Wnt Proteins Are Self-Renewal Factors for Mammary Stem Cells and Promote Their Long-Term Expansion in Culture

Yi Arial Zeng¹ and Roel Nusse¹,*
¹Howard Hughes Medical Institute, Department of Developmental Biology, Stanford University, School of Medicine, Beckman Center, B271, 279 Campus Drive, Stanford, CA 94305-5323, USA
*Correspondence: rrusse@stanford.edu
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SUMMARY

Adult stem cells have the ability to self-renew and to generate specialized cells. Self-renewal is dependent on extrinsic niche factors but few of those signals have been identified. In addition, stem cells tend to differentiate in the absence of the proper signals and are therefore difficult to maintain in cell culture. The mammary gland provides an excellent system to study self-renewal signals, because the organ develops postnatally, arises from stem cells, and is readily generated from transplanted cells. We show here that adult mammary glands contain a Wnt-responsive cell population that is enriched for stem cells. In addition, stem cells mutant for the negative-feedback regulator Axin2 and therefore sensitized to Wnt signals have a competitive advantage in mammary gland reconstitution assays. In cell culture experiments, exposure to purified Wnt protein clonally expands mammary stem cells for many generations and maintains their ability to generate functional glands in transplantation assays. We conclude that Wnt proteins serve as rate-limiting self-renewal signals acting directly on mammary stem cells.

INTRODUCTION

Stem cells are characterized by their ability to self-renew as well as to differentiate into specialized cells, properties critical for tissue maintenance and regeneration. The decision to self-renew or differentiate is regulated at multiple levels, including cell-intrinsic transcriptional programs and extracellular signals originating from a specialized niche (Morrison and Spradling, 2008). Cell-extrinsic control is important to prevent the unrestrained self-renewal of stem cells and their possible conversion into cancerous cells (Clarke and Fuller, 2006). A critical aspect of the niche model of stem cell regulation is that the availability of self-renewing factors is limited because of their local release. Understanding the nature of the signals and their effect on adult stem cells has been complicated because of the low abundance of these stem cells and the paucity of functional assays. Moreover, because adult stem cells tend to differentiate in the absence of the proper self-renewal signals, they are difficult to maintain in cell culture under defined conditions.

The mammary gland is an excellent model for studying adult stem cell behavior because of its accessibility and unique postnatal development (Deome et al., 1959; Kordon and Smith, 1998; Shackleton et al., 2006; Stingl and Caldas, 2007; Stingl et al., 2006; Visvader and Lindeman, 2006; Welm et al., 2003; Woodward et al., 2005). It is an epithelial organ with a highly branched ductal network, consisting of a basal layer of myoepithelial cells and an inner layer of luminal cells. During pregnancy, under the influences of hormones, luminal cells are induced to differentiate and develop into alveoli that produce milk at parturition. Alveoli regress at the end of lactation, and the mammary gland returns to a morphological state similar to that found in virgin mice (Richert et al., 2000). Transplantation assays have demonstrated the existence of a rare population of mammary stem cells (MaSCs) that are able to reconstitute a functional mammary gland (Deome et al., 1959; Young et al., 1971). These MaSCs are likely to be present in any of the branches, because each portion of the ductal system was shown to be capable of regenerating an entire epithelial tree upon transplantation (Kordon and Smith, 1998). More recently, a MaSC-enriched population of cells has been isolated based on specific cell surface markers (Lin⁻, CD24⁺, CD29hi) (Shackleton et al., 2006; Stingl et al., 2006). In spite of these advances, it has been difficult to study MaSCs in vitro because of loss of long-term self-renewal. Although short-term mammosphere cultures allow multilineage colonies to form in culture, over time these colonies lose their ability to self-renew and start to differentiate in vivo (Dontu et al., 2003). This is probably due to the absence of self-renewal factors in defined tissue culture media.

