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Report

Lgr5-Expressing Cells Are Sufficient and Necessary for Postnatal Mammary Gland Organogenesis

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SUMMARY

Mammary epithelial stem cells are vital to tissue expansion and remodeling during various phases of postnatal mammary development. Basal mammary epithelial cells are enriched in Wnt-responsive cells and can reconstitute cleared mammary fat pads upon transplantation into mice. *Lgr5* is a Wnt-regulated target gene and was identified as a major stem cell marker in the small intestine, colon, stomach, and hair follicle, as well as in kidney nephrons. Here, we demonstrate the outstanding regenerative potential of a rare population of *Lgr5*-expressing (*Lgr5*$^+$) mammary epithelial cells (MECs). We found that *Lgr5*$^+$ cells reside within the basal population, are superior to other basal cells in regenerating functional mammary glands (MGs), are exceptionally efficient in reconstituting MGs from single cells, and exhibit regenerative capacity in serial transplantations. Loss-of-function and depletion experiments of *Lgr5*$^+$ cells from transplanted MECs or from pubertal MGs revealed that these cells are not only sufficient but also necessary for postnatal mammary organogenesis.

INTRODUCTION

Adult stem cells are characterized by their ability to both self-renew and to differentiate into specialized cells. Unraveling the hierarchy of mammary stem and progenitor cells has been of great interest because the mammary gland (MG) undergoes extensive tissue expansion and remodeling at various phases throughout adult life. Moreover, deciphering the stem cell players contributing to normal mammary development is key to understanding subsequent pathologies, such as cancer transformation. During pubertal development, which happens between 3 and 8 weeks of age in mice, the mammary epithelium undergoes glandular expansion. This yields a branching network of ducts composed of two primary epithelial cell lineages: myoepithelial/basal, and luminal. During pregnancy, the epithelium goes through additional lobuloalveolar differentiation to allow lactation (Deome et al., 1959; Shackleton et al., 2006; Stingl and Caldas, 2007; Stingl et al., 2006; Visvader and Lindeman, 2006; Welm et al., 2003; Woodward et al., 2005). The MG can be regenerated efficiently by transplanting mammary epithelial cells (MECs) into cleared mammary fat pads. Serial transplantation and limiting dilution assays of primary cultures derived from clonal outgrowths have pointed to the existence of a rare subset of mammary cells that function as stem cells and reconstitute functional MGs (Kordon and Smith, 1998). This basal population, which includes mammary stem cells, is characterized by the surface antigen profile Lin$^-$CD24$^+$CD29^{high} or Lin$^-$CD24^{low}CD49f^{high} (Shackleton et al., 2006; Stingl et al., 2006) and is enriched in Wnt-responsive cells (Zeng and Nusse, 2010).

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). The Wnt coreceptor Lrp5 has been described as a marker of mammary stem cells (Badders et al., 2009), and secreted Wnt proteins are proposed as important self-renewal factors for MG stem cells (Zeng and Nusse, 2010). *Lgr5*, a downstream target of Wnt, was identified as a marker of adult stem cell populations in the small intestine, colon (Barker et al., 2007), stomach (Barker et al., 2010), and hair follicle (Barker et al., 2008), organs that undergo extensive postnatal regeneration. Recently, lineage-tracing experiments revealed that *Lgr5*$^+$ stem/progenitor cells also contribute to nephron formation during kidney development (Barker et al., 2012). Here, we unmask the regenerative potential of a rare *Lgr5*-expressing (*Lgr5*$^+$) mammary cell population and its indispensable contribution to pubertal mammary development.

RESULTS

Lgr5 Expression Is Restricted to a Rare Subpopulation of Cytokeratin 14+, Lin$^-$CD24$^+$CD49f^{high} Basal Cells

To identify cells that express *Lgr5* in the MG, we used the *Lgr5* knockin mouse model in which EGFP reporter gene expression is driven by the endogenous *Lgr5* regulatory sequences (Barker

