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Lgr5 marks cycling, yet long-lived, hair follicle stem cells

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In mouse hair follicles, a group of quiescent cells in the bulge is believed to have stem cell activity. Lgr5, a marker of intestinal stem cells, is expressed in actively cycling cells in the bulge and secondary germ of telogen hair follicles and in the lower outer root sheath of anagen hair follicles. Here we show that Lgr5$^+$ cells comprise an actively proliferating and multipotent stem cell population able to give rise to new hair follicles and maintain all cell lineages of the hair follicle over long periods of time. Lgr5$^+$ progeny repopulate other stem cell compartments in the hair follicle, supporting the existence of a stem or progenitor cell hierarchy. By marking Lgr5$^+$ cells during trafficking through the lower outer root sheath, we show that these cells retain stem cell properties and contribute to hair follicle growth during the next anagen. Expression analysis suggests involvement of autocrine Hedgehog signaling in maintaining the Lgr5$^+$ stem cell population.

The skin is the largest organ in the mammalian body, allowing interaction with and providing protection from the surrounding world. The interfollicular epidermis is constantly self-renewing, but the hair follicles undergo cyclic changes of growth, involution and resting phases[1]. Mouse hair follicle stem cells are thought to reside in the hair follicle bulge and are characterized by expression of the CD34 cell-surface marker, expression of cytokeratin 15 (K15)[2–5] and retention of either DNA or histone labels over long periods[6,7]. Keratinocytes with stem cell properties have also been isolated from other areas of the hair follicle[8,9]. At certain hair follicle stages, the lower portions of mouse vibrissa follicles contain cells that can reconstitute a complete hair follicle upon transplantation[10], arguing for a flux of stem cells retaining multipotency. Thus, the bulge might not be the only reservoir of stem cells.

We recently found that the leucine-rich G protein–coupled receptor 5 (Lgr5) marks rapidly cycling stem cells in the small intestine and colon[11]. Lgr5 is an orphan seven-transmembrane-domain receptor with similarity to thyroid-stimulating hormone, follicle-stimulating hormone and luteinizing hormone receptors[12]. *Lgr5* was originally identified as a Wnt/Tcf4 target gene expressed in colon cancer[13,14]. The control of self-renewal in intestinal crypts and hair follicles shares many regulatory characteristics, including a prominent role of the Wnt cascade[15]. The gene expression profiles of telogen bulge cells have been documented[3,5,7,16], and we noted that *Lgr5* was among the most highly enriched genes in bulge cells in at least one of these studies[5]. These findings suggested the existence of a common stem cell marker between these developmentally distinct tissues.

Here we show that Lgr5 is expressed in the lower bulge and secondary germ area of mouse telogen hair follicles and in the lower outer root sheath (ORS) of anagen hair follicles. These cells seem to be the first to proliferate at the induction of anagen and continue to proliferate until formation of the new hair follicle is complete. Notably, Lgr5-expressing cells isolated from mouse skin showed potent self-renewal and hair follicle reconstitution ability, contradicting the current view that hair follicle stem cells are relatively quiescent and located in the bulge of telogen hair follicles. Consistent with this, the progeny of Lgr5-expressing keratinocytes maintained hair follicles over more than 14 months and repopulated hair follicle compartments previously shown to contain hair follicle stem cells. The proliferative population of Lgr5$^+$ cells expressed genes characteristic of hair follicle stem cells as well as genes indicative of active Hedgehog (Hh) signaling.

RESULTS

Expression of Lgr5 in mouse hair follicles

To characterize expression of Lgr5 in hair follicles, we used two different knockin mouse models. In the first, termed *Lgr5*^{LacZ}, exon 18 of *Lgr5* contains an integrated *Ires-LacZ/Neo* fusion sequence, and in the second, termed *Lgr5-EGFP-Ires-CreERT2*, the endogenous *Lgr5* promoter controls expression of enhanced green fluorescent protein (EGFP) and the CreERT2 fusion protein[11] (**Fig. 1**). In late embryogenesis (embryonic day (E)18.5), we detected LacZ-positive cells (Lgr5$^+$) in the ORS of the larger forming hair follicles (**Supplementary Fig. 1a** online), whereas at postnatal day (P)14, when mouse hair follicles are in the growth phase (anagen), expression of LacZ was localized to the lower part of the ORS surrounding the hair follicle (**Fig. 1a**,**d**). At P20, when hair follicles are in the destructive (catagen) phase, Lgr5$^+$ cells linked the mesenchymal component of the hair follicle (the dermal papilla) with the bulge area (**Fig. 1b**,**e**), whereas in the hair follicle resting (telogen) phase, Lgr5 expression was

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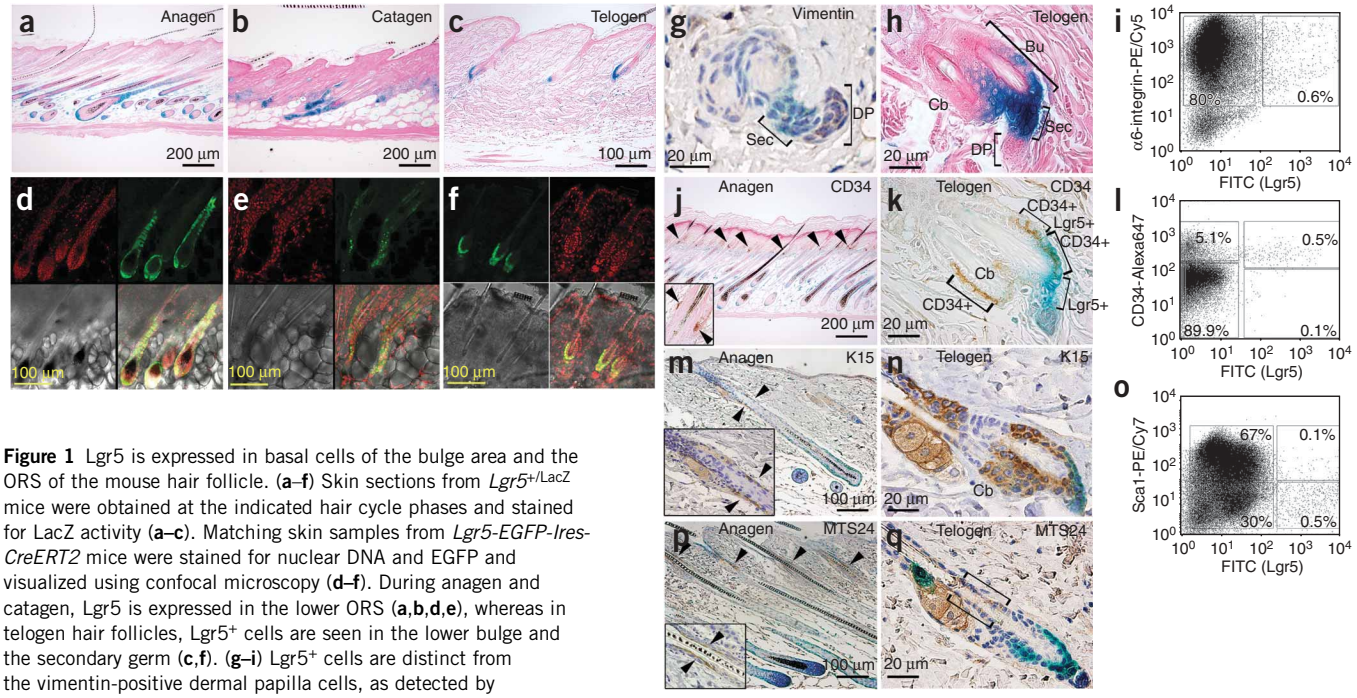

