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Hopx expression defines a subset of multipotent hair follicle stem cells and a progenitor population primed to give rise to K6$^+$ niche cells

Norifumi Takeda[1,2,3,*], Rajan Jain[1,2,3,*], Matthew R. LeBoeuf[1,4], Arun Padmanabhan[1,2,3], Qiaohong Wang[1,2,3], Li Li[2], Min Min Lu[2], Sarah E. Millar[1,4] and Jonathan A. Epstein[1,2,3,‡]

SUMMARY

The mammalian hair follicle relies on adult resident stem cells and their progeny to fuel and maintain hair growth throughout the life of an organism. The cyclical and initially synchronous nature of hair growth makes the hair follicle an ideal system with which to define homeostatic mechanisms of an adult stem cell population. Recently, we demonstrated that *Hopx* is a specific marker of intestinal stem cells. Here, we show that *Hopx* specifically labels long-lived hair follicle stem cells residing in the telogen basal bulge. *Hopx*$^+$ cells contribute to all lineages of the mature hair follicle and to the interfollicular epidermis upon epidermal wounding. Unexpectedly, our analysis identifies a previously unappreciated progenitor population that resides in the lower hair bulb of anagen-phase follicles and expresses *Hopx*. These cells co-express *Lgr5*, do not express *Shh* and escape catagen-induced apoptosis. They ultimately differentiate into the cytokeratin 6-positive (K6) inner bulge cells in telogen, which regulate the quiescence of adjacent hair follicle stem cells. Although previous studies have suggested that K6$^+$ cells arise from *Lgr5*-expressing lower outer root sheath cells in anagen, our studies indicate an alternative origin, and a novel role for *Hopx*-expressing lower hair bulb progenitor cells in contributing to stem cell homeostasis.

KEY WORDS: Hopx, Stem cell, Skin, Mouse

INTRODUCTION

The hair follicle (HF) has emerged as an informative system in which to study adult stem cell biology because it is characterized by predictable and precisely choreographed phases of growth (anagen), regression (catagen) and rest (telogen; Fig. 1A). Hair growth is, in part, maintained by HF stem cells (SCs) located at the base of the hair follicle in telogen (Alonso and Fuchs, 2006; Fuchs, 2009; Li and Clevers, 2010). The HF stem cell compartment is primarily composed of three layers: (1) basal bulge (CD34$^+$, integrin α6^{hi}) cells; (2) suprabasal bulge (CD34$^+$, integrin α6^{lo}) cells; and (3) cytokeratin 6-positive (K6) innermost cells surrounding the hair shaft (Blanpain et al., 2004; Hsu et al., 2011; Fig. 1A). Populations of stem cells have been elucidated outside the bulge (*MTS24*$^+$, *Lrig1*$^+$, *Lgr6*$^+$), although their contribution to hair follicle morphogenesis under physiological conditions appears limited (Nijhof et al., 2006; Jensen et al., 2009; Snippert et al., 2010). As telogen progresses, a rapidly cycling, biochemically distinct cohort of cells is established between the bulge and dermal papilla (DP), termed the secondary hair germ (HG). The secondary HG appears to house an activated subset of cells that provide the initial contributions to the growing hair follicle. These contributions combine with cells originating from the bulge during hair growth to migrate and proliferate during anagen, creating a linear trail of cells that will form the outer root sheath (ORS). The SC population of the outer bulge layer also includes quiescent cells that will contribute to subsequent rounds of hair follicle growth.

The growth phase (anagen) of the hair cycle is characterized by a stepwise differentiation of ORS cells into matrix progenitor cells of the hair bulb. As anagen progresses, matrix cells differentiate to produce the full complement of terminally differentiated cell lineages of the mature adult HF: the hair shaft, inner root sheath (IRS), and companion layer sandwiched between IRS and ORS (Fig. 1A). The growth phase is followed by catagen, which is characterized by involution of the lower half of the HF driven by widespread apoptosis (Botchkareva et al., 2006; Fig. 1A). A subset of matrix cells express sonic hedgehog (*Shh*) (St-Jacques et al., 1998; Kulessa et al., 2000; Blanpain et al., 2004), and initial studies posited that these cells survive catagen-induced apoptosis and contribute to the HG (Panteleyev et al., 2001). However, lineage studies in the adult do not support this hypothesis (Levy et al., 2005; Greco et al., 2009; White et al., 2011). The fate of *Shh*$^-$ matrix cells remains elusive, although it has been suggested that they may give rise to the companion layer (Winter et al., 1998).