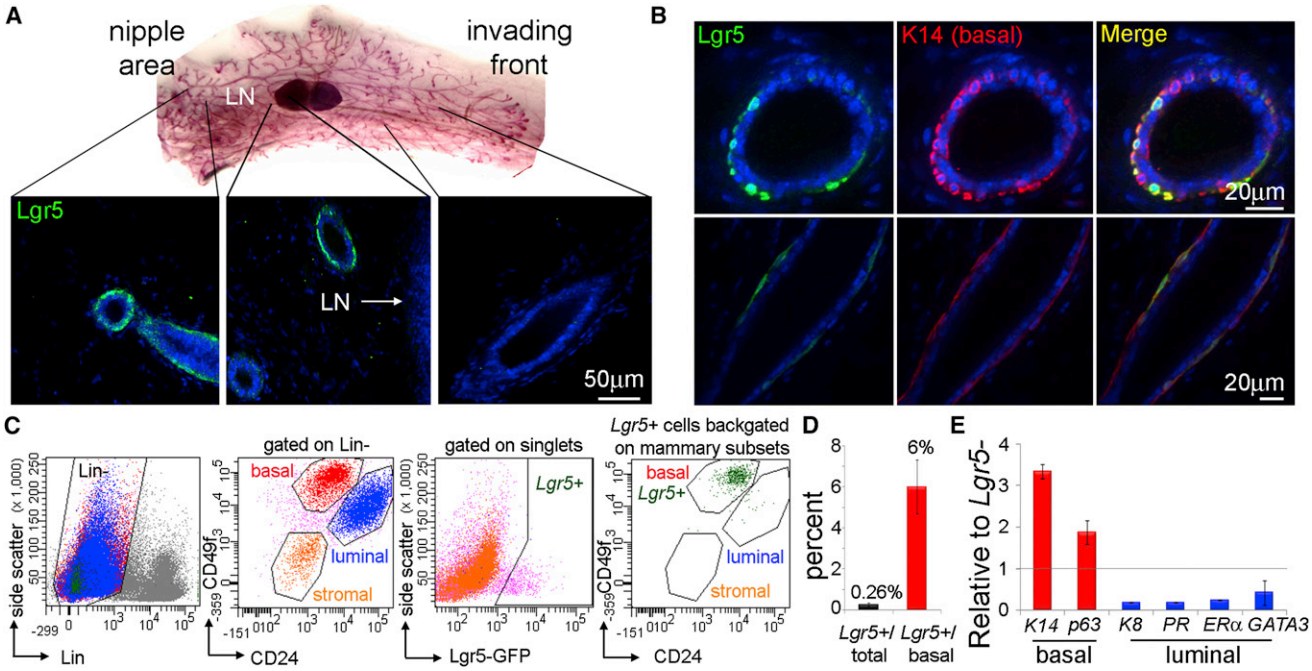

Figure 1. *Lgr5* Expression Is Restricted to a Rare Subpopulation of Cytokeratin 14+, Lin$^-$CD24$^+$CD49f^{high} Mammary Basal Cells

(A) The expression of *Lgr5* was examined in cryosections from 7-week-old *Lgr5*-EGFP MGs with an anti-GFP antibody (green). Carmine stain of a representative MG whole mount demonstrates that *Lgr5*$^+$ ducts are located to the nipple area, but not to the invading front. Around the lymph node (LN), there are some positive and negative ducts.

(B) Cryosections costained with anti-GFP and anti-K14. *Lgr5*$^+$ cells (green) are located to the suprabasal layer of the ducts and are a subpopulation of the myoepithelial K14$^+$ cells (red).

(C) MGs were isolated from *Lgr5*-EGFP mice and analyzed by flow cytometry for the expression of the cell surface markers Ter119, CD45, CD31 (Lin), CD24, and CD49f. *Lgr5*$^+$ cells (GFP$^+$) were part of the Lin$^-$CD24$^+$CD49f^{high} cells (stem cell-enriched population). *Lgr5*$^+$ cells are 0.3% of total mammary cells and 2.5% of Lin$^-$CD24$^+$CD49f^{high}basal cells. GFP$^+$ cells within the luminal population are 0.009% of total.

(D) Summary of flow cytometry data in Figure 1C, *Lgr5*$^+$ cells in 7.5-week-old pubertal female mice, percentage (%) of *Lgr5*$^+$ cells of total (n = 14), and of Lin$^-$CD24$^+$CD49f^{high}basal cells (n = 7). See also Figure S1.

(E) Real-time, quantitative PCR analysis of the *Lgr5*$^+$ cell population (relative to *Lgr5*$^-$ mammary cells) revealed that they are high for basal but not luminal markers. PR, progesterone receptor; ERα, estrogen receptor α. See also Table S1.

Bars represent SE.

et al., 2007). In adult pubertal MGs, only 14% (±2% SE) of ducts had *Lgr5*$^+$ cells, and they were all localized to the nipple side (taking the lymph node as a point of reference), as previously illustrated by Van Keymeulen et al. (2011). The nipple is where the fetal epidermis initially invaginates into the mammary fat pad and is the origin growth point of the mammary epithelium (Figure 1A). *Lgr5*$^+$ cells were a subset of cytokeratin 14-positive (K14$^+$) cells and were localized to the suprabasal position (Figure 1B), similar to that previously described for mammary stem cells by Sleeman et al. (2007). In MGs, adult stem cells have been defined by flow cytometry as a rare subset of Lin$^-$CD24$^+$CD29^{high} (Shackleton et al., 2006) or Lin$^-$CD24^{low}CD49f^{high} basal cells (Stingl et al., 2006), and a subpopulation of such cells exhibits the capacity to regenerate an entire MG in vivo. The vast majority of *Lgr5*$^+$ cells were basal, Lin$^-$CD24$^+$CD49f^{high} (Figure 1C and Figure S1) and were quite rare, comprising 0.26% (one *Lgr5*$^+$ cell per 386 cells) of total dissociated cells in pubertal MGs (Figure 1D). Previous studies have estimated the frequency of mammary stem cells or mammary repopulating units (MRUs) from adult virgin mouse MG to be 1 per 1,400 dissociated cells (for FVB background; Stingl et al., 2006); in contrast, 3%–7% of cells in intestinal crypts express *Lgr5* (Barker et al., 2007). In pubertal glands, among the mammary basal cells, only 6% were *Lgr5*$^+$ (Figure 1D); this was corroborated by the expression profile of *Lgr5*$^+$ cells, which showed high levels of basal but low levels of luminal epithelial markers (Figure 1E).

Within the Lin$^-$CD24$^+$CD49f^{high} Basal Population, *Lgr5*$^+$ Cells Are Highly Potent in Generating Functional Mammary Outgrowths

The analysis described above revealed that *Lgr5*$^+$ cells are a subset of the Lin$^-$CD24$^+$CD49f^{high} basal cells previously reported to include stem cells (Shackleton et al., 2006). To assess MG reconstitution competence, we challenged the *Lgr5*$^+$ cells for mammary regeneration and compared them to *Lgr5*-negative (*Lgr5*$^-$) basal cells in limiting dilution experiments (Figure 2A). In these experiments, we transplanted 10, 50, and 100 *Lgr5*$^+$ versus *Lgr5*$^-$ basal cells into cleared fat pads. The number of cells transplanted was chosen on the lower range to increase the stringency of the assay, focus on a small subset of basal

