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REVIEW

The dynamics of murine mammary stem/progenitor cells

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Abstract **The stem/progenitor cells in the murine mammary gland are a highly dynamic population of cells that are responsible for ductal elongation in puberty, homeostasis maintenance in adult, and lobulo-alveolar genesis during pregnancy. In recent years understanding the epithelial cell hierarchy within the mammary gland is becoming particularly important as these different stem/progenitor cells were perceived to be the cells of origin for various subtypes of breast cancer. Although significant advances have been made in enrichment and isolation of stem/progenitor cells by combinations of antibodies against cell surface proteins together with flow cytometry, and in identification of stem/progenitor cells with multi-lineage differentiation and self-renewal using mammary fat pad reconstitution assay and *in vivo* genetic labeling technique, a clear understanding of how these different stem/progenitors are orchestrated in the mammary gland is still lacking. Here we discuss the different *in vivo* and *in vitro* methods currently available for stem/progenitor identification, their associated caveats, and a possible new hierarchy model to reconcile various putative stem/progenitor cell populations identified by different research groups.**

Keywords mammary stem cell, cell hierarchy

Introduction

The adult murine mammary stem/progenitor cell has been a subject of intensive review by many research groups in recent years (Smith and Medina, 2008; Bruno and Smith, 2011; Fridriksdottir et al., 2011; Tiede and Kang, 2011; Visvader and Smith, 2011; Fu et al., 2014). Much of this increased interest in this subject is derived from the possible roles of these stem/progenitor cells in human breast cancer pathogenesis. For example, the mammary stem cells (MaSCs) may contribute to oncogenesis process in the MMTV-wnt-1 mouse model as the preneoplastic mammary tissue of this mouse model harbors an expanded pool of functional stem cells (Shackleton et al., 2006; Vaillant et al., 2008). Furthermore, recent studies have shown evidence for the luminal progenitors as the cell of origin for basal-like breast cancer (Lim et al., 2009; Molyneux et al., 2010). Our own recent findings also indicate that MaSCs are vulnerable targets for estrogen mimics such as bisphenol A during early developmental stages of mammary gland (Wang et al., 2014). Thus, various stem/progenitors may function as the initiating cells for different subtypes of breast cancer, and understanding the cell hierarchy within the normal mammary epithelia will help us gain insight into how breast cancer arises and develops (Visvader, 2011).

The presence of different types of cells within the mammary gland was first illustrated by ultrastructural examination where four types of epithelial cells were identified: primitive small light cells (SLC), undifferentiated large light cells (ULLC), differentiated large light cells (DLLC) and large dark cells (LDC). The LDC correspond to differentiated luminal and myoepithelial cells while the SLC have been described as having stem cell-like features based on the presence of mitotic chromosomes, lack of any specialized organelles, the ability to undergo symmetric and asymmetric division, and their disappearance in serial transplants that have reached growth senescence (Smith and Chepko, 2001; Smith et al., 2002). However, whether SLCs are truly MaSCs awaits future validation as to date there are no known specific markers identifying mammary epithelial stem cells.

In 2006, two landmark papers published in *Nature* identified a stem cell-enriched population on the basis of a

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high level of either CD49f (alpha 6 integrin) or CD29 (beta 1 integrin) and a moderate level of CD24 (heat stable antigen) on the cell surface (Shackleton et al., 2006; Stingl et al., 2006b). These cells can generate full ductal-lobular outgrowths and complete differentiation during pregnancy in the mammary fat pad reconstitution assay, indicating multi-lineage differentiation. Further, these cells can generate secondary outgrowth in serial transplants, indicating self-renewal *in vivo*. Both multi-lineage differentiation and self-renewal are hallmarks of stem cells. Thus, these studies suggest that the mammary epithelium is maintained by a rare population of multipotent stem cells. In 2011, a study using the *in vivo* genetic labeling techniques of lineage tracing revealed that multipotent stem cells only present in rudiment mammary gland in fetus prior to birth, and after birth the mammary gland development and homeostasis was controlled by two different lineage-restricted unipotent stem cells (van Keymeulen et al., 2011). Most importantly, this study raised the concern about using the mammary fat pad reconstitution assay to gauge the normal developmental potential of the mammary stem cells in their natural environment. In the years followed, various mouse models using the *in vivo* genetic labeling technique were employed to identify putative stem/progenitor cells under physiology conditions as reviewed below (also shown in Table 1). Despite these efforts, it is unclear whether unique cell populations identified in these different mouse models actually portray identical or different cell types for specific lineage(s), and the cell hierarchy within the murine mammary epithelium remains unsolved. In this review, we discuss the different experimental systems used to define the murine mammary epithelial stem/progenitor cells, and we also put forward a new cell hierarchy model that might help consolidate various stem/progenitors identified by different mouse models.