Wnt signaling has been implicated in different stages of mammary development as well as in mammary oncogenesis (Boras-Granic et al., 2006; Brisken et al., 2000; Chu et al., 2004; Lindvall et al., 2006, 2009; Nusse and Varmus, 1982; Roelink et al., 1990). Multiple Wnt genes are expressed in the mammary gland epithelium or stroma (Bühler et al., 1993; Gavin and McMahon, 1992; Huguet et al., 1994; Lane and Leder, 1997; Olson and Papkoff, 1994; Weber-Hall et al., 1994). Moreover, it has been suggested that Wnt signals expand mammary stem cells, as indicated by the fact that early tumorigenic lesion from transgenic mice overexpressing Wnt1 have increased numbers of stem cells (Nusse and Varmus, 1982; Shackleton et al., 2006; Vaillant et al., 2008). Whether Wnt proteins directly control normal mammary
stem cells is, however, not known. In this study, we use a combination of cell culture and in vivo transplantation experiments to show that Wnt proteins serve as important self-renewal factors for mammary gland stem cells.

RESULTS

Wnt Reporter-Positive Cells Are Enriched for MaSCs
To identify cells that are activated by Wnt/β-catenin signaling in the mammary gland, we used the Axin2-lacZ reporter mouse, which carries a lacZ gene inserted into the Axin2 locus (Lustig et al., 2002), a well-known Wnt/β-catenin target gene (Jho et al., 2002; Leung et al., 2002; Lustig et al., 2002). The expression of lacZ through the endogenous Axin2 promoter faithfully reflects sites of Wnt/β-catenin signaling in multiple tissues. In 8- to 12-week-old virgin animals, lacZ expression was detected in nearly all of the branches in the mammary gland (Figure 1A). Subsequent histological analysis indicated that the Wnt-responsive cells are located in the basal layer of the mammary ducts (Figure 1B; Baker et al., 2010; Teissedre et al., 2009) where mammary stem cells have been suggested to reside (Shackleton et al., 2006; Stingl et al., 2006).

By using a fluorogenic lacZ substrate to isolate live Axin2-lacZ reporter-positive cells in combination with the Lin−, CD24+, CD29hi markers, we confirmed that 5% of the Lin−, CD24+, CD29hi cells was positive for the Axin2-lacZ reporter (Figures 1C and 1D), as measured by X-gal staining. In contrast, no lacZ-expressing cells were present in the other epithelial cell population (Lin−, CD24−, CD29lo) (n = 0/1002; Figure 1E), which has not been found to contain mammary stem cells (Shackleton et al., 2006).

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expression of several genes, via quantitative reverse transcriptase-polymerase chain reaction (q-RT-PCR; Figure 1I). As expected, the Axin2 gene was expressed at higher levels in the lacZ+ cells compared to the lacZ− cells. The Wnt receptors Lrp5 and Lrp6 expression patterns were similar to that of Axin2 (Figure 1I), in line with the finding that stem cell populations can be enriched by selection for high Lrp5 expression (Badders et al., 2009). Conversely, expression of hey1, a Notch target, was significantly lower in the lacZ+ cells (Figure 1I). Notch signaling has been implicated in luminal cell-specific differentiation, whereas downregulation of Notch signaling in MaSCs can lead to increased reconstitution (Bouras et al., 2008).

To assess mammary gland reconstitution competence, we transplanted Lin−, CD24+, CD29hi cells that were either lacZ positive or negative into cleared fat pads. Notably, Wnt-responsive lacZ-positive cells generated mammary glands more efficiently than did lacZ-negative cells (Table 1). The resulting outgrowths contained lacZ-expressing cells (Figure 1H), indicating that they were derived from the donor mice. Taken together, the CD24+, CD29hi population can be further enriched for mammary stem cells by virtue of their Wnt/l-I-catenin responsiveness.

### MaSCs that Are More Responsive to External Wnt Signals Outcompete Wild-Type Stem Cells in Repopulation Assays

The Axin2 gene encodes a ligand-dependent negative-feedback regulator of the Wnt pathway that dampens signaling cell autonomously. Insertion of lacZ into Axin2 inactivates the gene (Lustig et al., 2002) and elevates Wnt signal strength in a ligand-dependent manner. To measure the impact of loss of the Axin2 gene on Wnt signaling activity in mammary cells, we transduced a TCF-dependent luciferase reporter construct (Fuerer and Nusse, 2010) into primary mammary epithelial cells isolated from wild-type and Axin2lacZ/lacZ mice. Luciferase activity was monitored after incubation with different concentrations of Wnt3A protein.