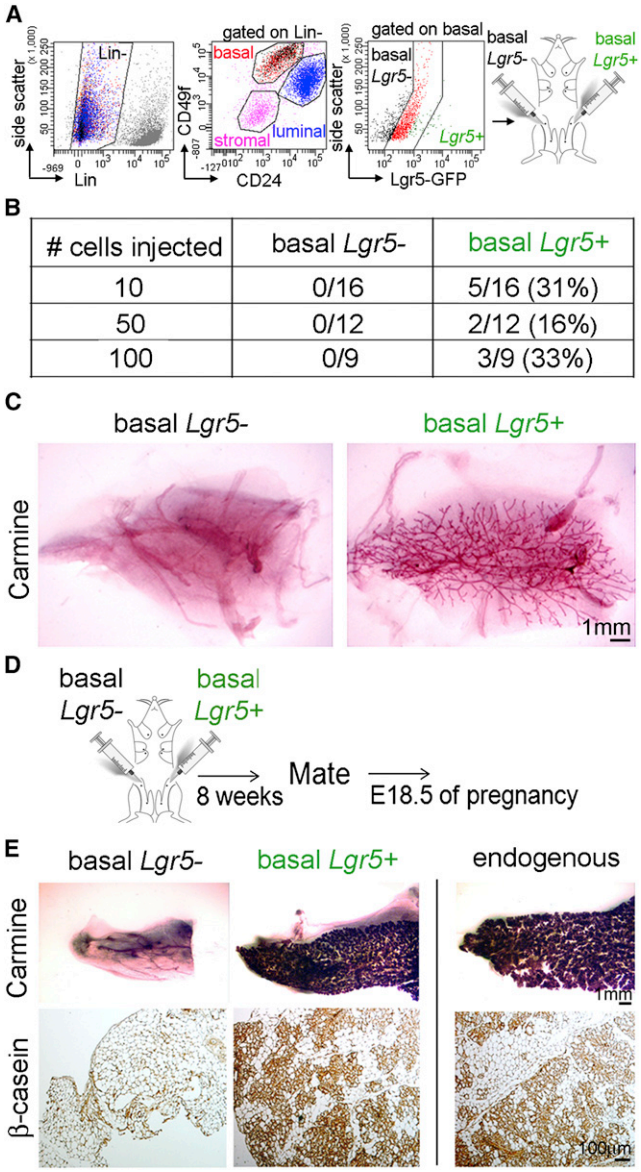

Figure 2. Within the Lin$^-$CD24$^+$CD49f^{high} Basal Population, *Lgr5*$^+$ Cells Are Highly Potent in Generating Functional Mammary Outgrowths

(A) *Lgr5*$^+$ (GFP$^+$) and nonexpressing (GFP$^-$) cells from *Lgr5*-EGFP were isolated by flow cytometry from the Lin$^-$CD24$^+$CD49f^{high} basal population and injected (10, 50, or 100 cells) into cleared mammary fat pads. Outgrowths were analyzed 6 weeks posttransplantation.

(B) Transplanted basal *Lgr5*$^+$ cells have higher numbers of outgrowths compared to the basal *Lgr5*$^-$ cells. Data are pooled from three different experiments.

(C) Whole-mount carmine-stained representative outgrowths show that ten basal *Lgr5*$^+$ cells are able to reconstitute a full MG versus no outgrowth for basal *Lgr5*-transplanted cells.

(D) Mice transplanted with ten *Lgr5*$^+$ cells were mated with males, and their MGs were analyzed on day 18.5 (E18.5) of pregnancy.

(E) Whole-mount carmine-stained mammary epithelial outgrowths from E18.5 pregnant female mice transplanted with ten basal *Lgr5*$^+$ cells that underwent full lobuloalveolar differentiation (basal *Lgr5*$^+$), comparable to the endogenous epithelium in MG #3 of the recipient mouse (upper panels). MG sections from the same mice stained positive for the milk protein, β-casein (lower panels; brown).

See also Figure S2 and Table S2.

cells, and avoid false negatives owing to *Lgr5*$^+$ cells that express low levels of GFP and could therefore be sorted into the *Lgr5*$^-$ group. We found that within the basal population, *Lgr5*$^+$ cells generated MGs far more efficiently than did basal *Lgr5*$^-$ cells. On average, 27% (±5% SE) of *Lgr5*$^+$ cells were able to regenerate a full MG, within the 10–100 cell range, or 1 MRU per 3.7 *Lgr5*$^+$ cells (Figures 2B and 2C). We then tested functionality upon pregnancy (Figure 2D) and found that these outgrowths were able to undergo full lactational lobuloalveolar differentiation and express the milk protein, β-casein (Figure 2E). Characterization of single basal *Lgr5*$^+$ cells versus basal *Lgr5*$^-$ cells revealed that the different functional mammary reconstitution abilities of the two subsets are based on differences in gene expression of lineage differentiation, stem cell, and pluripotency markers, demonstrating that these populations are distinct (Figure S2).

Lgr5$^+$ Cells Can Regenerate a MG from a Single Cell and Maintain a Regenerative Potential through Serial Transplantations

Because *Lgr5*$^+$ cells within the basal cell population were highly efficient in regenerating a full MG in limiting dilution experiments, we tested them for classical stem cell characteristics of multipotency and self-renewal. First, we assessed their ability to regenerate fully differentiated MGs from single cells (Figure 3A). We observed that 13 outgrowths were generated from 54 single *Lgr5*$^+$ transplanted cells (Figure 3B), demonstrating that 24% of *Lgr5*$^+$ single cells were able to regenerate a full MG equivalent to 1 MRU per 4.2 *Lgr5*$^+$ cells. These results are similar to those of the limiting dilution experiments (Figure 2). On close examination, we observed substantial epithelial outgrowth in the mammary fat pads (Figures 3C and S3) and demonstrated that these single transplanted *Lgr5*$^+$ cells were multipotent because they were able to differentiate into both mammary epithelial lineages (myoepithelial/basal K14$^+$ and luminal K8$^+$ cells) (Figure 3D). In addition, when we serially transplanted epithelial outgrowths from primary transplants of *Lgr5*$^+$ cells (Figure 3E), the *Lgr5*$^+$ outgrowths retained their regenerative potential through secondary and tertiary transplants, demonstrating a long-term, regenerative potential (Figures 3F and 3G).

