SCA-1 Labels a Subset of Estrogen-Responsive Bipotential Repopulating Cells within the CD24+ CD49fhi Mammary Stem Cell-Enriched Compartment

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SUMMARY

Estrogen stimulates breast development during puberty and mammary tumors in adulthood through estrogen receptor-α (ERα). These effects are proposed to occur via ERα luminal cells and not the mammary stem cells (MaSCs) that are ERα+. Since ERα+ luminal cells express stem cell antigen-1 (SCA-1), we sought to determine if SCA-1 could define an ERα+ subset of EpCAM+/CD24+/CD49fhi MaSCs. We show that the MaSC population has a distinct SCA-1+ population that is abundant in pre-pubertal mammary glands. The SCA-1+ MaSCs have less stem cell markers and less in vivo repopulating activity than their SCA-1neg counterparts. However, they express ERα specifically and enter the cell cycle at puberty. Using estrogen-deficient aromatase knockouts (ArKO), we showed that the SCA-1+ MaSC could be directly modulated by estrogen supplementation. Thus, SCA-1 enriches for an ERα+, estrogen-sensitive subpopulation within the CD24+/CD49fhi MaSC population that may be responsible for the hormonal sensitivity of the developing mammary gland.

INTRODUCTION

Estrogen exposure has long been associated with breast cancer (BCa) risk and early-life estrogen exposures can have impacts many decades later (Warri et al., 2008; Clemmons and Goss, 2001). Estrogen is also important for normal mammary gland development, with aromatase knockout (ArKO) and estrogen receptor-α (ERα)-deficient mice showing only rudimentary ductal structures (Fisher et al., 1998; Hewitt et al., 2010). It was therefore surprising to find that adult mouse mammary epithelial cells enriched for mammary stem cell (MaSC) activity (CD24+ CD29fhi/ CD24+ CD49fhi) were ERα negative (Asselin-Labat et al., 2006). However, MaSCs are extremely sensitive to estrogen and progesterone, as evidenced by their loss with ovariectomy and their increase during pregnancy (Asselin-Labat et al., 2010). It is then accepted that MaSC hormone sensitivity is mediated indirectly by the RANK/RANKL axis (Asselin-Labat et al., 2010; Joshi et al., 2010; Tanos et al., 2013).

Stem cell activity within the mammary gland was first described using mammary fat pad transplants (Daniel et al., 1968; Deome et al., 1959). Several investigators then used morphology to define these cells in tissue sections by nature of their small light appearance at the ultrastructural level, or by their localization within the cap cells of terminal end buds (TEBs) (Chepko and Smith, 1997; Williams and Daniel, 1983). More recent research has focused on trying to determine the identity of MaSCs using cell surface markers. Stem cell antigen 1 (SCA-1) has been used to isolate hematopoietic stem cells (HSCs) (Goodell et al., 1996; Spangrude et al., 1988), and Welm et al. (2002) assessed SCA-1 expression in the mammary gland. They showed SCA-1 was highest in the mammary populations that possessed in vivo stem cell activity. Following this study, a combination of cell surface markers, also adapted from the hematopoietic system, were used to enrich for MaSCs, including heat stable antigen CD24 and either integrin beta-1 (CD29) or integrin alpha 6 (CD49f) or SCA-1 negativity (Asselin-Labat et al., 2006; Sleeman et al., 2007). These markers led to a disagreement with the findings of Welm et al. (2002) and the role of SCA-1 in MaSC isolation has remained controversial.

Most recently, SCA-1 has been used to define ERα+ luminal mature cells and luminal progenitor cells within the mammary gland (Sleeman et al., 2007; Shehata et al.,...
To determine whether SCA-1 might identify a similar population within the CD49fhi MaSC-enriched population, we assessed the ERα transcript and protein levels in the SCA-1+ and SCA-1neg CD49fhi cells. We also sought to determine if this population of cells was responsive to estrogen exposure.

RESULTS

CD24+ CD49fhi SCA-1+ Cells Are Most Abundant in the Young Mammary Gland

The CD24+ CD49fhi isolation strategy (Figure 1A) is a superior MaSC isolation protocol compared with the use of CD24lo SCA-1neg (Asselin-Labat et al., 2006; Sleeman et al., 2007). The CD24+ CD49fhi population contains both an SCA-1+ and SCA-1neg subset (Figures 1A and 1B). Since MaSCs have been reported to be abundant in the young breast (Russo and Russo, 1978a; Russo et al., 1982), we analyzed the CD49fhi population for changes in SCA-1 with increasing age, after separation based on CD24 and CD49f. We used 4–11 independent pools, each containing mammary tissue from 20 mice at each time point to assess SCA-1+ cells with age. The total CD24+ CD49fhi population was least abundant in young mice and increased with age (Figure 1C). The CD24+ CD49fhi SCA-1+ subset was highly abundant in young mice and decreased with age (Figure 1D). The CD24+ CD49fhi SCA-1neg population followed the same trend as the parent CD24+ CD49fhi population (Figure 1E).