Stem/progenitor cell enrichment and isolation

In analogous to the hematopoietic system, the enrichment of mammary stem cells was initially attempted with the side population (SP), i.e., cells that are able to efflux the dye Hoechst 33342 (Welm et al., 2002; Alvi et al., 2003) and the stem cells antigen 1 (Sca-1) positive cells (Welm et al., 2002). Although SP and Sca-1$^+$ cells were reported to be able to generate ducts and alveoli, subsequent identification of stem cell-enriched basal compartment was characterized by CD29^{hi}CD49f^{hi}CD24$^+$Sca-1$^-$ with SP accounted for less than 10% in the population (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006b; dos Santos et al., 2013). This CD29^{hi}CD49f^{hi}CD24$^+$ cell population was isolated from the endothelial (CD31) and hematopoietic (CD45 and TER119) lineage-depleted mammary cells (Lin$^-$ MC), and was considered as the multipotent stem cells (also termed as mammary repopulating unit or MRU) because they display the stem cell hallmark features of multilineage differentiation and self-renewal when assessed by the mammary fat pad reconstitution assay. More recently, by using an inducible histone 2b promoter linked to a GFP report (K5tTA/H2B-GFP) mouse model, Hannon's group found that selection of cells with high levels of GFP (presumably these are slowly dividing cells) within the CD24$^+$CD49f^{hi} basal compartment resulted in further enrichment of MRU cells (dos Santos et al., 2013). Subsequent transcriptome profiling led to the discovery of CD1d, a cell surface glycoprotein, to be the marker of choice for isolation of highly enriched stem cells. This research group reported an MRU frequency of about 1/8 for CD1d$^+$ CD24$^+$CD29^{hi} cells compared with the 1/44 for total CD24$^+$CD29^{hi} cells. However, validation of this specific marker by others has not been reported.

Whereas the MaSCs can be defined by their ability to

Table 1 Stem/progenitors within the murine mammary gland identified by different model systems

Model system	Stem or progenitors	Niche	Lineage specification		MRU frequency	Reference
			in vivo labeling	Transplant		
WC/R26-*lacZ*	PI-MEC CD24$^+$CD49f^{hi}	Alveolar terminal ducts	Basal*	Bipotent	–	Wagner et al., 2002 Matulka et al., 2007
Tg11.5kb-GFP	s-SHIP$^+$	Cap cells	Basal	Bipotent	1/48	Bai and Rohrschneider, 2010
		Alveolar buds	Basal	Bipotent	1/79	
Axin2^{CreERT2}	*Axin2$^+$*	Fetal bud	Luminal	–	–	van Amerongen et al., 2012
		Pre-pubertal basal	Basal	Bipotent		
		Pubertal TEBs	Basal or luminal	–		
		Adult basal	Basal (virgin)	Bipotent		
		Adult basal	Bipotent (preg)	–		
Lgr5–EGFP–Ires–CreERT2	*Lgr5$^+$*	PND1	Luminal	–	–	de Visser et al., 2012
		PND12	Basal	Bipotent	1/1781	
Lgr5-EGFP	*Lgr5$^+$*	Nipple area	Basal	Bipotent	1/3.7	Plaks et a. 2013
K5tTA/H2B-GFP	CD24$^+$CD49f^{hi} GFP$^+$CD61$^-$	Tips of TEB	Basal	Bipotent	1/33	dos Santos et al., 2013
MMTVrtTA/H2B-GFP	CD24$^+$CD29^{lo} GFP$^+$	Luminal layer	Luminal*	Bipotent	1/350	Kaanta et al., 2013

Bipotent: give rise to both basal and luminal lineages; *Results based on flow cytometry analysis; Pregnancy-preg; PND-postnatal development day.

establish a fully functional mammary tree *in vivo*, the identification of progenitors, which are characterized by their ability to replicate and differentiate but not self-renew, requires different types of assays. In particular, the mammary colony forming cell assay was used for progenitor identification (Stingl et al., 2006a). The Lin^-MC characterized by $CD24^{hi}CD49f^+CD29^{lo}$ were identified as the luminal restricted progenitors, which were found to be able to form epithelial colonies when cultured *in vitro* on a feeder layer but failed to regenerate a new gland in the repopulation assay (Stingl et al., 2006a). Further, this $CD24^{hi}CD49f^+CD29^{lo}$ population can be separated into luminal progenitors and differentiated luminal cells based on the expression or absence of CD61 (β3 integrin), respectively (Asselin-Labat et al., 2006). However, Stingl's group recently observed that CD49b (α2 integrin) was a more selective marker of luminal progenitors than the CD61 as they found up to 47% of progenitors are of $CD61^-$ phenotype (Shehata et al., 2012). This discrepancy is due to the lack of general application of CD61 in different mouse strain as the most recent review indicated that CD61 can delineate progenitor cells from mice on a FVB/N background, but not those of C57BL/6 (Fu et al., 2014). The study by Stingl's group also revealed that the luminal cell compartment can be separated into three distinct cell populations based on the expression of Sca1 and CD49b (Shehata et al., 2012). Specifically, $Sca1^+CD49b^+$ luminal cells are ER^+ progenitors (or ductal-restricted progenitors) expressing higher transcript levels of luminal differentiation transcripts such as ER, FoxA1 and Gata3 and lower levels of Krt5 and Krt14, while $Sca1^-CD49b^+$ luminal cells are ER^- progenitors (or alveolar progenitors) that demonstrates lower levels of Krt18 and ER, and high levels of Elf5 and Lmo4, which are transcription factors that specify alveolar cell fate, as well as milk components Lalba and Mfg-e8. The $Sca1^+CD49b^-$ cells are mature ER^+ luminal cells (Shehata et al., 2012).

Although different stem/progenitors can be distinguished by cell surface markers listed above, it is worthy to note that these markers are not epithelial nor stem/progenitor specific and can only be used for isolation of stem/progenitor-enriched populations for subsequent function analysis. Our own analysis (Dong et al., 2013) on regenerated glands derived from MaSCs of GFP mice (thus all epithelial cells are GFP^+) showed that the cell surface markers of CD24 and CD49f can only be used for separation of cell populations between luminal and basal cells (Fig. 1A), thus enrichment of stem/progenitors illustrated above was a result of getting rid of most non-epithelial cells and the separation of two cell lineages. There was actually no or limited enrichment for stem/progenitors within each of the epithelial lineages. Therefore, further enrichment of stem/progenitors from the basal and luminal cell compartments require more specific markers.