Depletion Experiments Demonstrate that *Lgr5*$^+$ Cells Are Necessary for Postnatal MG Organogenesis

To determine whether *Lgr5*$^+$ cells are not only sufficient but also necessary for postnatal MG organogenesis, we used the *Lgr5*-DTR:GFP mice to deplete *Lgr5*$^+$ cells following administration of diphtheria toxin (DTx) (Figure 4A). This mouse model was used previously to demonstrate the dispensability of intestinal *Lgr5*$^+$ cells under steady-state conditions (Tian et al., 2011). However, depletion of *Lgr5*$^+$ cells from transplanted MECs immediately posttransplantation impaired the outgrowth of *Lgr5*-DTR:GFP donor epithelium, compared to the contralateral MG transplanted with WT MECs (Figure 4B). As an additional control, we found that the majority of MECs from *Lgr5*-DTR:GFP and WT mice not treated with DTx (i.e., in the presence of *Lgr5*$^+$ cells)

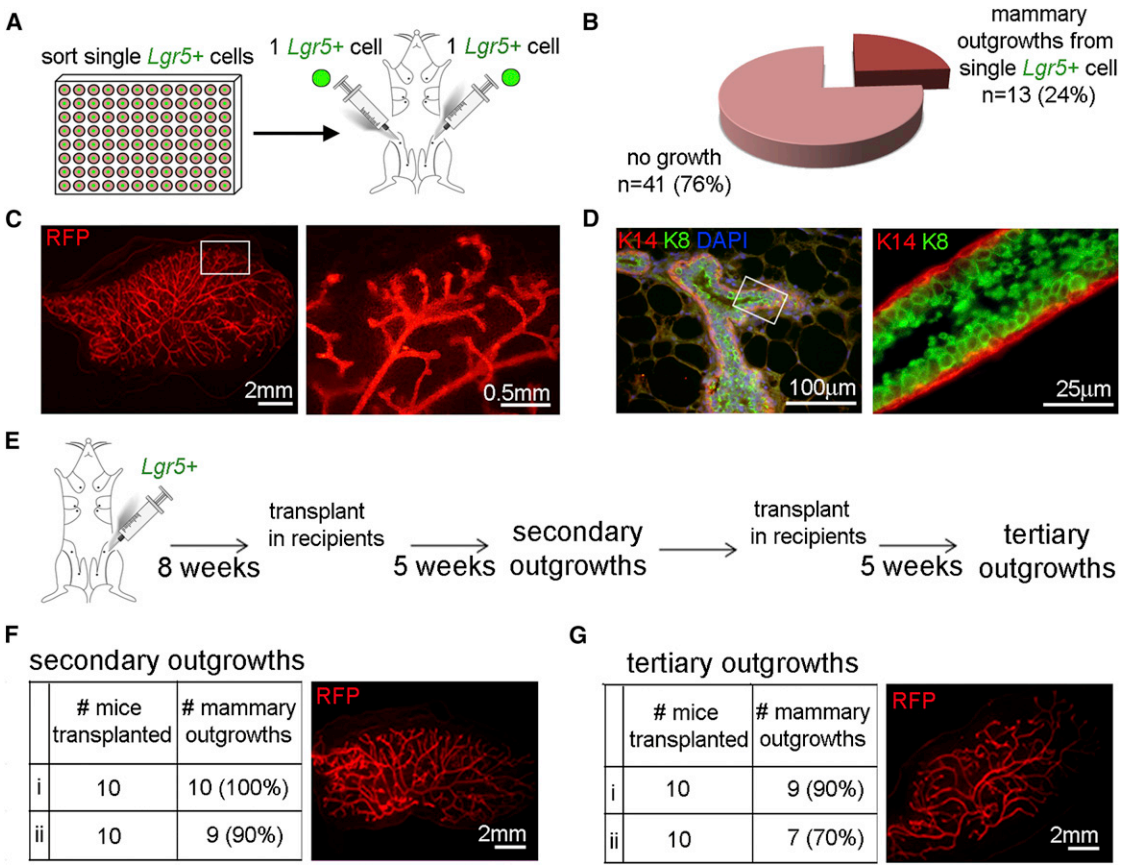

Figure 3. *Lgr5*$^+$ Cells Can Regenerate a MG from a Single Cell and Maintain Regenerative Potential through Serial Transplantations

(A) Single mammary *Lgr5*$^+$ (GFP$^+$) cells from *Lgr5*-EGFP crossed into the LifeAct-RFP mice were isolated by flow cytometry into 96-well plates and transplanted into cleared mammary fat pads. Outgrowths were analyzed at 8 weeks posttransplantation.

(B) From transplants of single adult mammary *Lgr5*$^+$ cells in 54 MGs, 13 mammary outgrowths were observed.

(C) A representative RFP+ mammary outgrowth from a single *Lgr5*$^+$ cell, exhibiting a full epithelial tree (left) with ductal structures at higher magnification of boxed area (right).

(D) Outgrowths from single *Lgr5*$^+$ cells differentiate into the myoepithelial (K14$^+$ in red) and luminal (K8$^+$ in green) lineages (left). Boxed area magnified (right). See also Figure S3.

(E) Mammary outgrowths from two mice transplanted with 100 *Lgr5*$^+$ cells (isolated from *Lgr5*-EGFP crossed into the LifeAct-RFP mice) were collected and retransplanted into ten mice each for secondary and the same for tertiary outgrowths.