Integrin α6^{hi} cells, a slowly cycling population located in the basal layer, and CD34$^+$, integrin α6^{lo} cells in the suprabasal layer have long been thought to mark the hair follicle stem cells (Albert et al., 2001; Trempus et al., 2003; Blanpain et al., 2004). More recently, rapidly cycling *Lgr5*$^+$ progenitor cells have been found to be in the secondary HG (Jaks et al., 2008). K6$^+$ cells in the innermost layer of the bulge maintain the quiescence of the basal and suprabasal layer stem cells (Hsu et al., 2011). The K6$^+$ layer is thought to share similarities with segments of the companion layer of anagen HFs (Higgins et al., 2009); both are adjacent to the ORS and express K6 (Winter et al., 1998; Wang et al., 2003; Hsu et al.,

[1]Department of Cell and Developmental Biology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA. [2]Penn Cardiovascular Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA. [3]Institute of Regenerative Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA. [4]Department of Dermatology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA.

*These authors contributed equally to this work
‡Author for correspondence (epsteinj@upenn.edu)

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2011), plasminogen activator inhibitor 2 (PAI2) (Lavker et al., 1998), calretinin (Poblet et al., 2005) and S100 calcium-binding protein A6 (S100A6) (Ito and Kizawa, 2001). However, the lineage relationship between the $K6^+$ inner bulge and companion layers remains a mystery.

Adult tissue-specific stem cells both self-renew and generate progeny that include components of their own niches and other functional derivatives (Morrison and Kimble, 2006; Hsu and Fuchs, 2012; Lander et al., 2012). The homeostatic mechanisms coordinating these activities are crucial to long-term regenerative potential, yet remain poorly understood. Defining lineage relationships and progression along alternative differentiation pathways is crucial for elucidation of this process. In many organs and tissues, the identities of various stem and niche cells remain controversial or unknown. In the intestinal crypt of adult mice, we recently demonstrated that *Hopx* (Chen et al., 2002; Shin et al., 2002) is strongly expressed by BrdU-retaining, +4 epithelial stem cells (Takeda et al., 2011). Interestingly, *Hopx*-expressing cells interconvert with the *Lgr5*-positive progenitor cell population found in the intestinal crypt base. Both *Hopx* and *Lgr5* are expressed in the HF; hence, we sought to examine the lineage progression and relationship of these cell populations in the skin.

Here, we show that *Hopx* is expressed in the basal layer of the telogen hair follicle and its expression is more restricted than previously reported hair follicle stem cell markers. Lineage-tracing experiments using inducible Cre-Lox technologies demonstrate that $Hopx^+$ cells give rise to all lineages of the hair follicle. Furthermore, we have identified a novel population of *Hopx*-expressing ($Lgr5^+$, Shh^-) lower hair bulb cells in the mature follicle that contribute to the $K6^+$ inner bulge layer of the stem cell niche in telogen. The $Hopx^+$ hair bulb progenitors are distinguished from $Lgr5^+$ lower ORS cells as the lower ORS does not express *Hopx*. Rather, they are Shh^- cells that escape catagen-induced apoptosis and give rise to $K6^+$ cells in the subsequent telogen.

MATERIALS AND METHODS

Generation of $Hopx^{3FlagGFP/+}$ mice

$Hopx^{3FlagGFP/+}$ mice were generated by homologous recombination in embryonic stem cells targeting a cassette containing *Exon3-3XFlag-Viral2A-eGFP-3′ UTR-Frt-PGK-neo-Frt* to the third exon of *Hopx*. An *Asc*I restriction enzyme site was introduced 70 bp upstream of exon 3 and sequential *Fse*I, *Eco*RI restriction enzyme sites were introduced immediately following the 3′ UTR. The *PGK-neo* cassette was removed by breeding initial progeny to mice expressing ubiquitous FlpE recombinase (*ACTFLPe*) (Rodríguez et al., 2000).