Figure 1 Lgr5 is expressed in basal cells of the bulge area and the ORS of the mouse hair follicle. (**a**–**f**) Skin sections from *Lgr5*$^{+/LacZ}$ mice were obtained at the indicated hair cycle phases and stained for LacZ activity (**a**–**c**). Matching skin samples from *Lgr5-EGFP-Ires-CreERT2* mice were stained for nuclear DNA and EGFP and visualized using confocal microscopy (**d**–**f**). During anagen and catagen, Lgr5 is expressed in the lower ORS (**a**,**b**,**d**,**e**), whereas in telogen hair follicles, Lgr5^{+} cells are seen in the lower bulge and the secondary germ (**c**,**f**). (**g**–**i**) Lgr5^{+} cells are distinct from the vimentin-positive dermal papilla cells, as detected by immunohistochemistry (**g**), are located mostly in the newly formed hair follicle but not in the club hair at 8 weeks of age (**h**) and express the basal cell marker α6-integrin, as detected by flow cytometry (**i**). FITC signal intensity reflects LacZ activity. (**j**–**n**) CD34^{+} and Lgr5^{+} populations do not overlap in anagen hair follicles (**j**) but show a partial overlap in telogen hair follicles, as detected by immunohistochemistry (**k**) and FACS analysis (**l**). Similarly, Lgr5^{+} cells overlap partially with K15^{+} cells in the bulge in telogen (**n**) but not in anagen (**m**). (**o**) The majority of Lgr5^{high} cells are negative for Sca1, as detected by FACS. (**p**,**q**) Lgr5^{+} cells and MTS24-positive cells represent distinct cell populations in anagen (**p**) and telogen (**q**). (**j**,**m**,**p**,**q**) Arrowheads and brackets indicate positively staining cells. (**j**,**m**,**p**) Insets show positively staining areas at higher magnification. Cb, club hair; DP, dermal papilla; Bu, bulge; Sec, secondary germ.

concentrated to the bulge area (**Fig. 1c**,**f**). Moreover, we detected strong LacZ expression in a small area between the vimentin-positive dermal papilla and the bulge, known as the secondary germ (**Fig. 1g**,**h**). These cells may be able to germinate the regrowth of the lower hair follicle in the next anagen phase and repopulate the bulge stem cell niche after injury[8], and thus may not yet be fully committed to a hair follicle fate.

To facilitate analysis of Lgr5-LacZ expression in the live keratinocyte population from mice in the telogen phase, we used FACS to detect and sort β-galactosidase expressing cells, which are denoted as Lgr5^{high} (ref. 17). Flow cytometry analysis showed that approximately 80% of keratinocytes isolated from 7- to 8-week-old mice expressed medium to high levels of the basal marker α6-integrin, whereas FITC-labeled Lgr5^{high} cells constituted around 0.6% of the cells, all belonging to the α6-integrin medium-high compartment (**Fig. 1i**). Expression of the stem cell marker CD34 showed substantial overlap with the Lgr5^{+} cell population in telogen hair follicles, as detected by immunochemistry and flow cytometry (**Fig. 1k**,**l**). However, although 80% of the Lgr5^{high} cells were positive for CD34, those cells constituted only 10% of the CD34^{+} cell population. In contrast, in the anagen hair follicle, CD34^{+} and Lgr5^{+} cell populations did not overlap, as the CD34^{+} population remained in the bulge area whereas Lgr5^{+} cells were found in the lower ORS (**Fig. 1a**,**j**).

K15 is a marker of hair follicle stem cells[5], and the telogen hair follicle bulge contained a well defined K15^{+} cell population that overlaps partially with the Lgr5^{+} cell population (**Fig. 1n**). Similar to CD34^{+} cells, the K15^{+} cell population remained in the bulge area when the hair follicle entered the anagen phase, and the overlap with Lgr5^{+} cells was lost (**Fig. 1m**). Whereas CD34^{+} and K15^{+} cells are situated in the bulge, the MTS24 antibody, which recognizes the Plet-1 glycoprotein[18], marks a proliferating cell population that is located at the level of the sebaceous gland and has been attributed stem cell properties[9]. As expected, Lgr5^{+} cells showed no overlap with the MTS24-positive cell population in any hair cycle phase (**Fig. 1p**,**q**). Sca1, a marker for hematopoietic stem cells, marks mainly basal cells with low clonogenic potential in the interfollicular epidermis and infundibulum[19–21]. Consistent with their differential localization, Lgr5^{high} cells were low in Sca1 expression (**Fig. 1o**). This is also in line with the observation that *Ly6a* mRNA, which encodes Sca1, was expressed at considerably lower levels (see below). Lgr5^{+} cells thus constitute a distinct cell population in the mouse hair follicle, which in telogen includes a subpopulation of the CD34^{+} and K15^{+} cell compartment.

Lgr5^{+} cells proliferate in response to anagen induction

Given that Lgr5 marks a cycling stem cell population in the intestine[11], it was of interest to investigate whether the Lgr5^{+} skin cells reside within a cycling cell population. To that end, we stained skin samples obtained from *Lgr5*$^{+/LacZ}$ mice at various hair cycle phases with an antibody recognizing the proliferation marker Ki67. In telogen hair follicles, Ki67^{+} cells could be detected in the isthmus, but the bulge areas were generally not proliferative (**Fig. 2a**). Notably, in some hair follicles presumed to be in the late telogen or pre-anagen state, the secondary germ area contained Ki67^{+}Lgr5^{+} cells (**Fig. 2b**), suggesting that Lgr5^{+} cells are the first to respond to the anagen-initiating signals originating from the dermal papilla or follicular environment[22]. In early anagen, proliferation started in the Lgr5-expressing secondary germ area (**Fig. 2c**), and these cells subsequently formed the anagen