Stem/progenitor cell identification

As mentioned above, identification of stem/progenitor cells has mainly relied on the mammary fat pad reconstitution assay (for stem cells) and the *in vitro* colony formation assay (for the progenitors). However, recently published lineage tracing data indicated a more restricted unipotent cell fate for MaSC tested in situ than those tested in transplantation systems (van Keymeulen et al., 2011; van Amerongen et al., 2012; Plaks et al., 2013). These findings raise significant concern about using the cleared fat pad transplant assay as the gold standard to define stem cell property, and whether lineage tracing should become the new standard to measure stem/progenitor cell potential under normal physiological conditions. Here we discuss the details of all available *in vivo* and *in vitro* assays and their associated caveats.

The mammary fat pad reconstitution assay

This assay involves clearance of endogenous epithelium from 21-day old recipient mice, the transplant of mammary fragments or cells of interest into the cleared fat pad, and assessment of the regenerated mammary outgrowth 8–10 weeks later in virgin or pregnant recipient mice (Deome et al., 1959). It has been widely used as the gold standard to define stem cell properties in murine mammary glands. In fact, most of our current understanding about the mammary epithelial cell hierarchy was based on the knowledge obtained from this cleared fat pad transplant assay. In particular, a common progenitor was proposed for both myoepithelial and luminal lineages in the adult mammary glands (Shackleton et al., 2006; Stingl et al., 2006b). Yet, a recent study that utilized lineage-tracing assays pointed to two different unipotent luminal and myoepithelial progenitors responsible for mammary gland expansion during puberty and pregnancy (van Keymeulen et al., 2011).

One explanation for this discrepancy is that transplantation unlocks the regenerative potential that is normally not utilized *in vivo*. This may be due to the microenvironment of the fat pad used for transplantation as previous published studies reported that even progenitor cells of a completely different origin could be reprogrammed to adopt a mammary stem cell fate (Boulanger et al., 2007; Booth et al., 2008). More recently, Fridriksdottir et al. (2011) also argued that "the lack of a positive outcome in a transplantation assay might not necessarily mean that the cells lack multipotency or are terminally differentiated but rather that the assay in question is not suitable for revealing their true characteristics." Thus, different regenerative capability among different cell populations may merely reflect their ability to respond to the niche signals in the transplantation system. The discovery of $CD24^+CD29^{lo}$ luminal cells capable of developing mammary outgrowths upon transplantation after co-injection with