Mice

$Hopx^{lacZ/+}$ (Chen et al., 2002), $Hopx^{ERCre/+}$ (Takeda et al., 2011), $Lgr5^{EGFP\text{-}ERCre/+}$ (Barker et al., 2007), $Shh^{ERCre/+}$ (Harfe et al., 2004), $K14^{ERCre/+}$ (Vasioukhin et al., 1999), $R26^{lacZ/+}$ (Soriano, 1999), $R26^{mT\text{-}mG/+}$ (Muzumdar et al., 2007) and $R26^{Tom/+}$ (Madisen et al., 2010) mice have been described previously. All mice were maintained on mixed genetic backgrounds. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols.

Observation and description of HFs

The first two HF cycles are synchronized in mice for the first 60 days of life. Anagen and catagen are subdivided into 6 and 8 stages, respectively, based on a variety of morphological criteria (Müller-Röver et al., 2001). They are further organized as follows: middle anagen (anagen III-IV), late anagen (anagen V-VI), middle catagen (catagen V) and late catagen (catagen VII-VIII). Because hair cycles slightly vary among strains and sexes, stages of mouse ages were evaluated instead. Single hair follicles were dissected with fine forceps and visualized on an Olympus MVX10 fluorescence dissecting microscope.

Lineage-tracing experiments

Mice were injected intraperitoneally with tamoxifen (100 mg/kg body weight; Sigma) dissolved in corn oil, as a single dose or daily for 5 consecutive days, as indicated.

BrdU-labeling experiments

To mark label-retaining cells (LRCs), $Hopx^{3FlagGFP/+}$ mice were injected intraperitoneally with BrdU (50 mg/kg body weight; Sigma) twice a day for 3 days at P3-5 (Braun et al., 2003; Jensen et al., 2009). Tissues were collected 7 weeks after BrdU administration, and then stained with an anti-BrdU monoclonal antibody (Rat, 1:20, Accurate).

β-Galactosidase staining

Tissues were fixed for 45 minute in ice-cold 2% paraformaldehyde (PFA) at 4°C, rinsed 3 times in PBS for 20 minutes at 4°C, and then incubated for 12-24 hours at 37°C in the staining solution [2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.01% NP40, 0.01% sodium deoxycholate and 1 mg/ml X-gal in PBS]. The stained tissues were fixed in 2% PFA, ethanol dehydrated and sectioned using standard methods.

Histology

Back skins were fixed in 2% PFA, ethanol dehydrated, embedded in paraffin wax and sectioned in the sagittal plane. Antibodies used were: GFP (goat, 1:100, Abcam) (rabbit, 1:200, Molecular Probes), RFP (recognizes tdTomato) (rabbit, 1:50, Rockland), AE13 (mouse, 1:25, Abcam), cytokeratin 5 (goat, 1:20, Santa Cruz) (rabbit, 1:200, Abcam), cytokeratin 6 (mouse, prediluted, Abcam) (rabbit, 1:200, Abcam), K15 (mouse, 1:50, Vector), Sox9 (rabbit, 1:100, Santa Cruz), Gata3 (mouse, 1:25, Santa Cruz), CD34 (rat, 1:25, BD Pharmingen), Lrig1 (goat, 1:50, R&D Systems), P-cadherin (mouse, 1:20, R&D Systems), Shh (rabbit, 1:50, Santa Cruz) and Flag (mouse, 1:250, Sigma). TUNEL staining was performed using standard protocols (Roche). Tissue sections were incubated with terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP at 37°C for 60 minutes. All immunohistochemistry was visualized on a Nikon Eclipse 80i fluorescence microscope, except the confocal images, which were evaluated on a Zeiss LSM 510 microscope. Radioactive *in situ* hybridization was performed using the previously described probe for *Hopx* (Chen et al., 2002). All images were analyzed using Adobe Photoshop (sizing, brightness or contrast adjustments, etc.). Brightness and contrast was adjusted linearly across the entirety of each image.