(F and G) *Lgr5*$^+$ outgrowths retain their regenerative potential through secondary (F) and tertiary (G) transplants. RFP images are representative of the mammary outgrowths.

were able to reconstitute mammary outgrowth (Figure 4C). Uncleared, endogenous mammary tissue from the WT recipient mice was not affected by DTx administration (Figure S4B). The total outgrowth area for *Lgr5*-DTR:GFP epithelial transplants (including impaired ducts, as shown in Figure S4A) was also significantly reduced in DTx-treated mice relative to the contralateral WT transplants (Figure 4D). These experiments indicate that, although all other epithelial cells were not depleted, the absence of *Lgr5*$^+$ cells was detrimental to adequate MG reconstitution. This protocol allowed targeted MG *Lgr5*$^+$ cell depletion because the recipient mice do not carry the *Lgr5*-DTR:GFP transgene. Mammosphere-forming assays in culture confirmed the indispensability of *Lgr5*$^+$ cells (Figure S4C).

To complement the results above, we examined the role of *Lgr5*$^+$ cells in postnatal MG organogenesis, in a more physiological setting, by injecting DTx to pubertal mice that were either *Lgr5*-DTR:GFP or WT littermates (Figure 5). Depletion of *Lgr5*$^+$ cells during pubertal MG development resulted in impaired ductal invasion (Figures 5A and 5B) and, interestingly, also in a significant reduction in the number of terminal end buds (TEBs) at the epithelial invading front (Figures 5C and 5D), even though *Lgr5*$^+$ cells (Figure 1A) and their lineage-specific progeny (Figures 5E and S5) are absent from the TEBs. In this context, although *Lgr4* has been shown to play a minor role in MG development (Oyama et al., 2011), *Lgr4*+ cells were not interchangeable with *Lgr5*$^+$ cells because a significant phenotype was observed upon *Lgr5*$^+$ cell depletion. These data show that under normal physiology, although all other cells (including additional progenitor cells) were not depleted, the presence of *Lgr5*$^+$ cells is necessary for MG pubertal

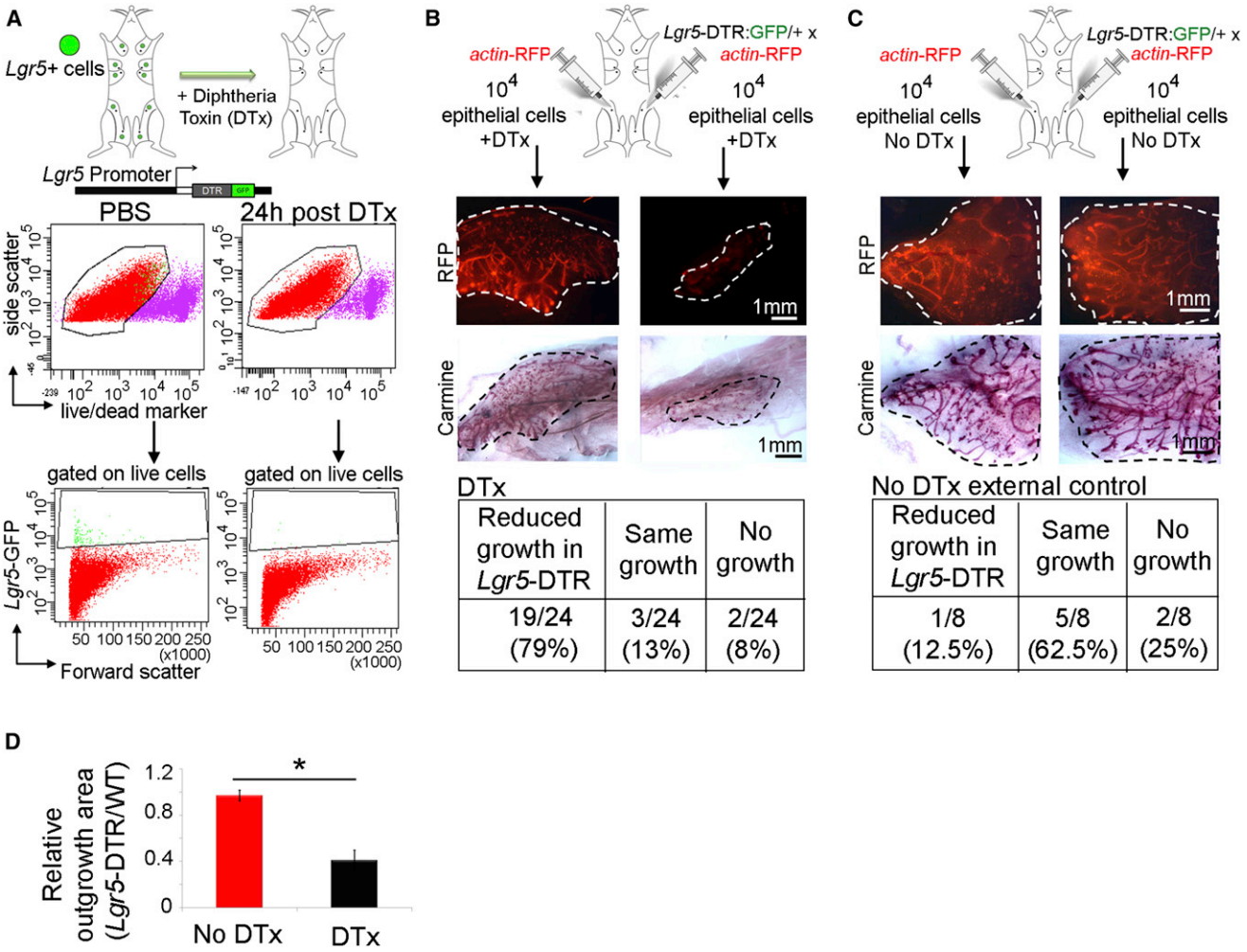

Figure 4. Depletion Experiments Demonstrate that *Lgr5*$^+$ Cells Are Necessary for MG Epithelial Reconstitution

(A) Depletion of *Lgr5*$^+$ cells was achieved utilizing *Lgr5*-DTR:GFP crossed into actin-RFP mice, injected with 50 ng/g BW DTx, analyzed 24 hr post-DTx i.p. (*Lgr5*$^+$ cells are 0.1% of total dissociated mammary cells versus 0% in DTx-injected mice).