To ensure that the SCA-1+ subset of CD24+ CD49fhi cells was not contaminating SCA-1+ luminal cells, we assessed epithelial lineages in Ly6A (SCA-1)-GFP mice (Alvi et al., 2003). Using EpCAM and CD49f, which has recently been shown to give similar results to CD24 and CD49f (Shehata et al., 2012), both SCA-1+ and SCA-1neg cells were detected within the EpCAM+ CD49fhi cells (Figures S1A–S1C). We backgated the luminal and CD49fhi
Gene Expression Analysis Characterizes CD24* CD49fhi SCA-1* Cells

To determine the lineage of the CD24* CD49fhi SCA-1* and SCA-1neg cells, we assessed the expression of several markers that have been directly or indirectly associated with MaSC (Asselin-Labat et al., 2006; Bouras et al., 2008; Kendrick et al., 2008; Cheng et al., 2000). These included Lgr5 (a Wnt regulated target gene), a-SMA (a cytoskeletal protein in basal cells, within which the stem cell population resides), Id4 (inhibitor of DNA binding 4 that maintains the stem cell pool), p63 (a p53 gene family member that regulates epithelial proliferation and differentiation), Delta1, Fzd7, and Jag1 (notch receptor ligands). Jag1 has been identified within MaSC (Asselin-Labat et al., 2006; Kendrick et al., 2008; Cheng et al., 2000) and luminal cells (Bouras et al., 2008). We assessed Hes1, which plays an important role in the Notch signaling pathway (Kageyama and Ohtsuka, 1999) and Mef2c, a transcription factor that regulates cell fate decisions and is present in human and mouse MaSC (Cheng et al., 2000) and long-term HSC (LT-HSCs) (Ficara et al., 2008). Also included was Aldh1a1 (a detoxifying enzyme responsible for the oxidation of intracellular aldehydes) since it has been used as a BCa stem cell (CSC) marker and is present in SCA-1* CSC. We also assessed the levels of luminal markers Keratin18, Notch1, Hey1, and Hey2 and the luminal progenitor markers Elf5 and C-kit to determine if the SCA-1* cells were progenitors rather than stem cells per se. Finally, we assessed the levels of the cell-cycle-related genes Cyclin D1 and Cyclin D2, which have been associated with luminal and basal cell populations, respectively (Joshi et al., 2010).

The CD24* CD49fhi SCA-1neg cells expressed high levels of stem cell markers Lgr5, a-SMA, Delta1, Id4, Fzd7, p63, and Aldh1a1 (Figure 2A). Consistent with this, Lgr5-GFP mice showed Lgr5+ cells within the CD24* CD49fhi SCA-1neg population (Figure S2). In contrast, the CD24* CD49fhi SCA-1* cells expressed only Delta1 in high levels, with moderate levels of p63 and Aldh1a1 (although these levels were higher than those observed in the luminal subsets). The CD24* CD49fhi SCA-1* cells had high Jag1 and Mef2c levels compared with the low levels observed in CD24* CD49fhi SCA-1neg cells. Hey1, Hey2, and Hes1 (notch target genes) were all higher in the CD24* CD49fhi SCA-1* cells, with some expression in the luminal cells. Expression of the luminal-specific marker K18 was higher in the CD24* CD49fhi SCA-1* cells compared with the SCA-1neg counterparts but lower than within the luminal populations (Figure 2B). Curiously, the expression of K18 was higher in CD24* CD49fhi SCA-1* cells at 3 weeks compared with 6 weeks of age. Notch1 was higher in CD24* CD49fhi SCA-1* cells compared with CD24* CD49fhi SCA-1neg cells, but appreciable levels were also observed in the luminal cells (Figure 2B). As expected, Elf5 and C-kit were highest in the CD24* CD49fhi SCA-1neg (ERneg luminal) population (Figure 2C). The CD24* CD49fhi SCA-1* cells expressed higher levels of Elf5 and C-kit compared with the CD24* CD49fhi SCA-1neg, although this was considerably lower than in the luminal cells. Similar to previously published results (Joshi et al., 2010), we found that Cyclin D1 was higher in the luminal cells and Cyclin D2 was higher in the MaSCs. The level of Cyclin D2 was slightly higher in the CD24* CD49fhi SCA-1* MaSCs compared with the CD24* CD49fhi SCA-1neg subset. Thus, the CD24* CD49fhi SCA-1* cells are not specific to either the luminal or basal lineage but rather express moderate levels of markers for both.