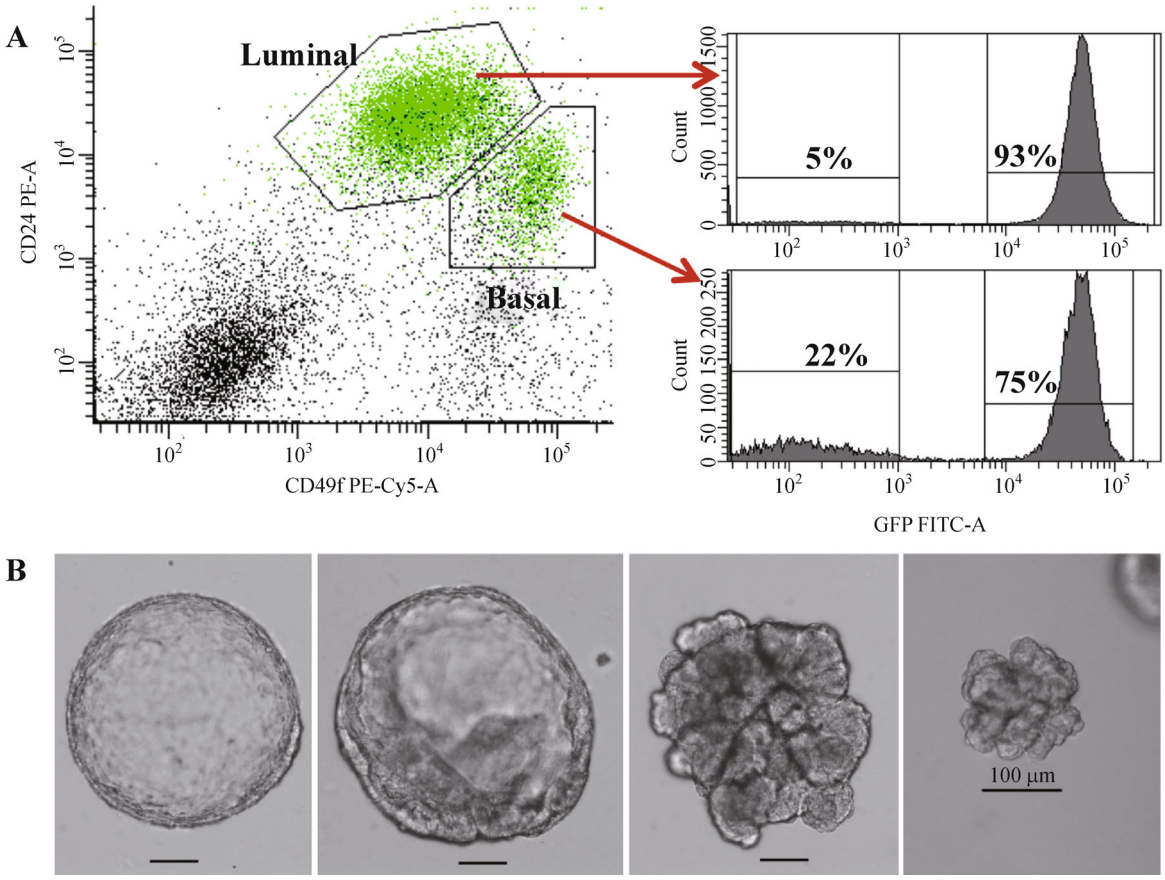

Figure 1 (A) Regenerated glands in virgin mice by GFP positive MaSCs showing non-epithelial cells (black) in the luminal ($CD24^{hi}CD49f^{+}$) or basal ($CD24^{+}CD49f^{hi}$) gates together with epithelial cells (green). Right panels show the histograms of %GFP negative (stromal) and positive (epithelial) cells in each gate. (B) Morphological distinct 3D-ECM organoids derived from single spheres formed by FACS sorted luminal ($CD24^{hi}CD49f^{lo}$) cells from C57BL6 mice aged 3 to 4 months. Scale bars, 100 µm. (Reprinted from Dong et al., Mammospheres from murine mammary stem cell-enriched basal cells: clonal characteristics and repopulating potential. Stem Cell Research, 2013 (10): 396-404 with permission from Elsevier.)

Matrigel (Jeselsohn et al., 2010; Vaillant et al., 2011), and the $H2B\text{-}GFP^{+}$ cells within the $CD24^{+}CD29^{lo}$ luminal compartment displayed increased MRU frequency when recipient mice were pregnant (Kaanta et al., 2013) all support this argument. Thus, mammary fat pad reconstitution assay may not be able to provide an accurate reflection of the actual stem/progenitor cell potency under physiologic conditions. On the other hand, it is still possible that this assay may accurately predict the intrinsic regenerative potential of stem/progenitors, thus can help delineate the relationship among these various stem/progenitors within the mammary epithelium.

in vitro colony formation assays

One widely accepted assay used as an *in vitro* readout for luminal progenitors was the mammary colony forming cell (Ma-CFC) assay, which involves plating luminal cells on a feeder layer of irradiated NIH3T3 cells and counting the epithelial colonies after 7–9 d culture (Stingl, 2009). However, we recently discovered that this assay varies with mouse strain. Unlike the C57BL/6 or FVB mice, luminal cells from Balb/c mice gave rise to not only fewer colonies (at least 10-fold less than that of C57BL/6) but also much smaller colonies (Wang et al., 2014). It is currently unknown what the cause for this strain variation, but this finding indicates that this Ma-CFC assay may not be adequate to evaluate mammary luminal progenitors of various mouse strains.

In analogous to the neurosphere assay used in neural stem cell research, we recently developed an *in vitro* sphere formation and differentiation (SFD) assay serving as an alternative in lieu of the mammary fat pad reconstitution assay (Dong et al., 2013). Our SFD assay involves mammosphere formation in suspension culture from sorted basal or luminal cells and sphere differentiation in 3-dimensional Matrigel culture. Spheres that differentiated into solid 3-dimensional organoids originated from MaSCs, and spheres that differentiated into hollow 3-dimensional organoids originated from luminal progenitor cells. Unlike the transplantation assay, where outgrowths derived from basal and

luminal progenitors exhibit similar morphology and contain both luminal and basal lineages (Shehata et al., 2012), the *in vitro* organoids formed by the basal and luminal cells in our SFD assay were distinctly different (Dong et al., 2013). More importantly, we also observed morphologically different organoids formed by the luminal cells (Fig. 1B), suggesting possible different progenitors within the hierarchy of the luminal lineage (see Discussion in the section "The mammary epithelial cell hierarchy").

Other *in vitro* colony formation assays involves directly plating primary or sorted cells in Matrigel culture for observing similar morphological organoids as our SFD assay (Stingl et al., 2006b; Joshi et al., 2010). However, our own tests with directly plating sorted cells into Matrigel culture, bypassing the mammosphere formation stage, revealed potential growth of contaminated non-epithelial cells such as preadipocytes in the luminal or basal populations from the flow cytometry sorting, which subsequently exerted unpredictable effect on the colony formation in the Matrigel culture system. Our SFD assay also employs the Matrigel culture on a low attachment plate to prevent cell attachment on the bottom of plastic plates, which might lead to reduced number of organoids formed. Similar to the caveats associated with the mammary fat pad reconstitution assay, all these *in vitro* culture systems reflect the capability of the stem/progenitors to form distinct colonies under these artificial conditions, and may not represent their true behavior *in situ*.

in vivo genetic labeling

The pitfalls outlined above for the repopulation assay as well as the *in vitro* colony formation assay emphasize the need to use methodologies that preserve tissue architecture for cell hierarchy delineation. In the last couple of years, there is a surge of using various mouse models to tag stem/progenitors in the mammary glands with genetic labeling (Table 1). However, a consensus of the epithelial cell hierarchy is lacking. Moreover, the use of same cytokeratin Krt5 promoter for lineage tracing in different studies generated different conclusions with the earlier study indicating that Krt5 marks unipotent long-living myoepithelial cell progenitors (van Keymeulen et al., 2011) and the more recent study indicating that Krt5 identifies bipotent stem cells (Rios et al., 2014). This discrepancy may arise from some inherent limitations of lineage tracing (Kretzschmar and Watt, 2012). For example, both transgenic and knock-in strains can be prone to insertion-site effects that affect the level and timing of gene expression, and ultimately determine whether cre expression is triggered in the stem or progenitor subset (Rios et al., 2014). Despite these associated caveats, identification of stem/progenitor cells using *in vivo* genetic labeling represents a better option for the measurement of the true fate of stem/progenitor cells *in situ*, and thus provides the best evidence for the reconstruction of cell hierarchy within the mammary epithelium.