(B) Isolated primary MECs of *Lgr5*-DTR:GFP mice or WT littermates transplanted into contralateral precleared mammary fat pads with or without DTx administration. MGs collected 3 weeks posttransplantation had significantly impaired outgrowths in the *Lgr5*-DTR:GFP transplants versus the WT controls.

(C) To assess the growth potential of the *Lgr5*-DTR:GFP and control littermate, mice transplanted with the same cells as in (B) but not treated with DTx reveal no difference between the two contralateral sides.

(D) Outgrowth area for *Lgr5*-DTR:GFP epithelial transplants (including impaired ducts) relative to the contralateral WT transplants is significantly reduced in DTx-treated mice (*p = 0.006). Bars represent SE.

See also Figure S4.

development and reinforce the depletion results in the transplantation setting.

DISCUSSION

Classically, stem cells are characterized by their ability to self-renew as well as to differentiate into specialized cells. According to these criteria, *Lgr5*$^+$ cells have been identified as adult stem cells in the small intestine, colon (Barker et al., 2007), stomach (Barker et al., 2010), and hair follicle (Barker et al., 2008). Our study now shows that *Lgr5*$^+$ cells are also adult stem cells in the MG. By transplantation assays, we demonstrated that most *Lgr5*$^+$ cells are a subset of the basal population previously shown to include the mammary stem cells, exhibiting superior reconstitution capabilities as compared to other cells within that population and are also extremely efficient in regenerating a MG from a single cell. The reconstituted MG epithelial tree was also functional because it was able to undergo adequate differentiation during pregnancy and produce a milk protein. *Lgr5*$^+$ cells were multipotent and maintain regenerative potential in serial transplantations and, therefore, sufficient for postnatal MG organogenesis. They were also necessary for MG organogenesis as shown in depletion assays in both transplantation and physiological settings.

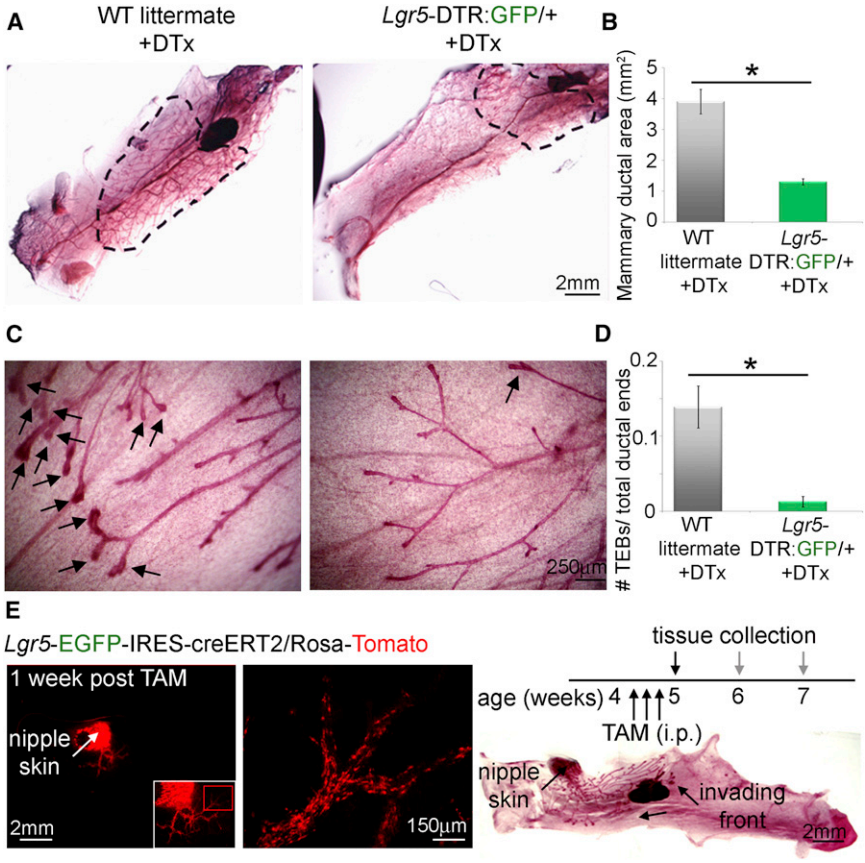

Figure 5. Depletion of *Lgr5*$^+$ Cells during Pubertal Development Results in Impaired Ductal Invasion and TEB Formation

(A) Carmine-stained MG of 4.5-week-old *Lgr5*-DTR:GFP mice (n = 6) or WT littermates (n = 4) that were i.p. injected with DTx demonstrates significantly reduced ductal invasion in the *Lgr5*-DTR:GFP mice.

(B) Quantification of data presented in (A). *p = 0.004.

(C) Depletion of *Lgr5*$^+$ cells from *Lgr5*-DTR:GFP mice resulted in significant reduction in the number of TEBs per MG versus WT littermates. Arrows indicate the TEBs.

(D) Quantification of data presented in (C). *p = 0.0015.

(E) Whole mounts of 5-week-old *Lgr5*-EGFP-IRES-creERT2/Rosa-Tomato mice 1 week past start of Tamoxifen (TAM) induction indicated that *Lgr5*$^+$ cell progeny is close to the nipple area (left) and, according to its localization and shape, mark myoepithelial cells (middle, enlargement of red boxed area in left) and not TEBs in the invading front (carmine-stained tissue on right).

Bars represent SE. See also Figure S5.