In the unstimulated state, wild-type and Axin2lacZ/lacZ cells had the same luciferase activity, whereas increasing Wnt3A concentrations activated the reporter to higher levels in Axin2lacZ/lacZ cells (Figure 2A). The difference between WT and Axin2lacZ/lacZ cells was prominent at lower concentrations of Wnt proteins (50–100 ng/ml), whereas higher concentrations saturated the response in either cell type.

The increased response of Axin2lacZ/lacZ mutant MaSCs to Wnt signals suggest that these cells would have a competitive advantage over wild-type cells under conditions in which Wnt proteins are limiting self-renewal factors. We tested this in repopulation assays in vivo (and, separately, in cell culture assays; see below) by isolating Lin−, CD24+, CD29hi cells from Axin2lacZ/lacZ mice that also carried an Actin-GFP marker, as well as wild-type Lin−, CD24+, CD29hi cells from Actin-DSRed mice. GFP- and DSRed-positive cells were mixed in different ratios and injected into cleared fat pads of recipient animals. As a control, MaSC-enriched populations from wild-type, Actin-GFP and wild-type, Actin-DSRed mice were mixed in a 1:1 ratio, resulting in an even distribution of GFP-positive and DSRed-positive mammary gland branches (Figure 2B; Figure S1A available online). In contrast, transplanting Axin2lacZ/lacZ, Actin-GFP cells in competition with twice as many Axin2lacZ/+; Actin-DSRed cells resulted in outgrowths that were predominantly GFP positive and therefore derived from Axin2lacZ/lacZ cells (Figure 2C; Figure S1B). Thus, the potentiated response of Axin2lacZ/lacZ mutant cells to a limiting Wnt source influences the ability of these stem cells to repopulate the mammary gland.

Of note, the Axin2lacZ/lacZ cells generated mammary glands with mild hypermorphic branching phenotypes (GFP-positive branches in Figure 2C), similar to glands in Axin2lacZ/lacZ mutant mice themselves (Figure 2G). However, these glands differentiate normally during parturition and regress after weaning (Figures 2H and 2I compared to WT glands in Figures 2E and 2F). Despite their elevated Wnt responsiveness, Axin2lacZ/lacZ mammary glands have normal ordered layers of cells. As demonstrated by histology and FACS analysis (Figure S2), the basal and luminal cell composition was similar to wild-type. There was no evidence of hyperplasia or tumors, suggesting that the cellular response to Wnt was not excessive. This is in contrast to the MMTV-Wnt1 mouse model where overexpression of Wnt itself causes cancer (Nusse and Varmus, 1982; Shackleton et al., 2006).

In the Presence of Wnt3A Protein, MaSCs Can Be Clonally Expanded In Vitro for Many Generations

Based on our observations in vivo, we next asked whether Wnt proteins could directly influence MaSCs in vitro by promoting their colony-initiating ability. To ensure that colonies originated from single cells rather than from aggregates, we isolated cells from wild-type; Actin-GFP mice and wild-type; Actin-DSRed mice, mixed the cells, and seeded them in Matrigel. As shown in Figure 3A, individual Lin−, CD24+, CD29hi cells formed monochromatic colonies. In Matrigel, the Wnt3A protein is active as Wnts Expand Mammary Stem Cells

### Table 1. Mammary Outgrowths Derived from Lin+, CD24+, CD29hi, lacZ− and Lin−, CD24+, CD29hi, lacZ+ Sorted Mammary Cells

<table>
<thead>
<tr>
<th>Number of Cells Injected</th>
<th>Lin−, CD24+, CD29hi, lacZ−</th>
<th>Lin−, CD24+, CD29hi, lacZ+</th>
</tr>
</thead>
<tbody>
<tr>
<td>500b</td>
<td>1/5</td>
<td>3/5</td>
</tr>
<tr>
<td>100b</td>
<td>0/5</td>
<td>1/5</td>
</tr>
<tr>
<td>100c</td>
<td>3/8</td>
<td>6/8</td>
</tr>
<tr>
<td>50d</td>
<td>5/16</td>
<td>11/16</td>
</tr>
</tbody>
</table>

p value < 0.01

Wnt-responsive (lacZ−) and non-Wnt-responsive (lacZ+) cells were FACS isolated via a LacZ fluorogenic substrate, in combination with the Lin−, CD24+, and CD29hi markers (Figure 1G), and injected into cleared mammary fat pads. The outgrowths were analyzed at 10 weeks post-transplantation. Implanted lacZ+ cells had higher numbers of outgrowths compared with the lacZ− cells. However, for those that were scored positive, there were no differences in the extent of the fat pad occupation. Data are pooled from four independent experiments.