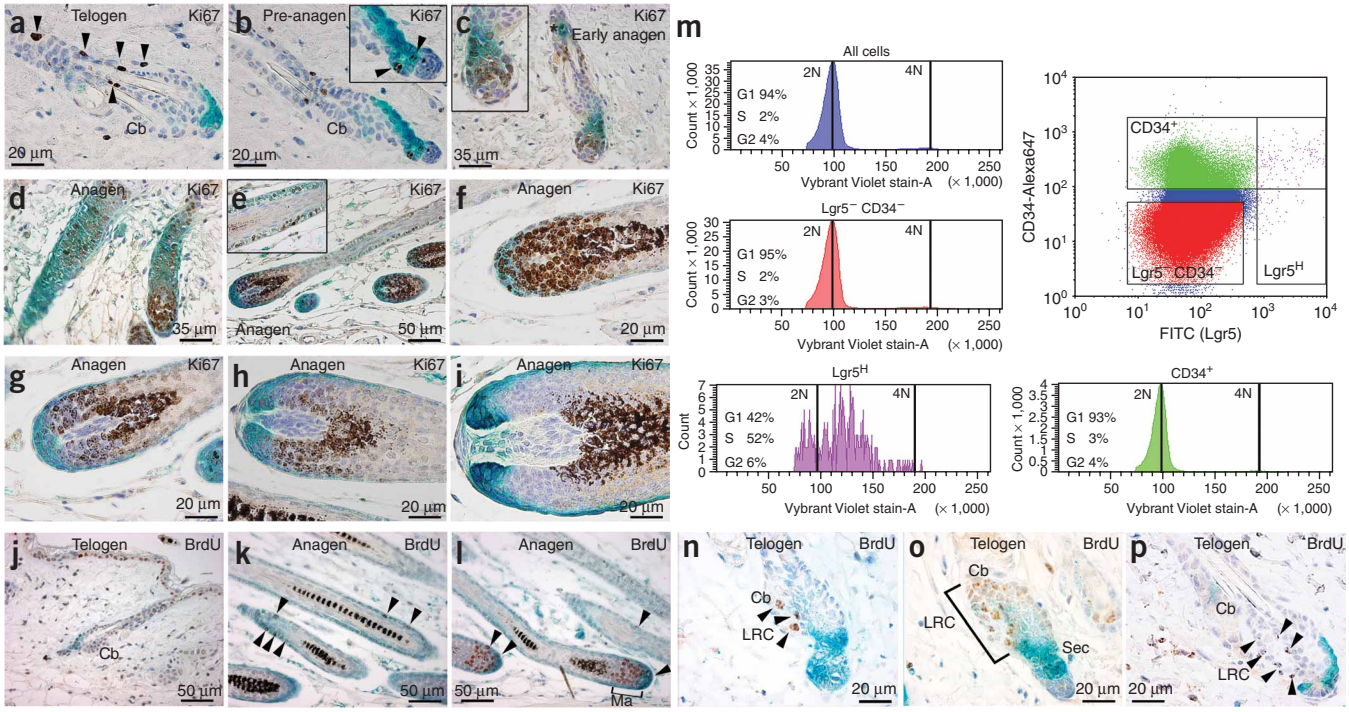

Figure 2 Lgr5$^+$ cells are the primary proliferating cells at the onset of anagen and are distinct from LRCs. (**a**–**l**) In the resting hair follicle (age 7 weeks), the cells in the bulge area do not express the proliferation marker Ki67 (**a**) and do not incorporate BrdU (**j**). Proliferative cells are located in the upper portion of the hair follicle (**a**, arrowheads). At the initiation of anagen, Ki67$^+$ nuclei appear among the Lgr5$^+$ cells above the dermal papilla in the secondary germ (**b**, arrowheads). In early anagen, the Ki67$^+$Lgr5$^+$ cell population expands (**c**) and forms the proliferating ORS of the growing hair follicle (**d**,**k**). During anagen, Ki67$^+$ cells are present in the Lgr5$^+$ ORS and in the matrix (**e**–**h**). Active proliferation is reduced first in the ORS (**h**) and then in the matrix (**i**) when the hair follicle reaches its maximal size. BrdU labeling of the proliferating cells for 1 h confirmed the lack of proliferation in the telogen hair follicles (**j**) and the presence of DNA-synthesizing Lgr5$^+$ cells in the ORS (**k**, arrowheads) and the matrix (**l**, arrowheads) of anagen hair follicles. (**m**–**p**) Cell cycle analysis of skin keratinocytes at the onset of anagen (10 weeks of age) shows that Lgr5^{high} (Lgr5H) cells are in G_1 and S phases, whereas CD34$^+$ and Lgr5$^-$CD34$^-$ cells are mainly in G_1 (**m**). LRCs marked by BrdU labeling at P10–12 (**n**,**o**) or at P3–5 (**p**) and detected at 8 weeks of age are distinct from Lgr5$^+$ cells. Cb, club hair; Ma, matrix; Sec, secondary germ. (**b**,**c**,**e**) Insets show positively staining cells at higher magnification.

hair follicle containing highly proliferative Lgr5$^+$ cells in the ORS (**Fig. 2d**,**e**). In anagen hair follicles, proliferative cells were concentrated in the bulb, where both Lgr5$^+$ cells in the outer cell layer and Lgr5$^-$ matrix cells expressed Ki67 (**Fig. 2f**,**g**). In late anagen, when the hair follicle reached its maximal size, proliferation stopped in Lgr5$^+$ and Lgr5$^-$ cells (**Fig. 2h**,**i**), preparing the hair follicle for subsequent involution.

To verify the data obtained by Ki67 staining, we injected bromodeoxyuridine (BrdU) 1 h before collecting skin samples. In telogen (8 weeks of age), the BrdU label was detectable only in the interfollicular epidermis, confirming the overall quiescence of hair follicle keratinocytes (**Fig. 2j**). As expected, during anagen, the Lgr5$^+$ lower ORS contained BrdU-positive cells all along its length (**Fig. 2k**,**l**), confirming those cells' proliferative status. This pattern of expression and proliferation of Lgr5$^+$ cells was markedly reminiscent of the previously proposed model for stem cell trafficking from the bulge to the lower part of vibrissa hair follicles[10]. The Lgr5 expression pattern in vibrissae is identical to that of anagen hair follicles in dorsal skin (**Supplementary Fig. 1b–f**), suggesting that analogous trafficking of stem cells occurs in dorsal hair follicles.

We next used cell cycle analysis to further assess proliferation. Consistent with the immunohistochemistry data obtained from telogen hair follicles, most cells in the populations analyzed were in the G_1 phase (**Supplementary Fig. 1g**). However, as soon as anagen was initiated, many Lgr5^{high} cells entered S phase (**Fig. 2m**), whereas CD34$^+$ and Cd34$^-$Lgr5$^-$ cells remained in G_1.

To analyze the potential overlap between Lgr5 expression and long-term label-retaining cells (LRCs), we used two different approaches. First, we injected pups with BrdU at two different time points during the first postnatal anagen phase (P3–5 and P10–12) and analyzed skin samples at the age of 8 weeks. BrdU label was retained mainly in the bulge area of the club hair, whereas the Lgr5 (LacZ)-positive area of the bulge was largely devoid of LRCs (**Fig. 2n–p** and **Supplementary Table 1** online).

Lgr5$^+$ cells are potent stem cells *in vitro* and *in vivo*

To evaluate the clonogenicity and hair follicle regeneration ability of Lgr5$^+$ cells, we used FACS coupled with *in vitro* and *in vivo* growth assays (**Fig. 3**). Sorted Lgr5^{high} cells isolated from telogen mouse skin (**Fig. 3a**,**b**,**i**) formed large colonies with high efficiency. After passaging, these cells maintained the growth of holoclones (55 of the initial 80 colonies, or ~68% efficiency; **Fig. 3c**), which are suggested to be derived from stem cells[23]. Lgr5^{high}CD34$^+$ keratinocytes showed enhanced colony-forming ability, whereas the colony-forming ability of the Lgr5$^-$CD34$^+$ population was similar to that of the Lgr5$^-$CD34$^-$ population (**Fig. 3d**,**e**). The *in vitro* growth properties of the Lgr5^{high} cells were thus consistent with a stem cell function.

One of the fundamental properties of adult stem cells is their ability to reconstitute all of the cells that form the tissue of origin. To test hair follicle reconstitution, we sorted Lgr5^{high}, Lgr5$^-$, CD34$^+$ and CD34$^-$ cells and combined them with wild-type fibroblasts, followed by transplantation onto the backs of nude mice. Again, the Lgr5^{high}

keratinocytes constituted the most potent population in regenerating hair follicles (**Fig. 3f**), whereas CD34$^+$ cells initiated hair follicle growth with approximately 10% of the efficiency of Lgr5^{high} cells. Donor origin was ascertained by sex-mismatched transplantations using donor keratinocytes from male mice, followed by hybridization with a Y-chromosome probe (**Fig. 3g**). Hair follicles formed from Lgr5^{high} cells contained all the main cellular layers, expressing markers for the ORS (K5); the Huxley, Henle and companion layers (K6); the inner root sheath (Gata3); and the hair shaft and cuticle (AE13; **Fig. 3h**). We concluded that the Lgr5^{high} cell population shows a marked enrichment for multipotent stem cells.