Wound healing assays

Mice that had been previously treated with tamoxifen at indicated days were anesthetized with ketamine and xylazine, and a 6 mm full-thickness excision of skin was made on the mid back (Ito et al., 2005). Skin samples were collected at indicated days after wounding and processed for β-galactosidase staining.

Keratinocyte isolation and FACS

Back skin keratinocytes were collected as previously described (Jensen et al., 2010). Briefly, mice were killed, dorsal hair was clipped and dorsal skin was taken and placed into Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS; Invitrogen). Subcutaneous tissue and fat were removed by gentle scraping, and the skin was cut into 1 cm strips and incubated at 37°C in 0.25% trypsin solution in HBSS for 70 minutes. The epidermis was then scraped into S-minimal essential medium (SMEM; Invitrogen) supplemented with 5% FBS. After gentle pipetting, the suspension was passed through a 70 μm filter (BD Biosciences), and keratinocytes were counted. Single cells were analyzed using BD FACS Calibur (BD Biosciences). Cells were pre-incubated for 15 minutes with Fc Block (BD Pharmingen), and 7-amino-actinomycin D (7-AAD; BD Biosciences) was used to exclude dead cells. The following antibodies were used in a solution of 5% FBS in PBS for 30 minutes on ice: CD34-eFluor660 (1:100, eBioscience) and CD49f -PE (1:100, BD Pharmingen).

RESULTS

Hopx expression characterizes hair follicle stem cells

To identify *Hopx*-expressing cells in the hair follicle, we generated a novel *Hopx* allele in which the final exon of *Hopx* was appended