a Shown as number of outgrowths per number of injected mammary fat pads.

b Transplantation with cells resuspended in 50% serum+PBS.

c Transplantation performed with cells resuspended in 50% Matrigel, which improves engraftment.
We next plated single sorted Lin\(^{-}\), CD24\(^{+}\), CD29\(^{hi}\) cells in the presence of either vehicle, Wnt3A protein, or the Wnt inhibitor Dkk1. The presence or absence of Wnt proteins had no apparent effect on cell proliferation; the size of the colonies under each of the three conditions was relatively constant, varying between 20 and 60 \(\mu\)m (Figure 3E) with colonies composed of approximated 15–60 cells (Figure 3F). Moreover, there were no discernible differences in staining for the cell cycle marker Ki67 (Figure 3G). When we examined the clonogenicity of the cells under these conditions, there were no significant differences in primary colony formation: the colonies occurred at a ratio of 1 colony per 15 cells plated (Figure 4A).

In contrast to the lack of a Wnt effect on primary colony formation, subsequent serial colony formation, by dissociation of primary colonies and replating, was dramatically influenced by the presence of Wnt protein (Figure 4A). Addition of Wnt3A of the conditions tested as detected by Annexin V staining (Figure S4). Wnt3A protein not only enhanced the clonogenicity of MaSCs but also lowered the expression of a differentiation marker, Gata-3 (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). Levels of Gata-3, a gene expressed and required during early luminal differentiation, were reduced 2- to 3-fold after exposure to Wnt3A, suggesting a possible role of Wnt-mediated suppression of MaSC differentiation (Figure S3C). However, the colonies contained similar numbers of terminally differentiated basal cells and luminal cells whether cultured with or without Wnt3A (Figures S5A–S5D), and expression levels of the markers K8 and K14 in early or late passage colonies were not significantly influenced either (Figure S5E).

By using colony-formation assays, we compared the clonogenic ability of Axin2\(^{lacZ/lacZ}\) mutant cells to wild-type cells over a range of Wnt protein concentration. In keeping with the
enhanced repopulating ability of the Axin\textsuperscript{2LacZ/LacZ} mutant cells when transplanted in vivo (Figures 2B and 2C), we found that the Axin\textsuperscript{2LacZ/LacZ} cells initiate secondary colonies at a higher rate than did wild-type cells (Figure 4D). In accordance with the luciferase Wnt reporter experiments (Figure 2A), the differences between Axin\textsuperscript{2LacZ/LacZ} and WT cells in colony-initiating assays were more pronounced at lower concentrations of the Wnt protein (50–100 ng/ml) than at saturating concentrations (200 ng/ml). Taken together, these experiments suggest that the clonogenicity of MaSCs is greatly increased by exposure to Wnt protein.

**Long-Term Cultured MaSCs Retain Full Developmental Potential In Vivo**

To test whether the clonally propagated cells had maintained stem cell characteristics—self-renewal and multipotency in vivo—we transplanted colonies from later passages into cleared fat pads to assess outgrowth. In contrast to vehicle-treated colonies, which failed to repopulate, Wnt-treated colonies displayed robust mammary gland reconstitution ability (Figures 5A–5C). Importantly, the repopulating frequencies remained relatively stable (1/34.5–1/48.8) with increasing passage (Table 2). The total colony number expanded with each successive passage, so this suggests that Wnt increases the absolute number of MaSCs.

Notably, single Wnt-treated colonies (derived from single cells in tertiary passage) were frequently able to reconstitute (17 out of 40 when analyzed in parturition recipients; Figure 5G; 4 out of 40 when analyzed in virgin recipients; Figure 5D, Table 2). Immunostaining for the markers Keratin 8 and Keratin 14 confirmed that the outgrowths had a regular morphology, composed of normally differentiated ductal structures with luminal and myoepithelial cells (Figures 5E and 5F). When recipient mice were in parturition, the mammary glands resulting from the transplanted colonies consisted of a dense ductal system ending in clusters of alveoli (Figure 5G) that contained abundant milk proteins (Figure 5H).