Progeny of Lgr5$^+$ cells maintain hair follicles

Stem cells in a tissue should be able to maintain the tissue over time by providing both differentiated cells and new stem cells. To investigate whether cells marked by Lgr5 fulfill such stringent criteria, we took advantage of the *Lgr5-EGFP-Ires-CreERT2* knockin mice[11]. These mice were crossed with the *Rosa26-LacZ* reporter strain, in which *LacZ* is preceded by a *lox*P-stop-*lox*P sequence. To mark Lgr5-expressing cells and their progeny, we injected *Lgr5-EGFP-Ires-CreERT2/Rosa26-LacZ* mice with tamoxifen to activate Cre recombinase at P21, when hair follicles transition from catagen to a short telogen (**Fig. 4a**). At this time point, Lgr5$^+$ cells were located in the bulge and secondary germ

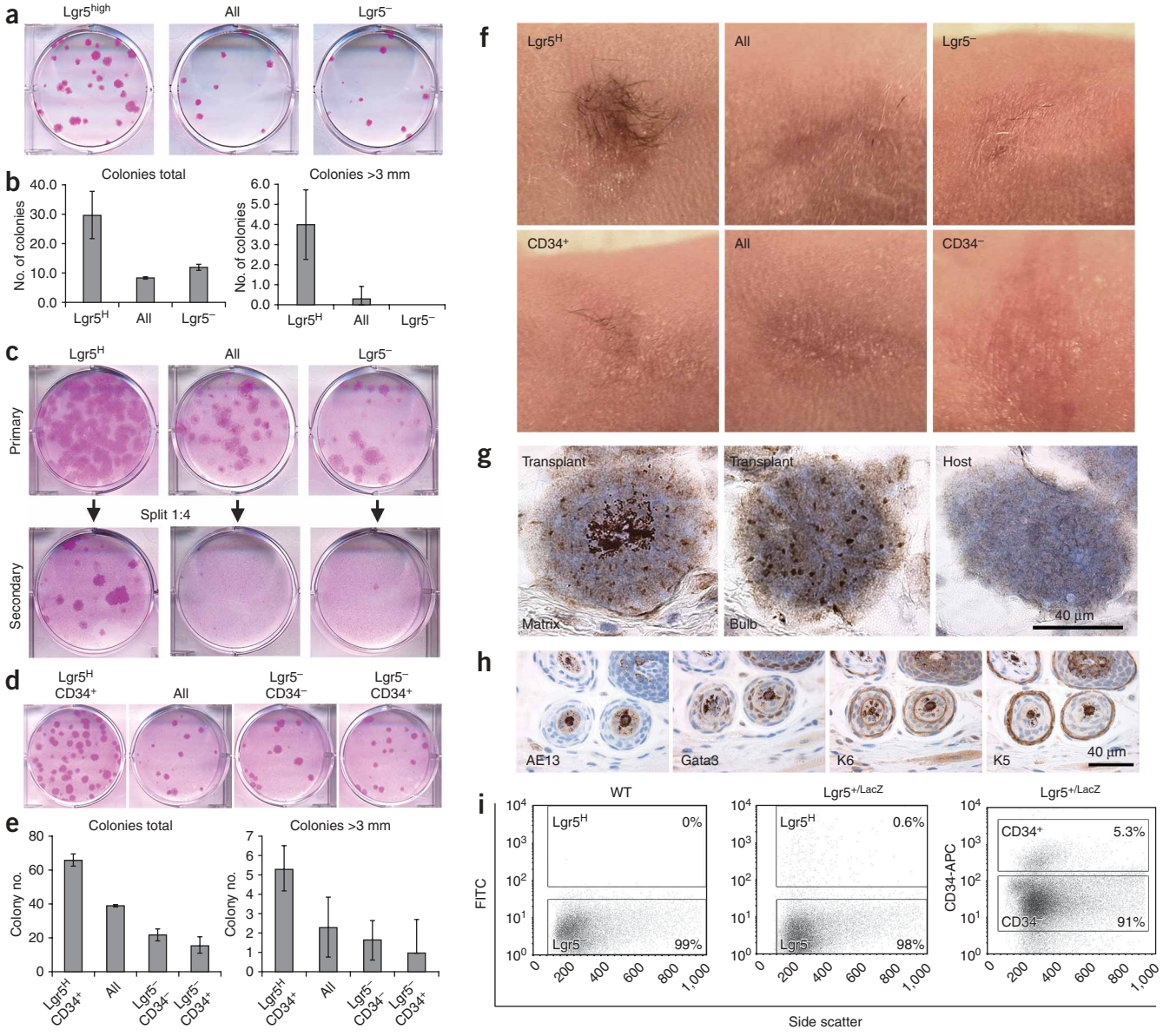

Figure 3 The Lgr5$^+$ cell population contains functional hair follicle stem cells. (**a**–**e**) Lgr5$^+$ cells efficiently form primary (**a**,**b**) and secondary (**c**) colonies *in vitro*. The CD34$^+$ cell population depleted of Lgr5^{high} (Lgr5^H) cells has substantially reduced colony-forming ability (**d**,**e**). The sorting gates used for **d** are shown in **Figure 1l**. (**f**) Lgr5^{high} cells reconstitute fully formed hair follicles with high efficiency. Lgr5^{high}, 78 hair follicles (mean of $n = 2$); CD34$^+$, 11 hair follicles (mean of $n = 2$). (**g**) Positive Y-chromosome staining in newly formed hair follicles confirms male donor origin. (**h**) Reconstituted follicles contain all major hair follicle components. AE13, hair shaft cuticle; Gata3, inner root sheath; K6, Huxley and Henle layers and inner inner root sheath; K5, ORS. (**i**) Sorting gates used for colony and transplantation assays (except **d**). Keratinocytes isolated from wild-type (WT) mice were used as a negative control for setting the sort gates. The gate 'All' encompasses all cells on the plot and is not shown. Error bars, s.d.