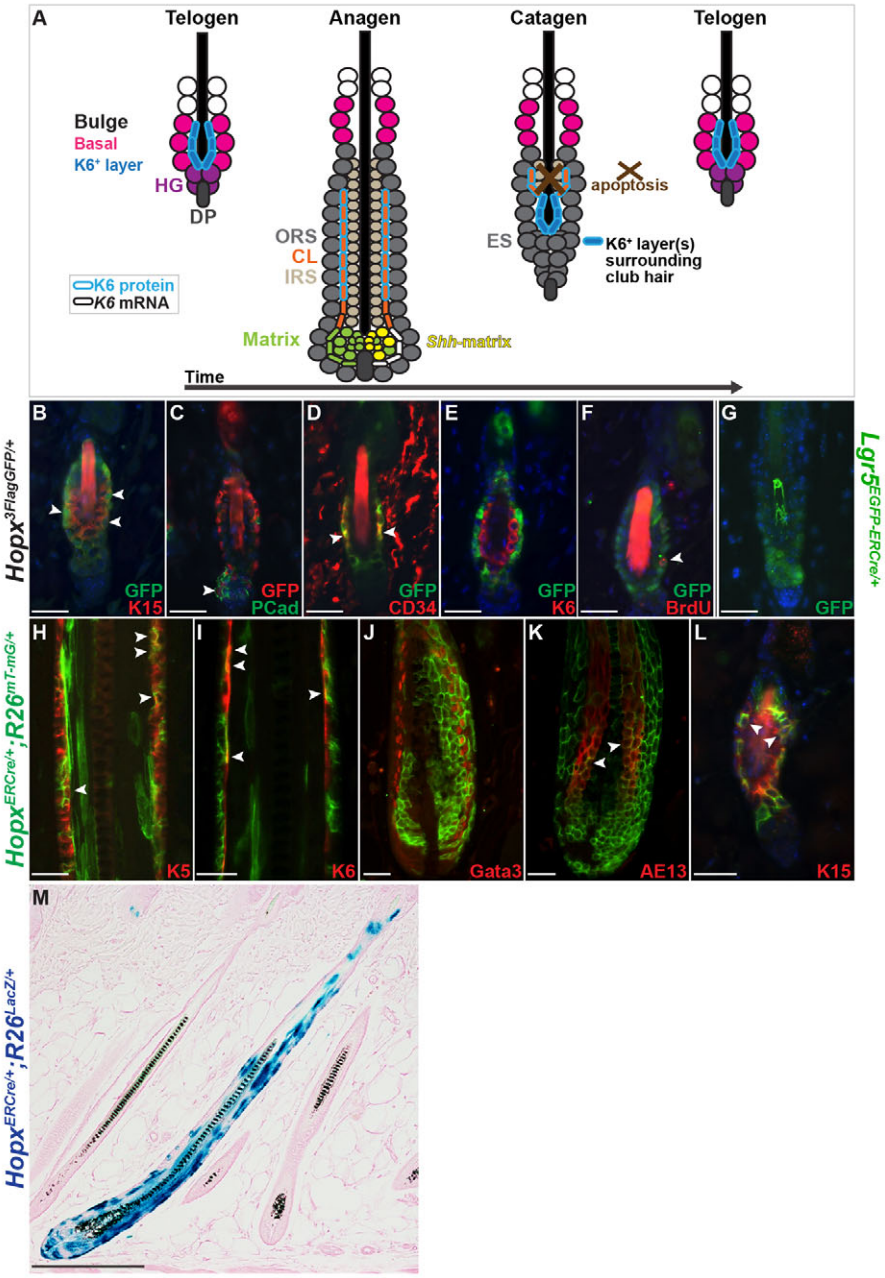

Fig. 1. *Hopx* specifically labels mouse hair follicle stem cells. (**A**) The hair cycle is characterized by successive phases of rest (telogen), growth (anagen) and regression (catagen). During anagen, *Shh*-expressing matrix progenitors in the lower hair bulb contribute to the IRS and hair shaft. During catagen, K6 protein-expressing cells surround the club hair. Some survive catagen-induced apoptosis and then home back to the innermost layer of the hair follicle. (**B-E**) Skin sections from $Hopx^{3FlagGFP/+}$ mice from P21, the first mouse telogen. K15, bulge cells; PCad, secondary HG; CD34, basal bulge stem cells; K6, niche cells. (**F**) Double staining of $Hopx^{3FlagGFP/+}$ skin for GFP and BrdU, demonstrating that LRCs labeled at P3-5 with BrdU express *Hopx* at P58. (**G**) GFP staining of $Lgr5^{EGFP-ERCre/+}$ skin at P21. (**H-L**) GFP staining of $Hopx^{ERCre/+};R26^{mT-mG/+}$ skin 3 months after a tamoxifen pulse at P50-54, with cell-specific markers as indicated. (**M**) β-galactosidase staining of $Hopx^{ERCre/+};R26^{lacZ/+}$ skin 15 months after labeling. Arrowheads indicate double-positive cells. HG, secondary hair germ; DP, dermal papilla; ORS, outer root sheath; CL, companion layer; IRS, inner root sheath; ES, epithelial strand. Scale bars: 25 μm in B-L; 250 μm in M.

to include a *3XFlag* sequence followed by *viral-2A-GFP* ($Hopx^{3FlagGFP/+}$; supplementary material Fig. S1A). GFP expression correlated with *Hopx* expression in the intestine and heart (Chen et al., 2002; Takeda et al., 2011) (supplementary material Fig. S1B-E). At postnatal day 21 (P21), the first adult mouse telogen, we detected strong *Hopx* expression in the basal layer of the hair follicle stem cell compartment, marked by cytokeratin 15 (K15; Krt15 – Mouse Genome Informatics) expression, and relatively weaker expression in the secondary HG, marked by P-Cadherin expression (Fig. 1B,C). $Hopx^{+}$ cells in adult telogen HFs co-expressed CD34, but did not express K6, a marker of the innermost bulge cells, unlike many other known stem cell markers [e.g. *Sox9* (Nowak et al., 2008), *Lhx2* (Rhee et al., 2006), *Tcf3* (Nguyen et al., 2006) and *K15* (Lyle et al., 1998; Liu et al., 2003); Fig. 1D,E]. *Hopx* expression was more restricted to the bulge than that of CD34, and overlapped with, but was not identical to, K15 expression. In addition, $Hopx^{+}$ cells retain BrdU 8 weeks after injection at P3-5, and hence we conclude they are LRCs (Fig. 1F). In contrast to *Hopx*, *Lgr5* expression was localized to the lower basal layer and HG, as has been previously described (Jaks et al., 2008) (Fig. 1G). Very few $Lrig1^{+}$ cells co-expressed *Hopx* (supplementary material Fig. S1F). FACS analysis during the second telogen (P55-P58) confirmed that *Hopx*-expressing cells are a subpopulation of $CD34^{+}$, integrin $\alpha6^{hi}$ and $CD34^{+}$, integrin $\alpha6^{lo}$ cells (data not shown).