SCA-1 Positivity Does Not Further Enrich CD24* CD49fhi MaSCs for Stem Cell Activity

The in vivo stem cell activity of the SCA-1neg and SCA-1* subsets of the CD24* CD49fhi population was assessed using limiting dilution mammary fat pad transplants. Donor mice were 6 weeks of age since, at this age, both populations are equally abundant (Figure 1). As shown in Figure 3, there were more positive outgrowths in the CD24* CD49fhi SCA-1neg cells (19,493, CI 5,377–16,761) compared with the CD24* CD49fhi SCA-1* cells (1,25,902, CI 10,618–63,178) (Figure 3A) equating to a 2.5-fold enrichment in stem cell activity (p = 0.042). Both populations possessed multipotent stem cell activity as they could generate all mammary lineages (Figures 3B–3I). Sections of each outgrowth were processed for H&E staining, ERα, and cytokeratin immunostaining to confirm that luminal, basal, and ERα+ epithelial cells could be generated. The size of the outgrowths generated by both SCA-1* and SCA-1neg cells was comparable (Figure S3). In vitro mammosphere analysis confirmed a 2-fold enrichment in stem cell activity in the SCA-1neg compared with SCA-1* (Figures 4A–4C). In contrast, very little in vitro progenitor activity was observed from either CD24* CD49fhi SCA-1* subpopulation, as measured by 2D colony-forming ability (Figures 4D–4G).

SCA-1 Positivity Does Not Further Enrich CD24* CD49fhi MaSCs for Quiescent Cells

As the SCA-1* subset of cells is abundant in the pre-pubertal mammary gland and declines when estrogen-driven mammary growth occurs, we reasoned these cells may represent a quiescent stem cell pool. To investigate, this cell-cycle activity was analyzed using Ki67 and Hoechst 33342 in 3- and 6-week-old FVB/n mice (Cheng et al., 2000; Mulally...
et al., 2013). As expected at 3 weeks of age, prior to the onset of ductal growth in the mammary gland, the total CD24⁺ CD49fhi MaSC population was predominantly in G₀ of the cell cycle (Figure 4A). The G₀ and G₁ gates were set using fluorescence minus one (FMO) controls lacking K67 (Figure S5). The luminal and nonepithelial populations were also predominantly quiescent at this age (data not shown). At 6 weeks of age, the CD24⁺ CD49fhi SCA-1neg...
Figure 3. SCA-1 Does Not Enrich for In Vivo MaSC Activity

(A) Limiting dilution mammary fat pad transplant data from CD24+ CD49fhi SCA-1+ (SCA-1+) and CD24+ CD49fhi SCA-1−/− (SCA-1−) cells. The fractions denote the number of positive outgrowths as a percentage of the total number of transplants. The percentage of positive outgrowths is given in brackets. Circles show the filled proportion of the fat pad. Stem cell enrichment was determined using the ELDA program.

(B–I) Mammary outgrowths were assessed using carmine-stained wholemount analysis (B and F), H&E (C and G), ERα (D and H), and CK14/CK18 immunostaining (E and I) to confirm the architecture and presence of cell lineages. Blue, DAPI; green, CK14; red, CK18. Arrowheads in (D) and (H) show ERα-positive cells; (B–E) show results from an SCA-1+ outgrowth and (F–I) from an SCA-1−/− outgrowth.
population was predominantly arrested in the G1 checkpoint, with a small percentage in G2/S/M stages (Figure 4B). In contrast, the CD24+ CD49fhi SCA-1+ cells were predominantly cycling (Figure 4C). Average values for three biological replicate pools of 20 animals are shown for 3 weeks (Figure 4D) and 6 weeks (Figure 4E). Together, these data indicate that SCA-1 does not enrich for a quiescent population of cells within the CD24+ CD49fhi population but instead enriches for a population of cells that is specifically activated to enter into the cell cycle at puberty.

**TEBs Are Enriched for SCA-1+ Cells**

To assess whether this specific activation of mitotic activity in SCA-1+ subset was due to their involvement of TEB proliferation, the proportion of CD24+CD49fhiSCA-1+ and SCA-1neg cells in TEBs was assessed (Figure 5). Using a gentle dissociation, TEBs were specifically isolated (Figure 5A) and enzymatically digested to single cells and analyzed by flow cytometry (Figure 5B). Ducts were isolated and processed in a similar manner for comparison (Figure 5C), as were the TEB and duct-depleted samples (Figure 5D). Within the CD49fhi population, there was a higher proportion of SCA-1+ cells in the TEB (80%) compared with the DUCT (10.5%) and TEB and DUCT-depleted (17.77%) samples (Figures 5E–5G). Consistent with these findings, analysis of the RNAs from isolated TEBs and ducts showed that the TEBs were enriched for cell-cycle pathways (Table S1) (Morris et al., 2006).