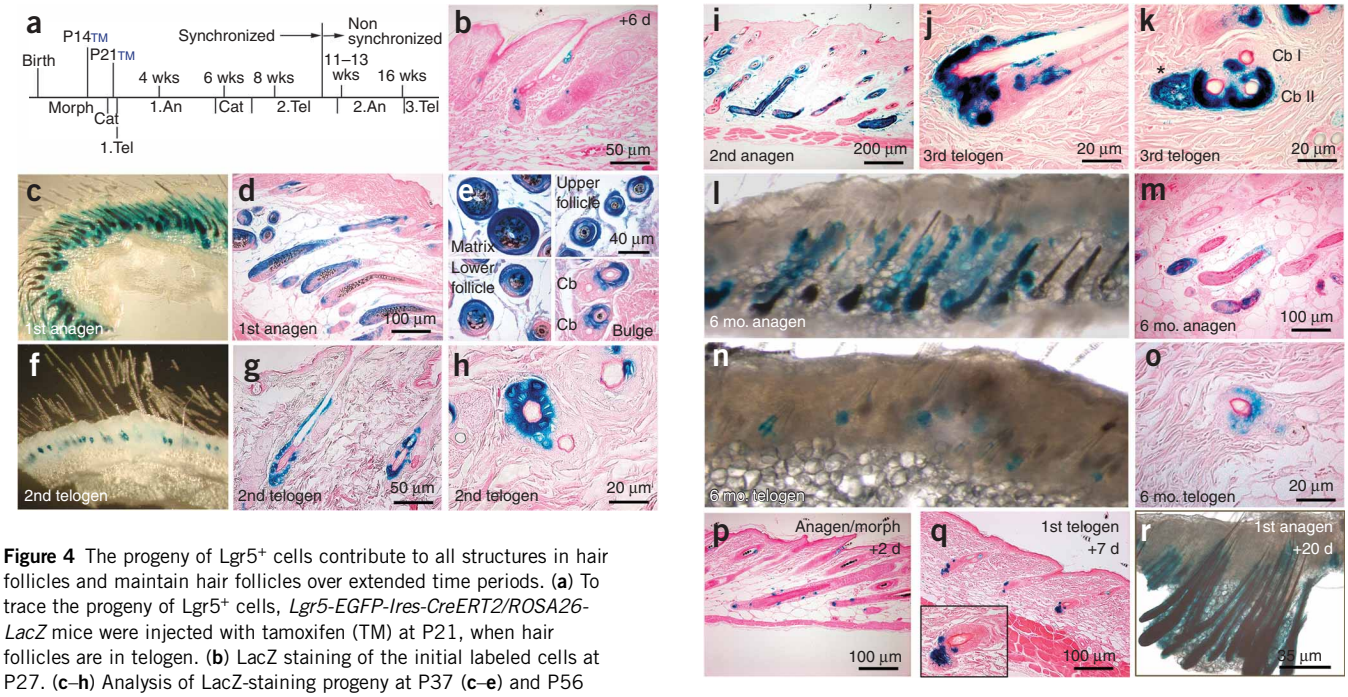

Figure 4 The progeny of Lgr5$^+$ cells contribute to all structures in hair follicles and maintain hair follicles over extended time periods. (**a**) To trace the progeny of Lgr5$^+$ cells, *Lgr5-EGFP-Ires-CreERT2/ROSA26-LacZ* mice were injected with tamoxifen (TM) at P21, when hair follicles are in telogen. (**b**) LacZ staining of the initial labeled cells at P27. (**c**–**h**) Analysis of LacZ-staining progeny at P37 (**c**–**e**) and P56 (**f**–**h**) revealed contributions to all parts of the hair follicles below the sebaceous gland, as assessed in longitudinal (**d**,**g**) and transverse (**e**,**h**) sections of hair follicles. **c**,**f** show whole-mount pictures of LacZ-stained skin. (**i**–**k**) Progeny of Lgr5$^+$ cells were present in the ORS of hair follicles at 13 weeks of age (**i**) and in the bulge area of the second club hair at 16 weeks (**j**, longitudinal section; **k**, transverse section). (**l**–**o**) Competence of Lgr5$^+$ cells for self-renewal is indicated by the presence of LacZ-stained progeny in anagen and telogen hair follicles of 6-month-old mice. Shown are whole-mount images (**l**,**m**) and longitudinal (**n**) and transverse (**o**) sections. (**p**–**r**) Progeny of anagen Lgr5$^+$ cells localized outside of the bulge area in mice injected with tamoxifen at P14. Initial labeling of hair follicles at P16 (**p**) contributes to the bulge of the hair follicle in the following telogen at P21 (**q**) and the first anagen at P34 (**r**). Morph, morphogenesis; An, anagen; Cat, catagen; Tel, telogen. Cb, club hair; *, nonspecific LacZ staining of the sebaceous gland.

areas of the hair follicle (**Figs. 1c**,**f** and **4b** and **Supplementary Fig. 2a** online). In the subsequent synchronized anagen phase at P37, most hair follicles were marked by LacZ expression from the bulge to the hair bulb (**Fig. 4c**–**e**), which is the part of the hair follicle that is newly formed during each hair cycle. Lgr5$^+$ cell progeny were sometimes also present in the hair follicle isthmus area. All hair follicle layers contained LacZ-positive cells, showing that the Lgr5$^+$ cells present in the telogen bulge and secondary germ can give rise to all the different hair follicle layers (**Fig. 4d**,**e**). The upper permanent part of the hair follicle (at the level of the sebaceous gland opening and higher) and the interfollicular epidermis never contained LacZ-positive cells, showing that Lgr5$^+$ stem cells during normal conditions do not contribute to these compartments. Analysis at the second postnatal telogen at P56 showed prominent labeling of the bulge area and secondary germ (**Fig. 4f**–**h**), consistent with self-renewal of Lgr5-marked stem cells. The progeny of Lgr5$^+$ cells marked at P21 contributed to hair follicles at the second anagen (age 13 weeks; **Fig. 4i**) and to the telogen hair follicle at the third telogen (age 16 weeks; **Fig. 4j**,**k**). When the Lgr5$^+$ cells were marked at P21, no contribution to the club hair was seen, as that hair follicle was formed during postnatal development of the hair follicle before the activation of Cre recombinase (**Fig. 4k**, CbI). In contrast, at the third telogen, the hair follicle formed during the second anagen contained progeny of the P21-marked cells (**Fig. 4k**, CbII). Longer-term maintenance was confirmed in a separate experiment by following mice up to 6 months after tamoxifen treatment during the second telogen (**Fig. 4l**–**o**), where progeny of Lgr5$^+$ cells were present both in anagen and telogen hair follicles (progeny of Lgr5$^+$ cells were present in hair follicles more than 14 months after tamoxifen administration; data not shown). Activity of Cre recombinase was very tightly regulated in this system, as no LacZ-expressing, lineage-traced cells were detected in 280-day-old mice not treated with tamoxifen (**Supplementary Fig. 2b**–**d**).

To address the question of whether the Lgr5$^+$ cells forming the ORS of the anagen hair follicle represent functional stem cells, we injected 14-day-old *Lgr5-EGFP-Ires-CreERT2/Rosa26-LacZ* mice with tamoxifen. At P14, Lgr5 was expressed in the ORS of the elongating hair follicle in dorsal skin (**Fig. 1a**). Two days after tamoxifen injection, labeled cells were seen in the ORS below the bulge region, consistent with the Lgr5 expression pattern (**Fig. 4p** and **Supplementary Fig. 2e**). Seven days later, the progeny of the labeled cells were present in the telogen hair follicles, predominantly in the secondary germ and the lower part of the bulge (**Fig. 4q**); moreover, we saw a notable contribution to the lower part of the hair follicle in the subsequent anagen 20 d after tamoxifen injection (**Fig. 4r**). This observation strongly suggests that Lgr5$^+$ cells are a dynamic population of stem cells migrating in the hair follicle during anagen and that the secondary germ contains stem cells originating in the lower ORS during the preceding anagen.

The progeny of Lgr5$^+$ cells can contribute to the permanent parts of the hair follicle up to the level of the sebaceous gland (**Fig. 4**), as previously reported for secondary germ cells in the case of injury[8]. To address this in more detail, we treated *Lgr5-EGFP-Ires-CreERT2/Rosa26-LacZ* mice with a low dose of tamoxifen at P21 to induce rare labeling of hair follicles (**Fig. 5a**–**c**). The rare nature of initial labeling was verified by serial sectioning of skin samples obtained from the same mouse (**Supplementary Figs. 2f** and **3** online). The percentage of labeled hair follicles did not change significantly during the course of the experiment (**Supplementary Table 2b** online), supporting the

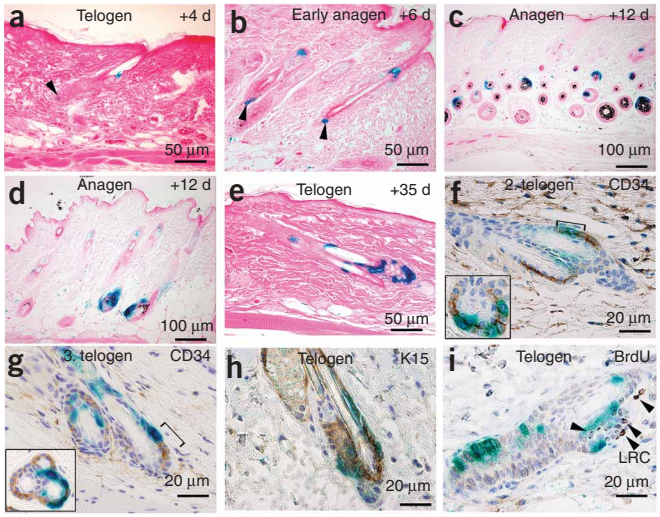

Figure 5 Progeny of the Lgr5$^+$ cells repopulate the permanent part, the bulge and the secondary germ of the hair follicle. (**a**,**b**) Mice were injected with a low dose of tamoxifen (0.5 mg) at P21, which overall resulted in one or no labeled cell per hair follicle. (**c**–**e**) Progeny of the labeled cells contribute to the ORS and the hair bulb of the anagen hair follicle (**c**, cross section; **d**, longitudinal section) and to various parts of the next telogen hair follicle (**e**). (**f**–**h**) The repopulated areas contain cells positive for CD34 (**f**,**g**) and K15 (**h**). (**i**) Lgr5-expressing cells and their progeny do not overlap with the LRCs labeled at P3–5 with BrdU.

conclusion that all labeled cells were revealed at day 4 after tamoxifen treatment. We observed apparent clonal expansion of Lgr5$^+$ cell progeny in anagen hair follicles 8 d after tamoxifen administration (**Supplementary Fig. 4a** online); at 12 d after injection, the progeny of Lgr5$^+$ cells were seen in the ORS of the anagen hair follicles (**Fig. 5c**,**d**). In the next telogen, labeled cells were found in the secondary germ, the bulge and the isthmus of the hair follicles (**Fig. 5e**).