To track the fate of telogen $Hopx^{+}$ cells, we crossed $Hopx^{ERCre/+}$ (Takeda et al., 2011) mice with $R26^{lacZ/+}$ (Soriano, 1999) and $R26^{mT-mG/+}$ (Muzumdar et al., 2007) indicator mice, in which β-galactosidase (β-gal) and membrane-bound GFP can be induced by Cre-mediated recombination, respectively. We injected mice with tamoxifen to activate Cre recombinase during the second synchronized telogen (P50-54). Examination at P55 revealed *Hopx*-derived cells in the basal layer and HG, consistent with the

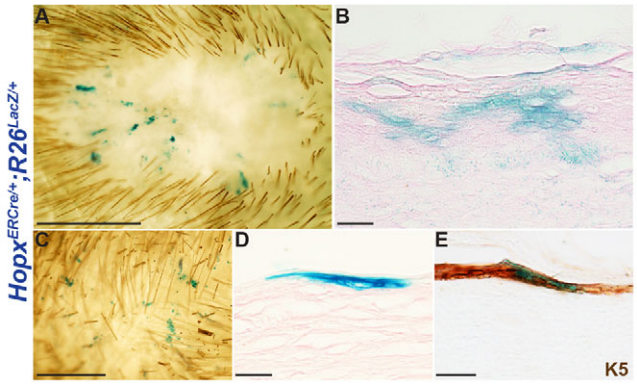

Fig. 2. *Hopx*$^{+}$ cells contribute to regenerating epidermis. (**A-E**) β-galactosidase staining of wounds and regenerated epidermis in *Hopx*$^{ERCre/+}$;*R26*$^{lacZ/+}$ mice induced during the secondary telogen, 2 weeks (A,B) and 9 weeks (C-E) after a full-thickness skin wounding. (E) Double staining of β-galactosidase and K5, demonstrating a K5^{+} basal layer of scar tissue is derived from *Hopx*$^{+}$ cells. Scale bars: 25 μm in B,D,E; 2 mm in A,C.