**The CD24+ CD49fhi SCA-1+ Cells Express ERα**

MaSCs have been shown previously to be ERαneg (Asselin-Labat et al., 2006; Sleeman et al., 2007), and yet we observed a specific activation of mitotic activity in the CD24+ CD49fhi SCA-1+ cells at puberty. We reasoned they may be responding to the hormonal surge at puberty and assessed the expression of hormone receptors. ERα mRNA levels and its target gene progesterone receptor (PR) were assessed using qPCR in three biological pools of 20 mice each. As anticipated, ERα was highest in the SCA-1+ luminal cells and absent in the CD24+ CD49fhi SCA-1neg MaSC population (Figure 6A) (Sleeman et al., 2007; Asselin-Labat et al., 2006). The CD24+ CD49fhi SCA-1+ cells had 20-fold higher levels of ERα at 3 weeks and 5-fold higher levels at 6 weeks compared with CD24+ CD49fhi SCA-1neg cells (Figure 6A).
We further assessed ERα protein levels by sorting cells from 4-week-old mammary glands directly onto slides. Nuclear ERα staining was observed in the CD24⁺ CD49⁺ SCA-1⁺ cells (Figure 6B) (14% ERα positive) but not in the CD24⁺ CD49⁺ SCA-1⁻ cells (Figure 6C). The positive control luminal SCA-1⁺ cells showed high levels of nuclear staining (Figure 6D) (49% ERα positive) compared with luminal SCA-1⁻ cells (Figure 6E) (5% ERα positive). PR transcript was relatively low in the luminal cells and CD24⁺ CD49⁺ SCA-1⁻ cells but increased 2-fold in 6-week-old CD24⁺ CD49⁺ SCA-1⁺ cells compared with the CD24⁺ CD49⁺ SCA-1⁻ cells (Figure 6F).

We also assessed the levels of Egfr and Rank/Rankl, as these have been proposed to mediate hormone action in MaSCs (Asselin-Labat et al., 2010). Egfr was highest in the CD24⁺ CD49⁺ SCA-1⁻ MaSCs at 3 weeks of age, while the CD24⁺ CD49⁺ SCA-1⁺ MaSC and luminal cells had lower levels. At 6 weeks of age, Egfr remained high in the CD24⁺ CD49⁺ SCA-1⁻ cells but was also expressed in SCA-1⁻ luminal cells at a similar level (Figure 6F). Rankl and Rank receptor (Tnfrsf11a) are reported to be expressed in the luminal and MaSC populations, respectively (Asselin-Labat et al., 2010). In our study, Rankl was absent at 3 weeks of age and present only in the luminal cells at
**G**

EREc: 5’ Pu  G G T C A N N T G A C C Py 3’

| Cell type     | Gene  | ERE   |
|---------------|-------|-------|
| SCA-1+ CD49hi | Asof1 | 0     |
| SCA-1+ CD49hi | Actn2 | 0     |
| SCA-1+ CD49hi | DBF1  | 0     |
| SCA-1+ CD49hi | Id4   | 0     |
| SCA-1+ CD49hi | Hes1  | 0     |
| SCA-1+ CD49hi | Lgr5  | 0     |
| SCA-1+ CD49hi | Fzd7  | GGGTCATTCTGTCC |
| SCA-1+ CD49hi | Tbx3  | GGGTCAATTACAG |
| SCA-1+ CD49hi | Ly6c  | AGGTCAATTGACCA |
| SCA-1+ CD49hi | Hey1  | GGGTCAATTGACCA |
| SCA-1+ CD49hi | Hey2  | GGGTCAATTGACCA |
| SCA-1+ CD49hi | Jagged1| AGCTCAACTGACCC |
| SCA-1+ CD49hi | Notch1| 0     |
| SCA-1+ CD49hi | Meox2 | 0     |
| SCA-1+ CD49hi | Meox2d| AGGTCAATCGATCT |

(legend on next page)
6 weeks of age. The Rank receptor was highest in the CD24+ CD49hi SCA-1neg cells at 3 weeks of age, with appreciable levels observed in CD24+ CD49hi SCA-1+ and SCA-1neg luminal cells. At 6 weeks of age, the pattern was similar; however, the levels were highest in the CD24+ CD49hi SCA-1+ cells. Together our data indicate that CD24+ CD49hi SCA-1+ cells present in the young mammary glands possess some ERα and show PR expression, which may render them sensitive to the pubertal hormone surge.

Since the CD24+ CD49hi SCA-1+ cells expressed hormone receptors, we determined if the genes identified to be present within the CD24+ CD49hi SCA-1+ and SCA-1neg populations contained estrogen response elements (ERE). We analyzed the mRNA sequence using both the dragon ERE program and mined the existing data from a genome-wide screen for high-affinity ERE in the mouse and human genomes (Bourdeau et al., 2004). The latter study used stringent cutoffs of two base deviations from consensus, also a location of the ERE within −10 to +5 kb of the transcriptional start site. The data were complementary. As shown in Figure 6G, EREs were not found within the genes Aldh1a1, α-SMA, Delta1, Id4, Hes1, or Lgr5, all of which were highly expressed in the CD24+ CD49hi SCA-1neg cells. Ly6a (Sca-1) was found to possess an ERE. EREs were also identified within the notch target genes Hey 1 and Hey2, which were both expressed at high levels in the CD24+ CD49hi SCA-1+ cells, and in the notch receptor ligand Jag1 (highest expression in CD24+ CD49hi SCA-1+) and in Fzd7 and p63, which were elevated in CD24+ CD49hi SCA-1neg cells (Figure 6G). There was no ERE in Mef2c, which was highly expressed in CD24+ CD49hi SCA-1+, but there was an ERE in the closely related Mef2d.