To determine whether Lgr5$^+$ cells can give rise to cells expressing known stem cell markers, we stained skin samples obtained from 8-week-old, tamoxifen-treated (at P21) *Lgr5-EGFP-Ires-CreERT2/Rosa26-LacZ* mice with antibodies recognizing K15, MTS24 and CD34. Labeled progeny of Lgr5$^+$ cells were observed in all parts of the bulge area and in the MTS24-positive isthmus region (**Fig. 5f–h** and **Supplementary Fig. 5b**,**c** online). A contribution of Lgr5$^+$ cell progeny to the CD34-expressing cell compartment was also evident in the bulge area in 16-week-old mice (**Fig. 5g**). To test whether an anagen growth phase is required for the repopulation of the bulge and isthmus, we injected 8-week-old mice with high doses of tamoxifen (5 mg/d for 2 d) and followed the mice for 4 weeks. During the course of the experiment, labeled cells appeared only in the area where Lgr5-expressing cells normally reside in telogen hair follicles, indicating that active cell proliferation during anagen is required for repopulation of the hair follicle bulge and isthmus by the progeny of Lgr5$^+$ cells (**Supplementary Fig. 5** and **Supplementary Table 2a**). Further evidence for a limited overlap between Lgr5$^+$ cells or their progeny and LRCs was obtained by lineage tracing of Lgr5$^+$ cells in skin labeled with BrdU at P3–5 (**Fig. 5i** and **Supplementary Table 1**), supporting the conclusion that the Lgr5$^+$ cell population is actively cycling.

Stem cell markers and active Hh signaling in Lgr5^{high} cells

To begin to define the properties of Lgr5$^+$ cells at the molecular level, we analyzed expression of genes with known importance in regulation of the hair cycle and hair follicle stem cells in Lgr5^{high} and basal Lgr5$^-$ keratinocytes (**Fig. 6a**). As expected, expression levels of *Lgr5* and *Cd34* mRNA were high in the Lgr5^{high} population, and levels of *Lgr5* mRNA were below the detection threshold in the Lgr5$^-$ population. Overall, the observed expression pattern was consistent with previous observations, including enhanced expression of *Tcf3*, no change in expression of the Wnt target gene *Axin2*, increased expression of *Lhx2* and reduced expression of *Klf5* in Lgr5^{high} cells[7,16]. Notably, the expression level of *Tcf4*, known to be a positive regulator of *Lgr5* in the intestine, was increased, consistent with a role for Wnt-pathway signaling in stem cell regulation.

Hh signaling is essential for hair follicle formation and growth during anagen[24,25]. The transcription factors Gli1, Gli2 and Gli3 are the terminal effectors of the Hh pathway, and Gli1 is itself a sensitive target gene for Hh signaling[26]. Lgr5^{high} cells expressed elevated levels of *Gli1* and *Gli2* mRNA, indicative of Hh pathway activity. This

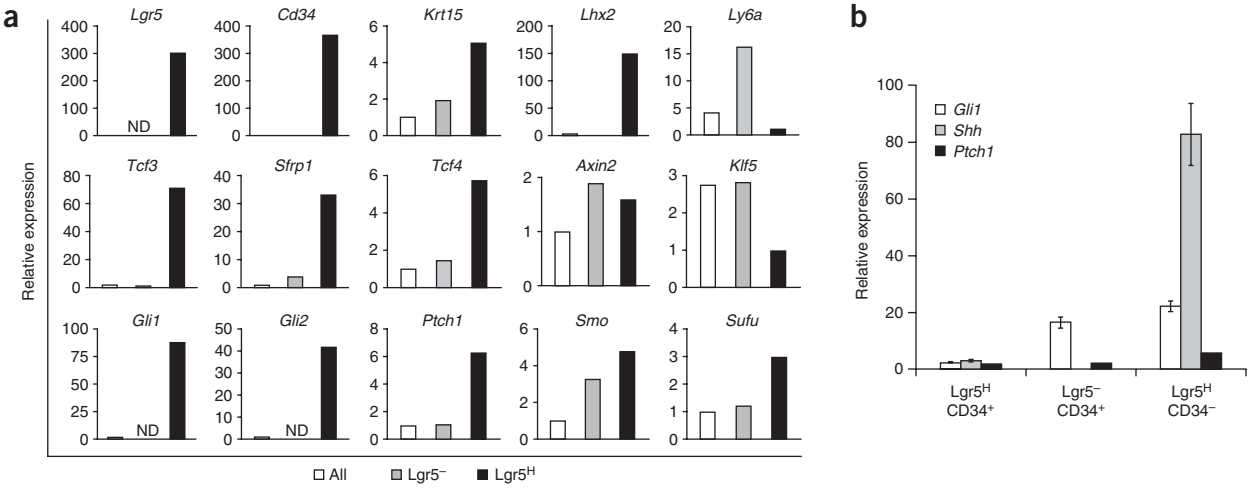

Figure 6 Lgr5$^+$ cells show a pattern of stem cell gene expression, including markers of active Hh signaling. (**a**) mRNA isolated from sorted Lgr5^{high}, Lgr5$^-$ and All keratinocytes (sort gates shown in **Fig. 1i**) was analyzed with regard to expression of known stem cell markers using real-time PCR. Data were normalized to *Arbp* expression and presented as fold change. (**b**) Expression of *Shh*, *Ptch1* and *Gli1* was analyzed using mRNA isolated from Lgr5- and/or CD34-expressing cell populations (sort gates shown in **Fig. 1l**). Data were normalized to the mean of *Hprt* and *Actb* expression. Error bars, s.d.

finding was further supported by expression of additional Hh pathway components, indicating competence in receiving and transducing Hh signals. To address a potential cellular source of Hh ligand, we tested the differentially sorted CD34 and Lgr5^{high} populations with regard to Hh ligand expression. Results from repeated experiments revealed that only *Shh*, specifically detected in the Lgr5^{high} population, was expressed (data not shown). To further refine the analysis, we took advantage of gene-specific amplification followed by real-time PCR. Expression of *Shh* was slightly increased in the CD34^{+}Lgr5^{high} cell population compared to the CD34^{+}Lgr5^{-} population, but highly increased in the CD34^{-}Lgr5^{high} population (**Fig. 6b**). These data and the corresponding alterations in *Gli1* expression suggest that Hh signaling is activated in an autocrine manner in CD34^{-}Lgr5^{high} cells, located adjacent to the dermal papilla, and in a paracrine manner in CD34^{+}Lgr5^{-} cells. The biological importance of Hh signaling in the Lgr5^{+} stem cells is as yet unknown, but it is possible that a low level of Hh signaling, along with enhanced expression of the Wnt signaling effector proteins Tcf3 and Tcf4, are required for stem cell maintenance and survival, and that high-level Hh signaling during the anagen phase drives proliferation and hair follicle fate.

DISCUSSION

Our study reveals the existence of a dynamic population of hair follicle stem cells marked by Lgr5. Under normal conditions, these cells maintain the cycling part of the hair follicle and can contribute to all hair follicle structures, including the bulge and isthmus, which are known to contain distinct stem and progenitor cell populations. Similar to previously isolated hair follicle stem cell populations, Lgr5^{+} cells can give rise to sebocytes in transplantation assays (**Supplementary Fig. 4d,e**), indicating their multipotential nature. The localization of Lgr5^{+} stem cells to the bulge area and secondary germ in telogen, as well as to the lower ORS down to the matrix in the growing hair follicle, is consistent with previously proposed models regarding the presence and flux of stem and progenitor cells[5,8,10]. Notably, Lgr5^{+} cells cycle regularly, in contrast to the LRC population commonly believed to harbor stem cells[2,7,27]. However, this observation is consistent with a recent report of active cellular turnover in the CD34^{+} cell compartment[28]. Furthermore, analysis of a mouse model lacking bone morphogenetic protein signaling suggests that CD34 expression and label retention are not obligatory markers of hair follicle stem cells[29]. Lgr5 defines such a cycling stem cell population; taken together, these observations call for a reinvestigation of the relationship between LRCs and hair follicle stem cells. As recently proposed for olfactory neural stem cells[30], stem cells within the LRC population, when present, may represent a reserve population activated after tissue damage, whereas under normal conditions, a cycling population of stem cells maintains homeostasis. Our data showing that Lgr5^{+} cells and their progeny proliferate during anagen, retain stem cell competence during hair follicle growth and contribute to the secondary germ upon catagen support the notion that the secondary germ contains an active stem cell population, as originally suggested by Dry *et al.*[31] and Chase *et al.*[32]. Additionally, our findings imply that stemness is an intrinsic property of hair follicle stem cells and does not depend on a specific niche. Such intrinsic or innate pluripotency was also recently described in mouse embryonic stem cells[33].