The mammary epithelial cell hierarchy

In recent years, several mouse model systems with various *in vivo* genetic labeling techniques were reported to tag different stem/progenitors (Table 1). To facilitate comparison among these different model systems, we will briefly discuss each model system first before mapping these different stem/progenitors on a proposed new cell hierarchy model.

The WC/R26-*lacZ* mouse model

In the WAP-CRE and Rosa26-lox-stop-lox-*lacZ* (WC/R26-*lac*Z) double transgenic mice, the transient expression of Cre recombinase (driven by the whey acidic promoter) during pregnancy permanently activates the ubiquitously expressing transgene, Rosa26-*lacZ*. By using this model, Smith's group identified a cell population in parous hosts that displayed the functional characteristics of a lobule-limited progenitor population. They termed these cells as parity identified mammary epithelial cells (PI-MEC) (Wagner et al., 2002). These cells were multipotent, self-renewing, and capable of retaining their activity through serial transplantations (Boulanger et al., 2005). Although they were found to locate at terminal ducts within alveolar units, they were exclusively $CD49f^{hi}$ cells (Matulka et al., 2007).

The s-SHIP model

The product of the novel *s-ship* gene was initially identified in embryonic and hematopoietic stem cells, but not in differentiated cells (Tu et al., 2001). Rohrschneider et al. subsequently generated a transgenic mouse model (Tg11.5kb-GFP) showing the 11.5-kb s-SHIP promoter-driven expression of GFP in many stem cell populations including fetal mammary bud cells (Rohrschneider et al., 2005). Later on, they found that GFP^+ cells only exist in cap cells and pregnancy-associated basal alveolar buds (Bai and Rohrschneider, 2010). These GFP^+ cells had a basal cell phenotype of $p63^+$, SMA^+, $K14^{lo}$, $laminin^+$, $E\text{-}cad^-$, $K8^-$, PR^-, $CD49f^{hi}$, $CD29^{hi}$, $Sca\text{-}1^{lo}$, $CD133^-$, $CD61^+$, and were also positive for proliferation markers of Ki67 and BrdU. In addition, GFP^+ cap cells and alveolar bud cells also had high regenerative capability with an MRU frequency of 1/48 and 1/79 respectively, and both cell populations exhibited self-renewal upon serial transplantation. These characteristics are consistent with the phenotype and stem cell nature of mammary epithelial cell fractions isolated by others (Stingl et al., 2006). Further fractioning of GFP^+ cells within the $CD24^+CD49f^{hi}$ basal compartment showed that $lin^-CD24^+CD49f^{hi}GFP^+$ cap cells possessed a significantly higher regenerative potential than $lin^-CD24^+CD49f^{hi}GFP^-$cells (1/71 vs. 1/333 MRU

frequency), suggesting that these GFP$^+$ cells may represent a more enriched MaSC fraction. However, it is worth to note that transplantation done by this group actually was assessed after mating, which would allow parity-activated progenitors to generate sizable outgrowths (Kaanta et al., 2013), thus the increased MRU frequency might in fact reflect the sum of different stem/progenitors.

The *Axin2^{CreERT2}* mouse model

Axin2 is a direct target gene of the Wnt/β-catenin pathway (Jho et al., 2002; Lustig et al., 2002), and *Axin2*$^+$ cells can regenerate mammary outgrowths in cleared fat transplant experiments (Zeng and Nusse, 2010). More recently, Nusse's group employs the *Axin2^{CreERT2}* mouse model for lineage tracing of the *Axin2*$^+$ cell during mammary gland development (van Amerongen et al., 2012). Depending on the developmental stage, *Axin2*$^+$ cells contribute differently to basal and luminal epithelial cell lineages of the mammary epithelium. *Axin2*$^+$ cells mark luminal progenitors in fetus (between E12.5 and E17.5), the basal lineage in prepubescent gland (between postnatal development day 14 and 16); independent basal and luminal precursors in puberty that give rise to basal and luminal lineage independently; preferentially basal cells in adult glands; and both basal and luminal cells in the alveoli that arise during the first pregnancy.

The *Lgr5-EGFP* knockin mouse model

Lgr5, a downstream target of Wnt, was identified as a marker of adult stem cell populations in the small intestine, colon, stomach, and hair follicle (Barker et al., 2008, 2007, 2010). Using this model, Werb's group found only 14% of ducts had *Lgr5*$^+$ cells and they were all localized to the nipple side (Plaks et al., 2013). Within the basal population, *Lgr5*$^+$ cells had high efficiency in generating mammary glands using limiting dilution transplantation assay (1 MRU per 3.7 cells). Depletion of *Lgr5*$^+$ cells during pubertal mammary gland development impaired ductal elongation and decreased the number of terminal end buds (TEBs) despite that *Lgr5*$^+$ cells and their lineage-specific progeny are absent from the TEBs. Earlier lineage-tracing experiments using an *Lgr5-CreER* line also confirmed that *Lgr5*$^+$ cells give rise mainly to myoepithelial cells in pubertal mammary glands (van Keymeulen et al., 2011). However, studies with *Lgr5-EGFP-Ires-CreERT2* mouse model showed that *Lgr5*$^+$ cells at postnatal development day 1 (PND1) contributed to luminal lineage in the postnatal and adult glands, yet *Lgr5*$^+$ cells at PND12 and later switched their fate and confined to basal lineage only (de Visser et al., 2012). This finding was in accordance with the observation of *Axin2*$^+$ cells in prepubescent glands between PND14 and PND16, where they are restricted to basal lineage (van Amerongen et al., 2012). This provides additional independent evidence for Wnt responsive cells to switch the fate at PND12 since both *Axin2* and *Lgr5* are downstream targets of Wnt. Together, these findings indicate that *Lgr5*$^+$ cells in postnatal development are mainly restricted to basal lineage, and they are required during physiologic pubertal development. Given these unique properties of *Lgr5*$^+$ cells, we suspected that *Lgr5*$^+$ cells represent the progenitors that control the basal lineage in puberty mammary gland development (Fig. 2).