Are Wnt signals continuously required to maintain stem cell identity? When we withdrew Wnt protein during secondary plating, we found a marked reduction in the efficiency of mammary gland reconstitution (2 small outgrowths, and 12 implants not generating any detectable tissue; Table 2). This result demonstrates that cells expanded in the presence of Wnt proteins are not transformed into an autonomously self-renewing state.

To confirm unambiguously that the cultured cells maintained full stem cell properties, we performed cell transplantsations starting from glands derived from late passage cultured cells. When analyzed by FACS, these primary outgrowths from Wnt-treated colonies displayed a normal CD24 and CD29 profile (Figure 5I, compared to Figure 1C). Moreover, the Lin\textsuperscript{−}, CD24\textsuperscript{+}, CD29\textsuperscript{hi} cells isolated from Actin-GFP mice were cultured in Matrigel, plus either Dkk1, Wnt vehicle control, or Wnt3A protein. Sizes of the colonies were measured after 1 week of culture in vitro. In all conditions, there are no differences in the average colony sizes (which ranged from 20 to 60 \( \mu \)m). Scale bar represents 50 \( \mu \)m. (F) DAPI staining of the colonies in (E) and cells numbers in each colony were quantified.

**DISCUSSION**

The ability to self-renew while simultaneously generating specialized cells is the critical property of adult stem cells. As currently understood, the choice of a dividing stem cell to self-renew or to differentiate is regulated by factors coming from
Implicit in all models of stem cell control is that differentiation is the default choice and that self-renewal depends on the presence of extracellular signals released by the niche. Among the signals implicated in the control of various types of stem cells, the Wnt pathway is prominent (Barker et al., 2007; Jaks et al., 2008; Kalani et al., 2008; Reya et al., 2003; Willert et al., 2003). For example, Wnt target genes, such as lgr5, are expressed in stem cells of the intestine and the hair follicle, and there are stem cell phenotypes associated with loss of Wnt signaling in these tissues (Barker et al., 2007; Jaks et al., 2008). Likewise, in the case of the mammary gland, there is genetic support for a role for Wnt signaling in the maintenance of MaSCs, such as defects in mammary gland development in mice mutant for Wnt pathway components (Boras-Granic et al., 2006; Brisken et al., 2000; Chu et al., 2004; Lindvall et al., 2006). The experiments described in this paper suggest that Wnt signaling, as monitored by the expression of the Wnt target gene Axin2, is a marker for enriching and identifying MaSCs in vivo. Compared to the Axin2-lacZ+ counterparts, Axin2-lacZ+ mutant cells gave rise to higher numbers of secondary colonies compared to wild-type cells (\text{y axis}), especially at limiting Wnt concentrations. At higher Wnt concentrations, the number of colonies plateaued irrespective of the genotype of the cells.

In spite of the evidence for a role of Wnt pathway in MaSC regulation, it remained unknown whether Wnt proteins acted...
directly on mammary stem cells and controlled their self-renewal properties. In this study, we provide multiple lines of evidence that this is indeed the case. In cell culture, we find that MaSCs are dependent on Wnt proteins for long-term expansion and maintenance of self-renewal under defined conditions. In these experiments, Wnt does not act as a mitogenic growth factor, as shown by the fact that the proliferation of the cells was not influenced by its presence. Rather, our data support a model of other known growth factors, such as EGF, as proliferative factors, while the Wnt protein prevents differentiation of the dividing cells and thereby leads to self-renewal. In line with this proposal, we find that the expression of Gata-3, a transcription factor that promotes differentiation of mammary stem cells into luminal cells, is suppressed by the Wnt signal (Figure S3C). The combined use of Wnt, EGF, and Matrigel has allowed us to expand MaSCs in cell culture, with full retention of their competence to regenerate an organ. This finding may have important implications for attempts to expand breast cancer stem cells (Al-Hajj et al., 2003) in a clonal fashion, as well as stem cells from other tissues. Expanding adult stem cells in culture holds great promise for regenerative medicine but has in general been difficult because of the lack of stem cell markers and knowledge about the required growth factors.