A point of particular interest is the repopulation of hair follicle compartments not containing Lgr5-expressing cells by progeny of Lgr5^{+} stem cells. The previously mentioned active proliferation of bulge cells during hair follicle cycling[28] is likely to be an underlying factor, consistent with our observation that completion of the anagen stage is required for repopulation to occur. We cannot determine with certainty whether a single Lgr5^{+} stem cell can give rise to progeny residing both in the lower part of the hair follicle and in the upper bulge area. However, we think this is a likely scenario given experiments using a low dose of tamoxifen, where traced cells are derived from one or a few cells in each hair follicle. Future experiments will be necessary to resolve this issue.

Even when only a few Lgr5^{+} cells are initially labeled after tamoxifen treatment during telogen or anagen, a substantial fraction of Lgr5^{+} cell progeny is present at the next anagen and telogen. This is likely to be a consequence of the fact that descendants of only around four

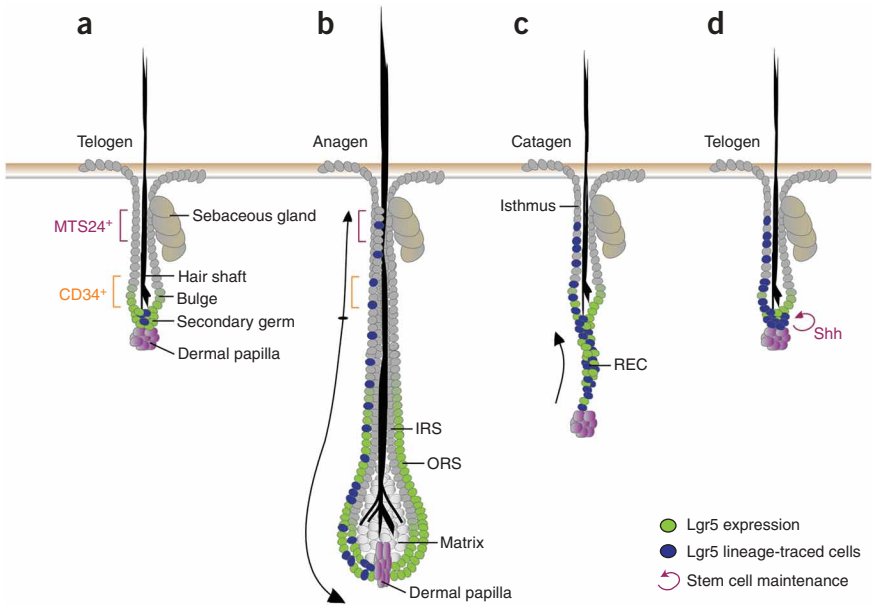

Figure 7 Proposed model showing migration and repopulation of the hair follicle by Lgr5-expressing stem cells and their progeny. (**a**) Lgr5-expressing cells (green) partly overlap with CD34^{+} cells but are distinct from MTS24^{+} cells. Lgr5 expression forms a gradient from the lower bulge to the secondary germ. Lgr5^{+} cells (blue) in the lower bulge and secondary germ initially undergo Cre-mediated labeling after tamoxifen treatment in telogen. (**b**) In anagen, the Lgr5^{+} cells start to proliferate and migrate, with progeny contributing to all structures of the anagen hair follicle. At this stage, Lgr5^{+} cells do not overlap with the CD34^{+} cell population. (**c**) In catagen, a subpopulation of the Lgr5^{+} cell progeny survive and move upwards; the regressing epithelial column (REC) and lower bulge now contain Lgr5^{+} cell progeny, which again express Lgr5. (**d**) In the following telogen, the secondary germ, bulge and permanent part of the hair follicle contain progeny of Lgr5^{+} cells. *Shh* is selectively expressed in Lgr5^{+}CD34^{-} cells, with a suggested role in stem cell maintenance and survival. Black arrows indicate cell migration. IRS, inner root sheath; ORS, outer root sheath.

progenitor cells are required to build a complete anagen hair follicle[34]. In support of this number, we observed in tracing experiments using a low dose of tamoxifen that Lgr5$^+$ cell progeny often contributed to either no part, one-quarter or one-half of a hair follicle (**Fig. 5c**). Assuming that the most potent progenitor cells expressed the highest level of Lgr5 (and thereby Cre recombinase), a contributing factor may be preferential labeling of this cell population as the dose of tamoxifen is lowered. An alternative explanation, which cannot be excluded, is that the cells did not initially accumulate detectable β-galactosidase activity but nonetheless underwent a recombination event at the *Rosa26* locus. However, based on the analysis of LacZ expression at various time points after tamoxifen treatment, we believe this is a less likely scenario.

In summary, our working model (**Fig. 7**) suggests that the progeny of the Lgr5$^+$ cells contribute to all layers of the anagen hair follicle, providing a constant flux of stem cells downward in the direction of the dermal papilla during the growth phase. When the hair follicle is regressing, the surviving cells move up, and one fraction loses Lgr5 expression and repopulates compartments known to contain other stem or progenitor cell populations (repopulation of these compartments by progeny of Lgr5$^+$ cells residing in the bulge is also possible). Another fraction reconstitutes the secondary germ, supporting the notion that Lgr5 expression defines a 'primitive' stem cell population able to give rise to all cell types in the mouse hair follicle.

METHODS

Transgenic mice and treatments. Transgenic mice were maintained within the animal facilities at Karolinska Institutet and the Hubrecht Institute, and experiments were carried out according to the national rules and regulations of Sweden and The Netherlands, respectively. The generation of *Lgr5*^{LacZ} and *Lgr5-EGFP-Ires- CreERT2* transgenic mice is described in detail elsewhere[11]. The *Rosa26-LacZ* reporter mice were obtained from The Jackson Laboratory. Mice were fed *ad libitum*.

To mark LRCs, 50 mg/kg BrdU was injected intraperitoneally six times, in 12-h intervals, at the indicated age. To mark replicating cells, 100 mg/kg BrdU was injected intraperitoneally 1 h before mice were killed. Cre recombinase was activated in *Lgr5-EGFP-Ires-CreERT2/Rosa26-LacZ* mice by injecting the indicated dose of tamoxifen (20 mg/ml dissolved in sunflower oil) once or twice in a 24-h interval.