The frequency of MRUs was previously estimated to be between 1 MRU per 8–17 cells using transplants of single cells or 1 per 64 cells within the Lin$^-$CD24$^+$CD29^{high} population of mammary cells (Shackleton et al., 2006) or 1 per 60 cells (for FVB background) and 1 per 90 cells (for C57BL/6 background) within the Lin$^-$CD24$^+$CD49f^{high} cells (Stingl et al., 2006) in limiting dilution experiments. More recently, the stem cell frequency within the adult Lin$^-$CD24$^+$CD49f^{high} population was estimated as 1 per 50 cells when coinjected with Matrigel (Spike et al., 2012). The reconstitution capabilities of one per four cells that we observed are remarkable, bringing us closer to obtaining a homogeneous population of MRUs.

Although previous transplant experiments suggested a common progenitor for both major mammary epithelial lineages (myoepithelial/basal and luminal) (Shackleton et al., 2006; Stingl et al., 2006), a recent study that utilized lineage-tracing assays pointed to two different progenitors for these lineages as early as birth (Van Keymeulen et al., 2011) and, therefore, suggested a more restricted fate for the *Lgr5*$^+$cells, which was reinforced in a recent study by de Visser et al. (2012) and also in our study. These data point to important differences between lineage-tracing and transplantation techniques. Indeed, individual stem cells can have different roles under physiological, homeostatic conditions visualized by lineage tracing (van Amerongen et al., 2012), compared to when they are challenged to regenerate an entire organ in the transplant assays (Keller et al., 2011). Thus, lineage-tracing experiments using an *Lgr5-CreER* line show that *Lgr5*$^+$ cells give rise only to myoepithelial cells in pubertal MGs (Van Keymeulen et al., 2011), whereas our transplant experiments demonstrated that a single *Lgr5*$^+$ cell is sufficient to regenerate a complete mammary epithelium and differentiates into both myoepithelial and luminal cells. The transplant assays might therefore uncover a regenerative potential of *Lgr5*$^+$ cells that would be inhibited during MG pubertal development. However, in all the previous studies, depletion of a specific cell population in the presence of all the other cells was not attempted. We now have demonstrated that, in the MG, the unique approach of specific *Lgr5*$^+$ cell depletion resulted in significantly impaired organogenesis, indicating that *Lgr5*$^+$ cells are required during both regeneration from transplanted MECs but also, and more importantly, during physiological pubertal development.

Previous studies indicate that mammary stem cells are likely to be present in any portion of the epithelial branches (Kordon and Smith, 1998). Our study showed that *Lgr5*$^+$ cells, although able to regenerate a full MG, are clustered toward the nipple area in pubertal MGs, where the branching of the epithelium originates, and they or their progeny is not found at the invading front of the ductal tree. However, *Lgr5*$^+$ cell depletion in the transplants resulted in significantly impaired reconstitution, although all other epithelial cells were not targeted for depletion. Moreover, *Lgr5*$^+$ cell depletion during physiological MG organogenesis also resulted in impaired ductal invasion and specifically was characterized by diminished TEBs. TEBs are essential to pubertal MG development and contain additional progenitor populations (as *Axin 2*+ cells; van Amerongen et al., 2012). Our data indicate that even if there are additional stem/progenitor cells that

contribute to MG organogenesis, *Lgr5*$^+$ cells are not only sufficient but also essential for this process and suggest a cross-talk between various stem/progenitor cells during normal MG development.

Stem cells are key for understanding both normal development as well as associated pathologies. In fact, *Lgr5* was first described as a gene expressed in colon cancer cells (van de Wetering et al., 2002). Moreover, it has since been postulated that transformation of *Lgr5*$^+$ stem cells drives malignant progression in the small intestine and colon (Barker et al., 2009), and stem cell activity has been demonstrated in *Lgr5*$^+$ cells in mouse intestinal adenoma (Schepers et al., 2012). *Lgr5* is also overexpressed in other cancers (McClanahan et al., 2006; Oskarsson et al., 2011; Yamamoto et al., 2003), including breast cancer (Oskarsson et al., 2011). The fact that *Lgr5*$^+$ cells are particularly efficient in regenerating a full MG suggests that they could also effectively play an active role in breast cancer once they are transformed. Because Wnt signaling has been implicated in different stages of mammary oncogenesis, future studies should explore the role of *Lgr5*$^+$ cells as breast cancer stem cells. Moreover, R-spondins were recently shown to potentiate Wnt/β-catenin signaling through Lgr5 (Carmon et al., 2011; de Lau et al., 2011; Gong et al., 2012). Because local epithelial R-spondin 1 signaling is required for normal development of the MG (Chadi et al., 2009), future studies evaluating the role of Lgr5 as a receptor for R-spondin during mammary development and cancer are worth pursuing.

EXPERIMENTAL PROCEDURES

Mouse Strains

C57BL/6J (Jackson Laboratories), β-actin-RFP (Long et al., 2005), LifeAct-RFP (Riedl et al., 2010), *Lgr5*-EGFP-IRES-creERT2 (*Lgr5*-EGFP) (Barker et al., 2007), *Lgr5*-DTR:GFP (Tian et al., 2011), and Ai14 Rosa-Tomato (Madisen et al., 2010) mice were bred and maintained in the UCSF animal facility according to IACUC guidelines. All mice were maintained in C57BL6J background. β-Actin-RFP and LifeAct-RFP reporter mice were used interchangeably to specifically identify and visualize mammary outgrowths from the donor mice.

Mammary Cell Preparations

MGs were dissected from pubertal (7- to 9-week-old) female mice. For flow cytometry and limiting dilution experiments, after mechanical dissociation with a scalpel, the tissue was placed in culture medium (DMEM/F12 with 5 ng/ml insulin and 50 ng/ml gentamycin (UCSF Cell Culture Facility) containing 2 mg/ml collagenase-1 (Sigma-Aldrich), and digested for 30 min at 37°C. The resulting suspension was sequentially resuspended in 2 U/μl DNase for 3 min at room temperature (RT), washed and dissociated with 2 ml 0.05% trypsin/EDTA (UCSF Cell Culture Facility) for 10 min at 37°C, and filtered through a 70 μm filter. Erythrocytes were lysed with Red Blood Cell Lysis Buffer (protocol ID PS00000002; Gilman et al., 2002) for 1 min at RT. For the DTx depletion experiments, epithelium-enriched organoids were prepared as described previously by Ewald et al. (2008), then dissociated with 2 ml 0.05% trypsin/EDTA and filtered as described above.