The second line of evidence for a role of Wnt signals as stem cell factors comes from the use of Axin2LacZ/lacZ mutant cells. Axin2 encodes a negative regulator of the Wnt pathway. Unlike other repressors of Wnt signaling, such as Axin1, APC, or GSK3, Axin2 is a target gene of Wnt signaling, implying that its expression and signal-dampening function are restricted to Wnt-exposed cells. Any consequence of Axin2 loss is therefore dependent on the presence of active Wnt proteins and indicates that cells showing a phenotype are actively receiving Wnt signals. In competitive repopulation experiments with wild-type cells, MaSCs isolated from Axin2LacZ/lacZ mice show a marked increase in repopulating the mammary fat pad and regenerating a functional mammary gland, indicating that the propagation of MaSCs in vivo is stimulated by external Wnt signals. While this result leaves open the possibility that Wnt acts as a proliferative signal, we suggest that it reflects the self-renewal ability of the Wnt protein, in part based on the increased ability of the Axin2LacZ/lacZ cells to generate colonies in cell culture (Figure 4D).

The Axin2LacZ/lacZ hypermorphic phenotype is unique compared to mutations in other Wnt signaling components, which mostly lead to hyperplasia or even tumors. Moreover, mammary glands originating from Axin2LacZ/lacZ MaSCs have normal ratios of basal and luminal cells. In addition, MaSCs colonies cultured in the presence of Wnt appeared to have normal expression levels of the basal lineage marker K14 and the luminal marker K8, suggesting that Wnt signals promote the self-renewal of MaSCs without altering cell fate specification.

The role of Wnt in the mammary gland is in agreement with current models of stem cell regulation, where the ability to sustain a limited pool of stem cells is a hallmark of the niche (Morrison and Spradling, 2008). This constraint is important in preventing unrestrained expansion of stem cells, which may otherwise lead to cancer (Clarke and Fuller, 2006). Our study suggests that local Wnt signals act as the self-renewal factors in the adult mammary gland. Indeed, previous studies reported that several Wnts, including Wnt2, Wnt4, Wnt5a, Wnt5b,
The minced tissue was placed in culture medium (RPMI 1640 with 25 mM [G4]. Mammary glands from 8- to 12-week-old virgin female mice were isolated. These were approved by the Animal Care and Use Committee of Stanford University. The DsRed/B6 and Nude strains were used in this study. Experimental procedures were performed with a FACSAria (Becton Dickinson). The purity of sorted cells was routinely more than 95%.

**Mouse Strains**
CD1, Axin2lacZ/+ (Lustig et al., 2002), Axin2lacZ/lacZ, Actin-GFP/B6, and Actin- 
DsRED/B6 and Nude strains were used in this study. Experimental procedures were approved by Animal Care and Use Committee of Stanford University School of Medicine.

**Primary Cell Preparation**
Mammary glands from 8- to 12-week-old virgin female mice were isolated. The minced tissue was placed in culture medium (RPMI 1640 with 25 mM [Hepes], 5% fetal bovine serum, 1% PSQ, 300U ml−1 Collagenase III [Worthington]) and digested for 5 hr at 37°C. After lysis of the red blood cells in NH4Cl, a single-cell suspension was obtained by sequential incubation with 0.25% trypsin-EDTA at 37°C for 5 min and 0.1 mg/ml DNase I (Sigma) for 5 min and gentle pipetting. Single cells were then replated in Matrigel as described above. In assays examining the requirement for Wnt protein in maintaining stem cell properties, Wnt3A protein was withdrawn for 7 days in secondary culture prior to transplantation.

**Cell Labeling, Flow Cytometry**
The following antibodies were used: biotinylated and APC conjugated CD31, CD45, TER119 (BD Pharmingen), CD24-PE/cy5, CD24-PE/cy7, CD29-FITC, CD29-APC (Biolegend), Streptavidin-PE/TexasRed, and Streptavidin-FITC (BD Pharmingen). Antibody incubation was performed for 1 hr in HBSS with 10% FBS and virus. At 48 hr after infection, cells were put under Puromycin selection. Wnt responsiveness was tested by treating cells with a limiting dilution of Wnt3A and luciferase activities were measured.