β-Galactosidase assay, immunohistochemistry, confocal imaging and Y-chromosome staining. Freshly obtained skin samples were fixed for 30 min at room temperature (22 °C) in PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde. Samples were then washed three times for 15 min each with rinse buffer (2 mM $MgCl_2$ and 0.1% Nonidet P40 in PBS) and stained for 36–48 h in a solution consisting of 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in rinse buffer. The samples were embedded in paraffin, sectioned at 4 μm and stained with eosin or subjected to immunohistochemical detection of various antigens. Sections were rehydrated, followed by antigen retrieval in 10 mM citrate buffer (30 min at 97 °C) and incubation with primary antibody for 1 h at room temperature. Subsequently, sections were incubated with the corresponding biotinylated secondary antibody (1:200, Vector Laboratories) and then with horseradish peroxidase–streptavidin conjugate (Zymed). Signal was detected using the Zymed DAB Plus Substrate kit according to the manufacturer's instructions, and counterstaining was carried out with Mayer hematoxylin where indicated. For immunohistochemistry with monoclonal mouse antibodies, the HistoMouse-SP kit (Zymed) was used according to the manufacturer's directions. The following primary antibodies were used: rat antibody to mouse CD34 (1:50; Abcam), monoclonal mouse antibody to Ki67 (1:50; Novacastra), goat antibody to mouse vimentin (1:100; Santa Cruz Biotechnology), mouse monoclonal antibody AE13 to acidic hair keratins (1:50; a gift from T.T. Sun, New York University School of Medicine), mouse monoclonal antibody to Gata3 (1:100; Santa Cruz Biotechnology), rabbit polyclonal antibodies to cytokeratins 5 and 6 (both 1:1,000; Babco), antibody to K15 (1:100; Abcam), MTS24 antibody (1:50; a gift from R. Boyd, Monash University) and mouse monoclonal antibody to BrdU (1:25; BD Biosciences).

For Y-chromosome detection, a biotinylated mouse-specific Y-chromosome probe (StarFISH, Cambio) was used on formaldehyde-fixed, paraffin-embedded sections according to the manufacturer's instructions.

For confocal imaging, skin samples were fixed in formalin for 30 min at room temperature and embedded in 4% low-melting agarose. Longitudinal sections between 100 and 200 μm thick were prepared using a vibratome. Sections were then permeabilized in PBS supplemented with 1% BSA, 1% dimethylsulfoxide and 0.1% Triton X-100, stained for 30 min with TO-PRO (1:500; Invitrogen) and embedded using Vectashield (Vector Labs). Sections were imaged with an SP5 confocal microscope (Leica) and processed using Volocity (Improvision) and Photoshop CS2 (Adobe) software.

Flow cytometry, cell sorting and colony-forming assays. Mouse dorsal skin keratinocytes were collected as described[35]. Briefly, 8-week-old mice ($n = 3$–5) were killed, dorsal hair was clipped and dorsal skin was taken and placed into calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS; Sigma). Subcutaneous tissue and fat were removed by gentle scraping, and the skin was cut into strips and incubated at 32 °C in 0.25% trypsin solution in HBSS for 2 h. The epidermis was then scraped into S-minimal essential medium (MEM; Invitrogen) supplemented with 0.2% BSA and 0.02% soybean trypsin inhibitor (Sigma). After 20 min of incubation at room temperature with gentle mixing, the suspension was filtered through a 70-μm cell strainer (BD Biosciences), and keratinocytes were counted using a hemacytometer. Cells were stained with a 1:5 dilution of PE- and Cy5-conjugated antibody to CD49f and a 1:50 dilution of FITC-conjugated antibody to CD34 (both from BD Biosciences) in a solution of 1% BSA in S-MEM for 1 h on ice.

For detection of intracellular β-galactosidase activity, a fluorescein di-β-D-galactopyranoside (FDG) staining kit (Invitrogen) was used according to the manufacturer's instructions. Keratinocytes were loaded with FDG using hypotonic shock and subsequently stained with the appropriate antibodies. Staining analysis was carried out using a FACSCalibur flow cytometer (BD Biosciences). Cell sorting was done using FACSDiva and FACSAria cell sorters at the Huddinge Hospital Center for Cell Analysis. Lgr5-positive cells were defined as having a signal higher than the cells isolated from nontransgenic mouse skin, and were termed Lgr5^{high}.

For cell cycle analysis, the Vybrant DyeCycle Violet stain kit (Invitrogen) was used according to the manufacturer's instructions. For the colony-forming assay, 3,000 viable cells per well were seeded using a six-well plate (BD Falcon). The cells were grown in defined keratinocyte serum-free medium (DK-SFM; Invitrogen) either on irradiated (50 Gy) NIH3T3 feeders or directly on plastic for 16 d. To assess formation of secondary colonies, the sorted cells were grown for 21 d, split 1:4 and grown for an additional 16 d. The growth medium was changed every second day. To visualize the keratinocyte colonies, cells were fixed with PBS-buffered 4% formaldehyde and stained with 0.5% rhodamine B. Three independent experiments were conducted.

Keratinocyte transplantation. For keratinocyte transplantation, cells isolated from male donors were stained for β-galactosidase activity and CD34 as described above and sorted into tubes containing 700,000 freshly isolated neonatal dermal fibroblasts. To prepare primary fibroblasts, female newborn C57.Bl6 mouse pups were killed, and dorsal skin was trypsinized overnight at 4 °C in HBSS containing 0.25% trypsin. The next day, the dermis and epidermis were carefully separated to avoid keratinocyte contamination. The dermal skin was minced and incubated in a 0.35% collagenase IV (Invitrogen) solution in HBSS for 30 min at 37 °C with constant stirring. The digested dermis was strained through a 100-μm cell strainer, centrifuged and resuspended in Eagle's MEM (Cambrex) supplemented with 8% of FBS and 0.6 mM $CaCl_2$. Before keratinocyte sorting, Eagle's MEM was replaced with DK-SFM. The suspension of sorted keratinocytes (100,000 cells of each population, except for Lgr5^{high}, of which 80,000 cells were used) and neonatal fibroblasts was centrifuged, resuspended in 100 μl of DK-SFM supplemented with gentamycin (10 μg/ml) and transferred into a silicone dome (cat. nos. 30266 and 30267, Renner Laborbedarf) implanted onto the back of a BALB/c$^{nu/nu}$ nude mouse. The chambers were removed after 1 week and the wounds left to

heal. Hair growth was seen 3–4 weeks after transplantation. Three independent transplantation experiments were conducted.

RNA isolation and real-time PCR analysis. Total RNA from FACS-sorted cells was purified using an RNAqueous-Micro kit (Ambion), treated with DNase for 10 min at room temperature when TaqMan probes were not used, quantified using Infinite 200 NanoQuant (Tecan) and quality-checked using the Bioanalyzer 2100 system combined with an RNA 6000 Pico assay (Agilent Technologies). SuperScript II (RNase H) reverse transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's instructions. cDNA amounts were normalized to expression of *Arbp* and confirmed by comparison with *Hprt1* and *Gapdh* levels. Real-time PCR analysis was conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems) using the Power SYBR Green PCR 2× Master Mix (Applied Biosystems). All primer pairs were designed for the same cycling conditions: 2 min at 95 °C for initial denaturing, 26–39 cycles of 15 s at 95 °C for denaturing, 15 s at 60 °C for annealing, and 30 s at 72 °C for extension. The sequences of the primers used for amplification are listed in **Supplementary Table 3** online. Two independent biological replicates (each from a pool of five mice) were analyzed and gave similar results. The gene expression changes were measured in triplicate (standard deviation (C_t) $\leq$ 0.51). To analyze *Shh* mRNA expression, FACS-sorted cells from four mice were pooled, and RNA was purified as described above. The cDNA samples were preamplified with a TaqMan Preamp Master Mix kit according to the manufacturer's instructions. For preamplification and real-time PCR, *Shh* (Mm00436527_m1), *Gli1* (Mm00494645_m1), *Ptch1* (Mm00436026_m1) and, for normalization, *Hprt* (Mm00446968_m1) and mouse *Actb* (VIC) TaqMan probes (Applied Biosystems) were used (standard deviation (C_t) $\leq$ 0.37 of technical triplicates). The differences between samples and controls were calculated based on the $\Delta\Delta C_t$ method and shown as fold change normalized to *Arbp* expression (for SYBR Green analysis) or as fold change normalized to the mean of *Hprt* and *Actb* expression (for TaqMan analysis). Controls lacking reverse transcriptase did not yield any products for the primer pairs or TaqMan probes used in this study.

Note: Supplementary information is available on the Nature Genetics website.

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