**CD24+ CD49hi SCA-1+ Cells Are Estrogen Responsive**

The number of CD24+ CD49hi SCA-1+ cells was highest in pre-pubertal mice (Figure 1), indicating that they are important in development. The high numbers in pre-pubertal mice decreased at puberty and decreased further in adult mice, consistent with estrogen responsiveness. We measured the levels of CD24+ CD49hi SCA-1+ and CD24+ CD49hi SCA-1neg cells in three mouse models of estrogen deficiency. The adult estrogen receptor α knockout mice (Ex3αERKO mice) have a rudimentary ductal structure (Hewitt et al., 2010) similar to that of a 3-week-old mouse. Compared with age-matched post-pubertal wild-type C57BL/6 mice with approximately 10%–20% CD24+ CD49hi SCA-1+ and 80%–90% CD24+ CD49hi SCA-1neg cells (Figures 7A–7C), the CD24+ CD49hi population of Ex3αERKO mice was composed almost entirely (98%) of CD24+ CD49hi SCA-1+ cells (Figures 7D–7F). Similarly, the CD24+ CD49hi MaSC populations from ERα AF2 region mutated mice (AF2ERKI) (Figure 7G) and from the aromatase-deficient mice (ArKO) (Figure 7H), which each have only a rudimentary ductal gland phenotype, also possessed largely CD24+ CD49hi SCA-1+ cells. This is summarized in Figure 7I. To see if the CD24+ CD49hi SCA-1+ cells were estrogen sensitive, we treated ArKO mice with 17β-estradiol pellets from post-natal day (P) 5 for 6 weeks. The 17β-estradiol supplementation led to increased mammary ductal growth in the ArKO mice (Figure 7J) and resulted in a reduction in the number of the CD24+ CD49hi SCA-1+ cells from 92% in placebo-treated ArKO mice to an average of 49% (Figure 7K). To complement these results, adult (8-week-old) wild-type C57BL/6 mice were treated with the ER antagonist tamoxifen (n = 5/group). At 12 weeks, the proportion of CD24+ CD49hi SCA-1+ cells was significantly increased in tamoxifen-treated versus control mice (p = 0.0036; Figure S6).

**DISCUSSION**

SCA-1 positivity identifies normal stem cells in many organs, cancer stem cells in the breast (Burger et al., 2005; Matsuura et al., 2004; Spangrude, 1989; Jo et al., 2009), and LT-HSCs in blood (Osawa et al., 1996; Zhao et al., 2000), yet its usefulness in isolating mouse MaSCs remains controversial. Here, we found that SCA-1 could identify a population within the CD24+/EpCAM+/CD49hi MaSC-enriched population that had less repopulating activity than the SCA-1neg counterparts but was Erα+ and estrogen responsive. This Erα+ population of MaSCs was abundant in young, but not old, mammary glands and was responsive to endogenous and exogenous estrogens.

The results of this study show the SCA-1+ subset of the CD24+ CD49hi population is highest in young mice and
decreases over time, in contrast to the CD49^hi SCA-1^neg and un fractionated CD49^hi cells, which are lowest in 3-week-old mice and increase with age. This appears to contradict the theory that MaSCs are highest in young mice, a theory based on the observation that TEBs, which house the MaSCs (Bai and Rohrschneider, 2010) are at their highest density in the mammary glands of young mice (Russo and Russo, 1978a; Russo et al., 1982). In this study, we have further challenged this theory by showing that MaSCs are not enriched within the TEBs, with a small proportion also found in the ducts of the mammary gland. The reason for an increase in CD24^+ CD49^hi cell numbers with age requires additional investigation, but is consistent with emerging data showing that MaSCs are increased in older animals (Huh et al., 2015; Plaks et al., 2013). We hypothesized that the reduction of the SCA-1^+ subset of the CD24^+ CD49^hi population with age is in response to estrogen signaling at puberty, stimulating a differentiation into CD24^+ CD49^hi SCA-1^neg cells.