**In Vitro Colony Formation Assay**
FACS-sorted cells were resuspended at a density of 4 × 105 cells/ml in chilled 100% growth factor reduced Matrigel (BD Bioscience), and the gels allowed to polymerized before covering with culture medium (DMEM/F12, 50 ng ml−1 EGF, plus either vehicle [1% CHAPS in PBS] or 200 ng ml−1 Wnt3A [Willert et al., 2003] or 200 ng ml−1 Dkk1). Culture medium was changed every 24 hr. Primary colony numbers were scored after 7–10 days in culture. For passages colonizing, media was aspirated and Matrigel was digested by incubation in 500 μl of Matrigel recovery solution (BD Bioscience) for 1 hr on ice. Colonies released from Matrigel were pelleted. Single cells were obtained through incubation with 0.25% trypsin-EDTA at 37°C for 5 min, followed by gentle pipetting. Single cells were then replated in Matrigel as described above. In assays examining the requirement for Wnt protein in maintaining stem cell properties, Wnt3A protein was withdrawn for 7 days in secondary culture prior to transplantation.

**Immunostaining**
Frozen sections were prepared by air-drying and fixation for 5 min in cold 4% paraformaldehyde. Tissue sections were incubated with primary antibodies at 4°C overnight, followed by washes, incubation with secondary antibodies for 1 hr at 25°C, and counterstaining with DAPI (Vector Laboratories). The following antibodies were used: rat anti K8 (1:250, Developmental hybridoma bank), rabbit anti K14 (1:1000, Covance), and rabbit anti milk (1:100, Nordic Immunological Laboratories).

**Mammary Fat Pad Transplantation and Analysis**
Sorted cells were resuspended in 50% Matrigel, PBS with 20% fetal bovine serum, and 0.04% trypan Blue (Sigma), and injected in ~5–10 μl volumes

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Number Colonies</th>
<th>EGF+Wnt3Aa</th>
<th>Repopulating Frequency</th>
<th>EGF+ Vehiclea</th>
<th>EGF+Wnt3A, Withdrawal of Wnt3A for 7 Daysa</th>
</tr>
</thead>
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<tr>
<td>2nd</td>
<td>100</td>
<td>16/18</td>
<td>1/41.5 (27.2–62.2)</td>
<td>1/10**</td>
<td>2/14**</td>
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<tr>
<td>3rd</td>
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<td>1/12**</td>
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<td>0/10</td>
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</tbody>
</table>

Lin−, CD24+, CD29hi cells isolated from Actin-GFP mice were cultured in Matrigel in the presence of Wnt3A or vehicle for 2–6 passages. Colonies (ranging from 100 to 1) were injected into cleared fat pads to test reconstitution. GFP outgrowths were observed for cells cultured with Wnt3A in each passage (Figure 5A). Implantation of single colonies yielded 17/40 GFP-positive outgrowths when examined in nulliparous recipients and yielded 17/40 GFP-positive outgrowths when examined in recipients in parturition. In vehicle or withdrawal of Wnt3A conditions, a small percentage of partial reconstituted glands were detected (Figure S6), as indicated by double asterisk.

*a Shown as number of outgrowths per number of injected mammary fat pads.**

**Table 2. Mammary Outgrowths Derived from In Vitro Cultured Colonies**

[53x203]The minced tissue was placed in culture medium (RPMI 1640 with 25 mM [G4]. Mammary glands from 8- to 12-week-old virgin female mice were isolated. These were approved by the Animal Care and Use Committee of Stanford University. The DsRed/B6 and Nude strains were used in this study. Experimental procedures were performed with a FACSAria (Becton Dickinson). The purity of sorted cells was routinely more than 95%.

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Mammary glands from 8- to 12-week-old virgin female mice were isolated. The minced tissue was placed in culture medium (RPMI 1640 with 25 mM [Hepes], 5% fetal bovine serum, 1% PSQ, 300U ml−1 Collagenase III [Worthington]) and digested for 5 hr at 37°C. After lysis of the red blood cells in NH4Cl, a single-cell suspension was obtained by sequential incubation with 0.25% trypsin-EDTA at 37°C for 5 min and 0.1 mg/ml DNase I (Sigma) for 5 min with gentle pipetting, followed by filtration through 40 μm cell strainers.