Cell Labeling, Flow Cytometry, and Sorting

Antibodies against the mouse antigens CD45, CD31, TER119, CD49f, and CD24 were purchased from eBioscience. For the single-cell transplants, single *Lgr5*-GFP$^+$ cells were sorted into 96-well plates in minimal medium plus 2.5 nM FGF2 (Ewald et al., 2008). Flow cytometry was performed with the use of LSRII for data analysis and FACS ARIA for cell sorting (BD Biosciences).

Mammary Fat Pad Transplantation

Cleared fat pads from 3-week-old female nude mice (Simenson) were transplanted with 1–100 MECs in 50/50 Matrigel/minimal medium plus 2.5 nM FGF2 (Ewald et al., 2008). The tissues cleared from the MGs were carmine stained as described below to validate adequate clearing of the native epithelium (to ensure that the native epithelium had not yet reached the lymph node). The transplanted mammary epithelium was allowed to grow from 3 to 8 weeks, and mammary outgrowths were analyzed by whole-mount staining with carmine, whole-mount fluorescence, or flow cytometry. For the secondary and tertiary transplants, pieces of mammary fat pad containing epithelium were transplanted into cleared fat pads from 3-week-old female nude mice. Mammary outgrowths were analyzed 5 weeks after transplants. Outgrowths were considered positive when the epithelium invaded at least half of the fat pad. For single-cell transplants and serial transplantation experiments, *Lgr5*-EGFP-IRES-creERT2 mice were crossed into the LifeAct-RFP reporter mice, and for *Lgr5* depletion experiments, *Lgr5*-DTR:GFP mice were crossed into the β-actin-RFP reporter mice to allow easier and reliable detection of outgrowths.

Histochemistry, Immunohistochemistry, and Immunofluorescence

Mammary whole mounts were stained with carmine Alum (Sigma-Aldrich). Cryo- or paraffin sections from the inguinal (#4) MGs of *Lgr5*-EGFP mice or from mammary outgrowths were labeled using the following primary antibodies: GFP (Abcam; ab5450, 1:200); cytokeratin 14 (Covance; PRB-155P, 1:500); cytokeratin 8 (Troma 1, Developmental Studies Hybridoma Bank, Iowa; 1:50); and β-casein (ABBIOTEC; #250558, 1:200).

Real-Time PCR

Sorted cell populations were lysed, and RNA was extracted using a QIAGEN mini kit (74104). cDNA synthesis was performed using the Invitrogen SuperScript III system (18080-051), and quantitative reverse-transcription PCR was done via the SYBR Green (Applied Biosystems; 4309155) method and an Eppendorf Realplex Mastercycler. Primer sequences are listed in Table S1. Primers were purchased from SABiosciences. Relative quantification of gene expression was calculated according the Pfaffl method. Target gene expression in each cell subpopulation was normalized to HPRT and GAPDH reference gene expression. The data reported are one representative experiment of three independent sorting and quantitative reverse-transcription PCR experiments.

DTx-Mediated Cell Depletion

Mammary fat pads from 3-week-old female nude mice (Simenson) were cleared to remove all endogenous epithelium, and the recipient mice were allowed to grow bigger before transplantation and therefore become more resilient to DTx toxicity. Four to 5 weeks later, 10^4 MECs from *Lgr5*-DTR:GFP or WT littermates were contralaterally transplanted into precleared fat pads in Matrigel/minimal medium plus 2.5 nM FGF2, 1:1 (Ewald et al., 2008) containing 1 μg/ml DTx (Sigma-Aldrich), or no DTx in external controls, to achieve immediate but local *Lgr5*$^+$ cell depletion. After 6 days, mice were injected intraperitoneally (i.p.) with 50 ng/g body weight (BW) DTx, three times/week for 1.5 weeks to maintain *Lgr5*$^+$ cell depletion throughout the experiment. Mammary tissue was collected 3 weeks posttransplantation, which is sufficient time to yield mammary outgrowths. Due to possible DTx toxicity at the concentration of 50 ng/g BW, which allows full *Lgr5*$^+$ depletion in the mammary, the treatment regimen above could not be prolonged further to allow outgrowths to fully progress, so the internal controls of outgrowths from WT cells, which are also subjected to DTx, serve as a reference to the *Lgr5*-DTR:GFP outgrowths. The external control group was i.p. injected with PBS under a similar regimen.

In a separate set of experiments, 4.5-week-old *Lgr5*-DTR or WT littermates were injected i.p. with 50 ng/g BW DTx, three times per week for 1.5 weeks. Inguinal MGs were retrieved, carmine stained, and the ductal-invaded area was calculated. Calculation was done using ImageJ software—the ductal area calculated is demarcated (the lymph node is the point of reference for ductal invasion). Additionally, TEBs were manually counted directly from MG whole mounts. Due to the possible effect of DTx depletion on additional organs in *Lgr5*-DTR:GFP mice and DTx toxicity, these experiments could not be prolonged beyond the current endpoint.

In Vivo Tamoxifen Induction

Four-week-old *Lgr5*-EGFP-IRES-creERT2/Rosa-Tomato female mice were i.p. injected with 5 mg of Tamoxifen (Sigma-Aldrich) diluted in sunflower oil (Sigma-Aldrich) every other day for a total of 3 days (15 mg total), as indicated in Van Keymeulen et al. (2011). MGs were collected at 5, 6, and 7 weeks of age, and Cre induction was assessed by whole-mount fluorescence while epithelial outgrowths were visualized by carmine staining.

For further details, see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.12.017.

LICENSING INFORMATION

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