To confirm the SCA-1^+ cells were not a contaminating luminal population, we used SCA-1-GFP (Ly6A-GFP) mice and assessed the gene expression profile of the SCA-1^+ subset compared with the SCA-1^neg subset. We showed these cells are not luminal progenitors, as they expressed predominantly basal markers. The CD24^+ CD49^hi SCA-1^+ cells showed expression of Delta1, α-SMA, and p63, all absent in the luminal cell populations. Aldh1a1, a marker of SCA-1^+ cancer stem cells, was higher in CD24^+ CD49^hi SCA-1^neg cells than in the CD24^+ CD49^hi SCA-1^+ cells. The CD24^+ CD49^hi SCA-1^+ cells did show high expression of Notch receptor ligands and target genes (Jagged 1, Hes1, Hey 1, Hey2), which are all more associated with differentiated cells in the breast, but Hes1 has also been linked to LT-HSCs. Mef2c is a transcription factor with identified roles in cardiac morphogenesis and myogenesis. It is present in human and mouse MaSCs (Cheng et al., 2000) and is also present in LT-HSCs (Ficara et al., 2008) and was highly expressed in the CD24^+ CD49^hi SCA-1^+ cells. In additional, the CD24^+ CD49^hi SCA-1^+ cells express high levels of Notch1, which has been associated with differentiated cells, but do not express high levels of the luminal marker Keratin18. Furthermore, the CD24^+ CD49^hi SCA-1^+ cells did not express high levels of Elf5 and C-kit and thus cannot be luminal progenitor cells. Collectively, our gene expression data indicate that the CD24^+ CD49^hi SCA-1^+ cells are basal cells with some expression of the previously identified MaSC markers that we have found to be associated with the CD24^+ CD49^hi SCA-1^neg cells that have the highest in vivo transplant activity. The expression of some MaSC markers is in agreement with the limited stem cell activity of these cells. Statistical significance was not reached in many cases due to the nature of the analysis (three individual pools of 20–30 mice). This high number of animals yielded only picogram amounts of RNA that required amplification for gene expression analysis. With only three samples, nonparametric analysis was used, as we were unable to test for normal distribution of the data. Our analysis is consistent with previous research using limited samples of MaSCs and differentiated luminal cells (Bouras et al., 2008).

When we compared the in vivo MaSC activity using the gold standard mammary fat pad transplant assay, we found the CD24^+ CD49^hi SCA-1^neg cells had higher stem cell activity than the CD24^+ CD49^hi SCA-1^+ subset. Although the stem cell activity of the CD24^+ CD49^hi SCA-1^+ cells was limited (in vitro mammospheres and in vivo transplants), they were able to generate all the lineages, thereby fulfilling a major requirement of an MaSC. However, they did not have progenitor activity in vitro. Our data showing that the SCA-1^neg compartment has higher in vivo stem cell activity is consistent with the work of Stingl et al. (2006) and Shackleton et al. (2006) but contrasts with the work of Welm et al. (2002), who showed SCA-1 enriched for MaSCs. However, these early studies compared the SCA-1^+ versus negative epithelial cell compartments without prior selection on lineage or epithelial cells and so may be complicated by the inclusion of nonepithelial cells. They were unable to calculate the stem cell enrichment, as only six transplants were completed and the cell inoculum varied. Direct comparisons in the same laboratory with numerous replicates at each dose are required to determine why these differences occur.

In line with the aforementioned hypothesis that changes with the SCA-1^+ subset of the CD24^+ CD49^hi population with age is in response to estrogen signaling, we showed that the CD24^+ CD49^hi SCA-1^+ cells are mitotically active
at 6 weeks of age compared with the CD24+ CD49fhi SCA-1neg cells. Interestingly, neither population has greater than 0.1% of cells in G0 of the cell cycle at 6 weeks, indicating that like the hematopoietic system (Cheng et al., 2000), adult stem cells within the mammary gland are not dormant.

ERα expression was confirmed to be significantly increased in the SCA-1+ compared with the SCA-1neg CD24+ CD49fhi population using both mRNA and protein analysis, indicating direct estrogen responsiveness. Previous studies were unable to show ERα transcripts and very limited protein in CD49fhi stem cells (Asselin-Labat et al., 2006; Sleeman et al., 2007). We propose this was a consequence of using 10- to 12-week-old mice, in which the CD24+ CD49fhi SCA-1+ subset is quite low. However, Asselin-Labat et al. (2006) did report that 0.01% of the MaSC cells that they assessed had ERα expression, and thus it may be that these rare cells were the SCA-1+ cells in the MaSC pool.

As the CD24+ CD49fhi SCA-1+ cells decrease at puberty but paradoxically express ERα, it was proposed that they were negatively regulated by estrogen. This was confirmed when estrogen-deficient mouse models (Ex3ERKO, AF2ERKI, and ArKO) were assessed as adults (10–12 weeks of age) and showed a high proportion of CD24+ CD49fhi SCA-1+ cells. Adding support to this, treatment of ArKO mice with estrogen shifted the proportion of CD24+ CD49fhi SCA-1+ cells into CD24+ CD49fhi SCA-1neg cells, leading to an increase in mammary gland growth. Due to this negative regulation of SCA-1+ cells by estrogen overlapping with a period of high mitotic activity, we postulate that these CD24+CD49fhi SCA-1+ cells are important in ductal expansion of the mammary gland at puberty. To investigate this point further, lineage tracing will be required. Furthermore, since these cells are so sensitive to changes in estrogen and are in such high abundance in the young mammary gland, it implicates them in the understanding of how hormone exposure can mediate BCa risk many decades after exposure. If the CD24+ CD49fhi SCA-1+ cells are also shown to be the most carcinogen sensitive, it may explain why carcinogenic exposure in younger women has the most deleterious impact on BCa risk later in life. Intriguingly, HSCs, which are also SCA-1-, undergo more frequent self-renewing divisions in females compared with males, which Nakada et al. (2014) recently proved could be mediated by estrogen. Estrogen was also shown to be critical for the mobilization of proliferating HSCs to the spleen and expansion of splenic erythropoiesis during pregnancy (Nakada et al., 2014).