**Cell Labeling, Flow Cytometry**
The following antibodies were used: biotinylated and APC conjugated CD31, CD45, TER119 (BD Pharmingen), CD24-PE/cy5, CD24-PE/cy7, CD29-FITC, CD29-APC (Biolegend), Streptavidin-PE/TexasRed, and Streptavidin-FITC (BD Pharmingen). Antibody incubation was performed on ice for 15 min in HBSS with 10% fetal bovine serum. LacZ fluorogenic substrate staining was allowed to polymerized before covering with culture medium (DMEM/F12, 50 ng ml−1 EGF, plus either vehicle [1% CHAPS in PBS] or 200 ng ml−1 Wnt3A [Willert et al., 2003] or 200 ng ml−1 Dkk1). Culture medium was changed every 24 hr. Primary colony numbers were scored after 7–10 days in culture. For passages colonizing, media was aspirated and Matrigel was digested by incubation in 500 μl of Matrigel recovery solution (BD Bioscience) for 1 hr on ice. Colonies released from Matrigel were pelleted. Single cells were obtained through incubation with 0.25% trypsin-EDTA at 37°C for 5 min, followed by gentle pipetting. Single cells were then replated in Matrigel as described above. In assays examining the requirement for Wnt protein in maintaining stem cell properties, Wnt3A protein was withdrawn for 7 days in secondary culture prior to transplantation.

**Lentiviral Vector and Infection**
The 7xTcf-FFluc/SV40 viral construct contains seven Tcf/Lef-binding sites, the 5’ UTR of the pSuperTopflash reporter plasmid, firefly luciferase gene, and a SV40-Puro cassette for Puromycin selection (Fuerer and Nusse, 2010). Lentivirus was produced by transient transfection in 293T cells. Mammary cells were isolated from 8- to 12-week-old virgin female glands as described above, followed by preplating for 30 min to deplete stromal cells. The suspending epithelial cells were collected and plated in DMEM/F12/10% PBS with virus. At 48 hr after infection, cells were put under Puromycin selection. Wnt responsiveness was tested by treating cells with a limiting dilution of Wnt3A and luciferase activates were measured.

**Immunostaining**
Frozen sections were prepared by air-drying and fixation for 5 min in cold 4% paraformaldehyde. Tissue sections were incubated with primary antibodies at 4°C overnight, followed by washes, incubation with secondary antibodies for 1 hr at 25°C, and counterstaining with DAPI (Vector Laboratories). The following antibodies were used: rat anti K8 (1:250, Developmental hybridoma bank), rabbit anti K14 (1:1000, Covance), and rabbit anti milk (1:100, Nordic Immunological Laboratories).

**Mammary Fat Pad Transplantation and Analysis**
Sorted cells were resuspended in 50% Matrigel, PBS with 20% fetal bovine serum, and 0.04% trypan Blue (Sigma), and injected in ~5–10 μl volumes
into 3-week-old female cleared fat pads. Reconstituted mammary glands were harvested after 6–10 weeks. All transplanted cells were labeled with either GFP or LacZ. GFP outgrowths were detected under a fluorescence dissection microscope. LacZ outgrowths were detected by whole-mount X-gal staining.

**Competitive Repopulation Assays In Vivo**

In controls, Lin+CD24+, CD29hi cells from WT; Actin-GFP mice and from WT; Actin-DsRed mice were isolated, respectively, and mixed in a 1:1 ratio, 3000 WT; Actin-GFP cells + 3000 WT; Actin-DsRed cells per injection. In Actin-DsRed mice were isolated, respectively, and mixed in 1:2 ratio, 2000 Actin2+/+; Actin-GFP cells + 4000 Actin2+/+; Actin-DsRed cells per injection. Mixed cells were injected into cleared fat pads of Nude mice as described above.

**Statistics**

Limiting dilution analyses used the “statmod” software package for the R computing environment (http://www.R-project.org). MaSc frequencies were estimated with a complementary log-log generalized linear model. Two-sided 95% Wald confidence intervals were computed, except in the case of zero outgrowths, when one-sided 95% Clopper-Pearson intervals were used instead. Student’s t test was used. Significance is indicated on the figures with the following convention: p < 0.01. Error bars in all panels represent the standard deviation (SD).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and one table and can be found with this article online at doi:10.1016/j.stem.2010.03.020.

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**REFERENCES**


Badders, N.M., Goel, S., Clark, R.J., Klos, K.S., Kim, S., Bafico, A., Lindvall, C., Williams, B.O., and Alexander, C.M. (2009). The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. PLoS ONE 4, e5694.