The K5tTA/H2B-GFP mouse model

In this system, the expression of the H2b histone (linked to GFP) is regulated by a tetracycline responsive element (TRE) and a tet-controlled transcription activator (tTA) under the endogenous keratin K5 promoter. Thus, the transgene expression can be repressed in a doxycycline-dependent fashion, allowing isolation of slowly dividing cells with retained nuclear GFP signal. By using this model, the Hannon group showed that the H2b-GFP^{hi} cells within the CD24$^+$CD29^{hi} basal compartment yield higher mammary gland reconstitution ability (MRU frequency: 1/33) (dos Santos et al., 2013) than the CD24$^+$CD29^{hi} cell population (1/70) reported earlier (Shackleton et al., 2006). These cells are located at the tips of TEBs and are CD61$^-$. Gene expression analysis of these cells showed enrichment of G protein-coupled receptors and pathways involving Wnt/β-catenin signaling. These authors thus concluded that H2b-GFP^{hi} label-retaining cells represent a subset of MaSCs.

The MMTVrtTA/H2B-GFP mouse model

Unlike the K5tTA/H2B-GFP model used above, studies using the MMTVrtTA line revealed no evidence that label retention enriches for MaSC activity in the basal compartment though the authors acknowledged that the length of the 'chase' in their experiment might have been insufficient to isolate a mammary population of interest (Kaanta et al., 2013). Instead, they found that H2B-GFP$^+$ cells within the CD24$^+$CD29^{lo} luminal compartment contain a population of multipotent progenitors that give rise to outgrowths upon transplantation. Although the regenerated glands were much smaller than glands derived from CD24$^+$CD29^{hi} cells, they contained all mammary lineages and were able to produce milk. More interestingly, the reconstitution efficiency of these H2BGFP$^+$CD24$^+$CD29^{lo} cells scored much higher when recipient mice were pregnant (1/350) than those kept as virgin (1/1600). These cells share transcriptional characteristics with MaSC and luminal-restricted progenitors and are also CD14$^+$, which was shown to be an alveolar phenotype *in vitro* (Stingl et al., 2006b). These data suggest that the H2BGFP$^+$CD24$^+$CD29^{lo} cells are multipotent pregnancy-activated progenitors. However, unlike the PI-MEC identified earlier (Wagner et al., 2002), these cells do not give rise to serially transplantable mammary outgrowths, indicating