In line with their estrogen responsiveness and activation of the cell cycle at puberty, the CD24+ CD49fhi SCA-1+ cells were found to be enriched in TEBs. TEBs are highest in the pubertal animal and are considered the proliferative unit of the developing gland (Russo and Russo, 1978a, 1978b, 1980). This agrees with our data showing that the SCA-1+ TEBs are abundant and actively cycling at puberty. TEBs have been associated with the carcinogen sensitivity of the mammary gland, yet whether the stem cells or the proliferative nature of the TEBs is responsible is unclear (Russo et al., 1979). Our data indicate that the proliferating SCA-1+ subset of MaSCs within the TEBs are likely mediating the carcinogen sensitivity.

Here, we have characterized an ER-positive and estrogen-sensitive population within the CD24+ CD49fhi MaSC-enriched compartment. Mammary repopulating activity, while present, does not appear to be a predominant function, instead their abundance in the young mammary gland and estrogen sensitivity indicate a role in pubertal mammary development and hormonal sensitivity early in life.

**EXPERIMENTAL PROCEDURES**

**Animals and Breeding Experiments**

Animal experiments were completed under approval from Monash University Animal Ethics Committee, Peter MacCallum Cancer Centre Animal Ethics Committee, Animal Ethics Committee of Florey Institute or under the UK Animals Scientific Procedures Act 1986. MaSCs were isolated from fourth mammary fat pads of FVB/n mice at 3, 6, 9, and 25 weeks of age, transplant, and qPCR experiments. LGR5-GFP mice (B6.129P2-Lgr5tm1(cre/ERT2)Cle/J) were a kind gift from Hans Clevers (Barker et al., 2007). ERα knockout (Ex3ERKO), ERα AF2 domain dysfunctional (AF2ERKI) (Arao et al., 2011), aromatase-deficient (ArKO) (Fisher et al., 1998), and Ly-6A (SCA-1) GFP transgenic mice (Alvi et al., 2003) all have been described before. All mice were housed under specific pathogen-free conditions. ER- and aromatase-deficient mice were maintained on a soy-free diet (Specialty Feeds), with undetectable levels of isoflavones (Soyfree), as dietary phytoestrogens have been shown to be estrogen in estrogen-deficient mice (Britt et al., 2002).

**Isolation, Staining, and Flow Cytometric Analysis of Mouse Mammary Cells**

MaSCs were isolated using mechanical and enzymatic digestion followed by fluorescence-activated cell sorting (FACS), as detailed previously (Asselin-Labat et al., 2006; Sleeman et al., 2007). Cells were stained with either anti-CD24 Pacific Blue (catalog no. 582583; BD Biosciences) or EpCAM BV605 (catalog no. 563214; BD Biosciences), CD31 (catalog no. 561410)/CD45 (catalog no. 557853) PE Cy7 (BD Biosciences), Ly6A-PE (catalog no. 553108, BD Biosciences), and CD49f-APC (catalog no. 552848)/TER119 (catalog no. 552848)/TER119 (catalog no. 557853) PE Cy7 (BD Biosciences), and Ly6A-PE (catalog no. 553108, BD Biosciences), and CD49f-APC (catalog no. 552848)/TER119 (catalog no. 557853).
Isolation of Mammary TEBs and Ducts

TEB and duct isolation was performed as previously (Morris et al., 2004) with modifications. Mammary glands were collected from FVB/n mice at 5–6 weeks of age and coarsely chopped in a Petri dish using scalpel blades before incubation in digest mixture (1 mg/mL collagenase 1 [Worthington] in 10 mL of serum-free L15) at 37°C for 25 min. Following this, the tube was vigorously shaken, and the digest mix was split into two 50 mL Falcon tubes. Tubes were made up to 50 mL with serum containing L15. The tubes were then briefly centrifuged, and the pellets from each tube were combined and centrifuged again before placing onto a gridded 35 mm Petri dish (Thermo Scientific) under a dissection microscope. Using a 10 μL pipette set to 2 μL, TEBs and ducts were gently sucked up the pipette tip and placed into serum containing L15. Within an hour, TEB and ducts were gently trypsinized to obtain single cells and stained for flow cytometry.

