different, and somewhat conflicting, populations of stem and progenitor cells. Despite the discrepancies, these studies have begun to fine-tune our understanding of MaSCs and how they drive development of the gland and maintain homeostasis of the resulting arboreal architecture. Additionally, the results of a number of recent studies are revealing the role MaSCs play in the many cycles of proliferation and apoptosis needed both to expand and maintain the gland form and functions during pregnancy and to return it to a quiescent state after involution.

Many questions remain. Even the most fundamental puzzles—for example, what signals drive cells down a particular lineage path; and why does this go wrong in cancer?—are not clearly delineated. Of course, interactions and signaling between cells are important, but a major driver of differentiation appears to be signaling from the tissue microenvironment, especially from the ECM in general and the BM in particular. For decades, the ECM and BM were thought to be the inert ‘bricks and mortar’ of a tissue, simply providing physical structure. We now know that correct tissue architecture, including the organization and stiffness of the ECM, together with the reservoir of growth factors, cytokines and proteinases within which the stem cell niche nestles, are essential for mammary glandular tissue to develop and function properly.

Competing interests
The authors declare no competing or financial interests.

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References
Bruno, R. D. and Smith, G. H. (2012). The mouse mammary microenvironment redirects mesoderm-derived bone marrow cells to a mammary epithelial progenitor cell fate. Stem Cells Dev. 21, 948-954.


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alveolar cell fate decisions, differentiation, involution, and mammary tumor formation. Stem Cells 28, 928-938.