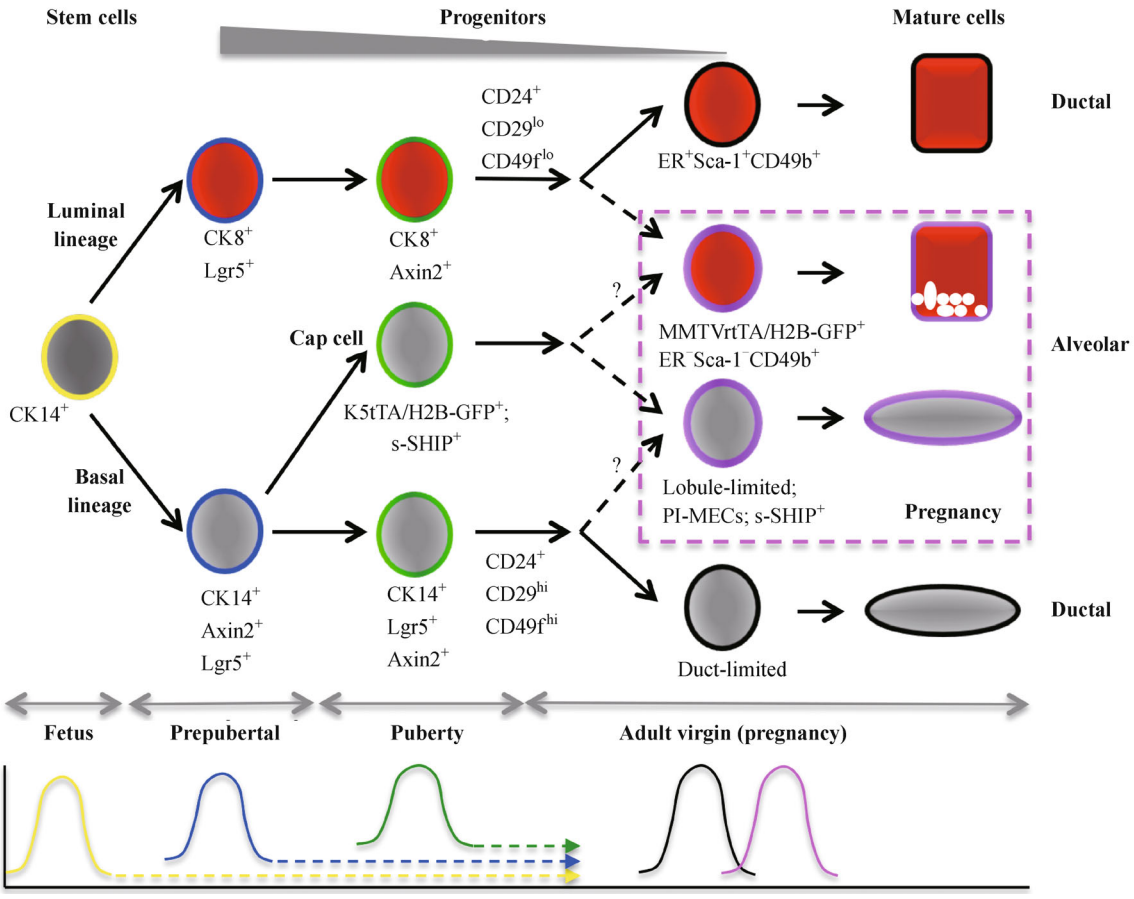

Figure 2 Schematic model of the murine mammary epithelial hierarchy: bipotent stem cells in fetus give rise to luminal (filled in red color) and basal (filled in gray color) progenitors in prepubertal and pubertal glands. The luminal and basal lineage can be further divided into ductal (shaped with black outline) and alveolar-restricted (shaped with purple outline) progenitors. The luminal and basal alveolar progenitors are to be activated during pregnancy. The triangle bar indicates progenitor cells with decreasing multi- or uni-potency during the development from prepubertal to adult glands. Curves represent different stem/progenitors that are predominant in the epithelial cell population at different developmental stages. The dashed lines indicate potential possibilities. Cell markers labeled under each stem/progenitor cells correspond to published studies (see Text for details).

limited self-renewal. Because these cells are identifiable in early puberty, they may arise during or before pubertal development and remain dormant until pregnancy.

Our hypothetical hierarchy model

Previously, two different models were proposed to explain the cell hierarchy in the mammary epithelium with one model assumes the existence of independent ductal and lobular progenitors, which can both give rise to basal and luminal lineages, and a second model proposes an early separation between basal and luminal lineages (Visvader and Smith, 2011). However, the first model is based on the evidence that limiting dilution transplantation experiments with the primary mammary cells showing the existence of lobule-limited or duct-limited progenitors and both can give rise to basal and luminal lineages in transplants (Bruno and Smith, 2011). As we discussed earlier that the mammary fat pad reconstitution assay may not necessarily reflect the true cell fate of stem/progenitors *in situ*, it is thus important to only look into evidence derived from *in vivo* genetic labeling for cell hierarchy delineation. Here we propose a new cell hierarchy model (Fig. 2) that can be built on top of the second model described above. First, multiple lines of lineage tracing evidence (e.g, the *Axin2* and *Lgr5* mouse models) all point to the existence of two distinct lineages that remain as separate arms throughout the mammary gland development (van Keymeulen et al., 2011; van Amerongen et al., 2012; Plaks et al., 2013). We predict that separation of these two lineages may occur as early as in the fetus since *Axin2*$^+$ cells in fetus marked luminal progenitors only. Second, both the basal/myoepithelial and luminal arms can be further divided into the ductal and alveolar sublineages in adult glands. Thus, there will be two basal progenitors (ductal vs. alveolar) and two luminal progenitors (ductal vs. alveolar) in the adult glands with progenitors responsible for alveolar development only activated by pregnancy.

The presence of two basal progenitors (ductal vs. alveolar)

with the alveolar basal progenitor only activated during pregnancy in our model may help accommodate the duct-limited and lobule-limited progenitors reported previously (Bruno and Smith, 2011). Because the duct- or lobule-limited progenitors were identified based on transplantation studies using limiting dilutions of unfractionated mammary epithelial cells, we believe these progenitors must reside in the basal compartment because only basal cells had high engraftment efficiency in the mammary fat pad transplant system (Stingl et al., 2006b). Our model specifying that these two basal progenitors can only give rise to their specific lineages also matches well with the cell fates described for duct-limited and lobule-limited progenitors. Our model may also help explain the limited self-renewal capability observed for MaSCs obtained from pregnant mice (Asselin-Labat et al., 2010) as this pregnancy-associated MaSC pool expansion may result from increased alveolar progenitors, which exhibit limited self-renewal when transplantation was done in virgin recipient mice. Future studies can validate this by conducting serial transplantation experiments in pregnant recipient mice. Additional evidence for the presence of more than one progenitor within the basal compartment comes from in vitro culture of human breast epithelial cells where myoepithelial cell-only colonies were found (Stingl et al., 1998). The cells give rise to this particular myoepithelial cell colonies could be our ductal basal progenitors. Unfortunately, it has proven difficult to segregate different progenitors within the basal compartment as they show a common cell surface phenotype and gene expression profile (Stingl et al., 2006b). If our model holds true, we believe the s-SHIP$^+$ cells reside in the pregnancy basal alveolar buds are the alveolar basal progenitors since these cells are characterized by basal phenotype and had high regenerative potential upon transplantation when assessed in pregnant recipient mice. Thus, we may be able to use gene signatures derived from s-SHIP$^+$ cells to fractionate the basal compartment into ductal and alveolar progenitors for the adult glands.