Limiting Dilution Fat Pad Transplantation Assays and Outgrowth Confirmation

MaSCs were isolated from 6-week-old nulliparous animals for comparison of the CD24+ CD49fhi SCA-1neg and CD24+ CD49fhi SCA-1+ populations. MaSCs were collected into serum containing L15 medium (Invitrogen) and immediately resuspended in serum-free L15 at the required concentration. Fresh cells were transplanted into cleared fat pads of 3-week-old mice as described previously (Britt et al., 2009). After 10 weeks, the reconstituted fat pads were removed and assessed by carmine-stained wholemounts. All outgrowths were dissected and processed into paraffin blocks for confirmation of structure and cell lineage analysis by immunohistochemistry. Keratin 14/18 dual immunofluorescence was completed as described previously (Britt et al., 2009). All sections were examined on a Nikon C1 confocal microscope, Invert, based on Nikon Eclipse Ti and captured using the NIS Elements Nikon acquisition software. Single antibody-stained control sections, where either the primary antibody was absent or was combined with an inappropriate secondary antibody, were used to confirm the specific staining. Erα staining was completed as described previously (Britt et al., 2009). The MaSC enrichment within this population was determined using the extreme limiting dilution statistical analysis program (Hu and Smyth, 2009).

Cell-Cycle Analysis Using Hoechst 33342 and Ki67

To distinguish cells in G0/G1 from cells in G2/S/M, the samples were stained with the DNA binding dye Hoechst 33342 and Ki67 to delineate G0 from G1 since Ki67 is not present in noncycling (G0) cells (Mullally et al., 2013). All cells were immunostained for lineage markers mentioned above before fixing and permeabilizing with a Fix and Perm Kit (Invitrogen). Simultaneous staining of Ki67 (clone B56, catalog no. 556003; BD Biosciences) 1:20 in reagent B of the Fix and Perm Kit was completed at room temperature for 30 min. Cells were washed in PBS/0.1% NaAzS/5% fetal bovine serum (FBS), spun down at 3500 × g for 5 min, and resuspended in Hank’s balanced salt solution with 20 mM HEPES, 10% FBS, 1 mg/mL glucose, and 6.16 μg/mL Hoechst 33342 (catalog no. H1399; Molecular Probes) and incubated for 30 min at room temperature. Finally, the cells were washed and resuspended in Lebovitz’s 10% FBS and analyzed by flow cytometry on an LSRIIb (BD Biosciences).

RT-PCR Analysis

RNA was isolated from flow-cytometry-sorted cell populations using the RNeasy Micro Kit (Qiagen) following the manufacturer’s instructions. RNA content was quantitated using the Qubit 2.0 Fluorometer (Invitrogen) and quality was determined using a 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized by reverse transcription using Superscript III, random primers, and dNTPs (Invitrogen) and pre-amplified with TaqMan PreAmp master mix (Applied Biosystems) as per the manufacturer’s instructions. qRT-PCR was completed using TaqMan probes on a 7900HT Thermocycler (Applied Biosystems) in a reaction consisting of 50 ng of cDNA, 0.5 μL of TaqMan probe, 5 μL of TaqMan Advance Mastermix, 3 μL of H2O under the following conditions: 50°C for 2 min, 96°C for 20 s, 95°C for 1 s, and 60°C for 20 s, with 40 cycles of the last two conditions. The data were analyzed using SDS version 2.3 software. Relative expression of each probe to housekeeping probe 18S in each sample was determined using the 2^ΔΔCt method. Samples were normalized and compared with the 6 week CD24+ CD49fhi SCA-1neg control. The relative gene expression levels were analyzed using GraphPad Prism. A normality test could not be completed (three biological replicates of n = 20 pooled mice), and thus a nonparametric Kruskal-Wallis test with Dunn’s multiple comparison was used. p Values less than 0.05 were considered statistically significant. Table S2 shows the TaqMan primers used for these studies.

ERα Immunofluorescence Staining

FACS-sorted cells were cytospon onto Superfrost Plus glass slides (Menzel-Gläser) at 600 rpm for 3 min using a Shandon Cytospin 3 (Thermo Scientific). The cells were then fixed onto the slides in PBS/4% paraformaldehyde for 10 min at room temperature. Cells were then permeabilized for 2 min in PBS/0.15% Triton-X 100 (Sigma) and blocked O/N in PBS/1% BSA (Amresco) at 4°C. The following day, ERα anti-mouse monoclonal antibody (ID5 clone, catalog no. M7047; Dako) was added at 1:50 in PBS and incubated for 1 hr at room temperature. Cells were then washed in Tris-buffered saline (TBS)/0.1% Tween 20, before secondary antibody (anti-mouse immunoglobulin G1, S55) was added and incubated at room temperature for 1.5 hr. Cells were then washed in TBS/Tween 20 0.1%, counterstained with 5 μg/mL DAPI (3 × 5-min washes), mounted in Vectashield mounting-media (Vectorlabs), and cover-slipped for imaging.

Stem Cell Analysis in Estrogen-Deficient Mice

To determine if the CD24+ CD49fhi SCA-1+ cells were estrogen sensitive, we assessed the MaSC populations present in the ERα-deficient Ex3sERKO mice (Hewitt et al., 2010), in the ERα dysfunctional AF2ERKI mice (mutations in the AF2 activation function domain of Erα) (Arao et al., 2011), and in estrogen-deficient ArKO mice (Fisher et al., 1998). ArKO mice and wild-type mice were treated with 0.2 mg of silastic 17β-estradiol or saline placebo pellets implanted into the dorsal flank from P5 to P47.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found
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