The presence of two luminal progenitors (ductal vs. alveolar) in our model will allow us to accommodate the ER$^+$ progenitor (Sca1$^+$CD49b$^+$) and ER$^-$ progenitor (Sca1$^-$CD49b$^+$) identified recently (Shehata et al., 2012). These two progenitors were isolated from the luminal compartment characterized by EpCAM^{hi}CD49f^{lo}, thus in agreement with our assignment of luminal lineage for these two progenitors. Because the Sca1$^+$CD49b$^+$ luminal cells express higher levels of ER and FoxA1, which have been demonstrated to be essential for ductal but not lobular morphogenesis during mammary gland development (Bernardo et al., 2010), the authors suspected that these ER$^+$ cells are probably ductal-restricted progenitors (Shehata et al., 2012). Likewise, they found that the Sca1$^-$CD49b$^+$ luminal cells express high levels of Elf5 and Lmo4, transcription factors that specify alveolar cell fate (Sum et al., 2005; Oakes et al., 2008), and milk components Lalba and Mfg-e8 (Maningat et al., 2009), they thus deduced that these ER$^-$ cells could be the alveolar progenitors. Thus, these ER$^+$ and ER$^-$ progenitors correspond to the ductal and alveolar luminal progenitors outlined in our model.

When viewed structurally, our model thus has two alveolar progenitors with one contributes to basal lineage (give rise to mature alveolar myoepithelial cells) and the other contributes to luminal lineage (give rise to mature alveolar luminal cells). This can help accommodate the PI-MEC and MMTVrtTA/H2B-GFP$^+$ cells discussed above as both cell populations were activated by pregnancy, yet the former exhibit CD24$^+$CD49f^{hi} basal phenotype while the latter display CD24$^+$CD29^{lo} luminal phenotype. In addition, recent lineage tracing study using K5-rtTA/TetO-cre/R26R-Confetti glands at mid-pregnancy showed that alveolar and myoepithelial cells within a given alveolus often expressed different fluorescent proteins (Rios et al., 2014), indicating different progenitors for each cell type, supporting the presence of two alveolar progenitors proposed in our model.

Although we propose that there are ductal and alveolar progenitors under each lineage, it is possible that the basal and luminal alveolar progenitors may derive from a common ancestor. Studies using *Axin2*^{CreERT2} mouse model showed that labeled basal and luminal alveolar cells can be detected side by side within the same cell cluster during multiple pregnancies (van Amerongen et al., 2012). In contrast, studies with the K5-rtTA/TetO-cre/R26R-Confetti glands showed separate progenitors for the basal and luminal cells within the same alveolus (Rios et al., 2014). Together, these findings imply a common ancestor for the basal and luminal alveolar progenitors, and van Amerongen et al. implicated that it should be one bipotent Wnt/β-catenin-responsive cell that can generate an entire alveolar structure. We suspect that this common ancestor could be derived from the cap cells in the puberty gland. The cap cell region of TEBs is believed to be one of the MaSC niches (Kenney et al., 2001). Recent studies using the K5tTA/H2B-GFP mouse model revealed that H2B-GFP$^+$ label retaining cells were located at the tips of TEBs, had high regeneration potential, and were enriched in Wnt/β-catenin signaling (dos Santos et al., 2013). Another piece of evidence connecting the alveolar progenitors with the cap cells is the s-SHIP mouse model where s-SHIP was only expressed in the cap cells and the alveolar bud cells (Bai and Rohrschneider, 2010). Unfortunately, lineage tracing was not conducted in this model. Future studies tracing the s-SHIP$^+$ cells in cap cells at the puberty will help answer whether these cells eventually give rise to alveolar cells during pregnancy.

In our model, we also propose two progenitors (basal vs. luminal) in the prepubertal glands and three progenitors (basal, luminal, and cap cells) in the puberty glands (Fig. 2). The studies with both Wnt signal responsive mouse models (*Axin2* and *Lgr5*) suggest the presence of both basal and luminal progenitors in prepubertal and puberty, yet cap cells seem distinct from those two progenitors. Interestingly, both the *Axin2* and *Lgr5* models revealed a fate switch from luminal to basal lineage for the Wnt-signal responsive cells at

PND12 during prepubertal stage (de Visser et al., 2012; van Amerongen et al., 2012). This finding also suggests that Wnt signal controls different progenitors during development. We suspect that these progenitors may function as transient amplifying cells during the mammary gland development. In light of the recent lineage tracing study showing the presence of bipotent MaSCs both at the pubertal and adult glands (Rios et al., 2014), it is possible that bipotent MaSCs from fetal stage still maintain a pool of cells throughout all developmental stages, and signals/cues associated with each developmental stage may ultimately decide which stem/progenitors prevail the whole epithelial cell population at any particular time point.

Future outlook

In conclusion, many progresses have been made in identifying different stem/progenitor cells in the murine mammary epithelium at different developmental stages by using cell surface markers, mammary fat pad reconstitution assay, and *in vivo* genetic labeling techniques such as lineage tracing. Yet, a precise picture of the epithelial cell hierarchy is still lacking. It is important for newcomers to this field to be aware of the limitations of mammary fat pad reconstitution assay, especially when such evidence is needed to interpret the complex relationship among various stem/progenitor cells under physiological conditions. Further delineation of the murine mammary epithelial cell hierarchy requires more specific markers that allow better separation of different stem/progenitors as well as stem or progenitor specific promoters that can be used for cell-fate mapping.

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Compliance with ethics guidelines

Qiaoxiang Dong and Lu-zhe Sun declares that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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