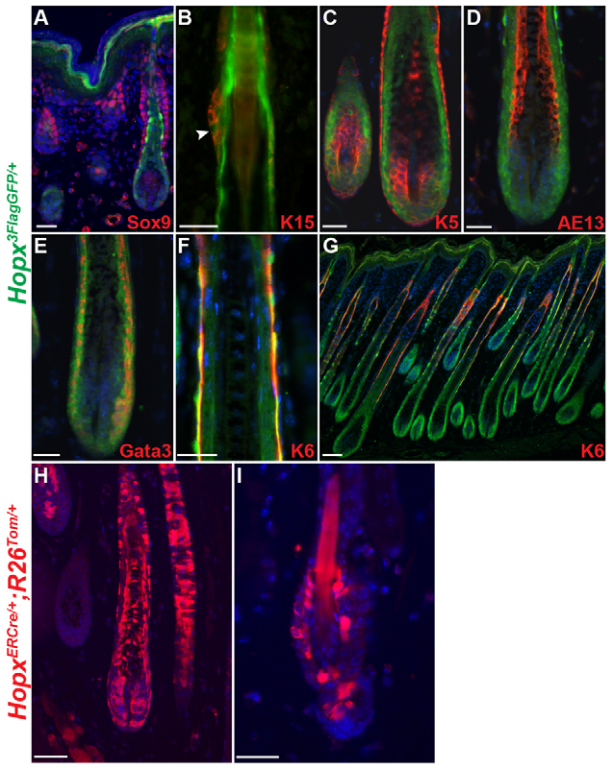

Fig. 3. Lineage tracing of *Hopx*-expressing precursors. (**A-G**) GFP staining of *Hopx*$^{3FlagGFP/+}$ skin at P0 (A) and P5 (B-G), the morphological middle anagen, with cell-specific markers. (**H**,**I**) TdTomato staining of *Hopx*$^{ERCre/+}$;*R26*$^{Tom/+}$ skin 80 days after labeling at P5. Arrowhead indicates a double-positive cell. Scale bars: 25 μm.

expression pattern of *Hopx*$^{3FlagGFP/+}$ mice (supplementary material Fig. S1G). All HF compartments of the mature anagen stage follicle, including the ORS (K5^{+}), companion layer (K6^{+}), IRS (Gata3^{+}), hair shaft (AE13^{+}) and matrix contained derivatives of *Hopx*-expressing cells (Fig. 1H-K). Fated cells were also found in the subsequent telogen bulge and HG (Fig. 1L). Fifteen months after tamoxifen induction, *Hopx* derivatives were still present in all cell types of the mature follicle, suggesting that *Hopx*$^{+}$ cells in adult telogen undergo self-renewal (Fig. 1M). The inefficiencies of tamoxifen-inducible Cre-Lox technologies probably account for, at least in part, the lack of labeling in every hair follicle. No ectopic reporter activity was detected in uninduced control mice (*Hopx*$^{ERCre/+}$;*R26*$^{mT\text{-}mG/+}$, data not shown).

Hair follicle SCs have been shown to contribute to the interfollicular epidermis during wound healing (Ito et al., 2005; Cotsarelis, 2006; Snippert et al., 2010; Brownell et al., 2011). We pulsed *Hopx*$^{ERCre/+}$;*R26*$^{lacZ/+}$ mice with tamoxifen during the second telogen phase (P50-54), and then created a full-thickness wound 48 hours later (P56). Two weeks after injury, streams of β-gal^{+} *Hopx* derivatives were seen within the wound in a radial pattern extending from hair follicles on the edge of the wound towards the wound center (Fig. 2A). Histological analysis revealed that *Hopx* derivatives contributed to the re-epithelialized skin (Fig. 2B). Nine weeks after wounding, clones of *Hopx*-derived cells were found within the newly formed cytokeratin 5 (K5)-positive epidermal cells and more differentiated suprabasal and granular layer cells (Fig. 2C-E). We could not detect β-gal expression in any uninjured areas of epidermis or ectopic reporter activity (data not shown). Taken together, these data suggest that *Hopx*$^{+}$ cells of the telogen hair follicle can self-renew, give rise to all lineages of the hair follicle during subsequent cycles of hair growth, and contribute to the interfollicular epidermis upon injury. Hence, *Hopx* is a marker of resident adult HF stem cells.

Hopx-expressing adult HF SCs derive from a *Hopx*-expressing precursor

The precise cellular origin and timing of hair follicle stem cell specification remain poorly defined. We sought to determine whether *Hopx* is expressed during postnatal hair follicle maturation and stem cell specification. We could not detect *Hopx* expression at P0 in the developing bulge, whereas *Sox9*$^{+}$ cells were found in the ORS and primitive bulge (Fig. 3A). In immature P5 anagen stage follicles (anagen III) (Müller-Röver et al., 2001), *Hopx* expression was present in the K15^{+} upper ORS cells, but absent in the lower ORS (Fig. 3B,C). *Hopx* was expressed in the hair shaft, matrix, IRS and companion layer (Fig. 3D-G). Lineage-tracing experiments using *Hopx*$^{ERCre/+}$;*R26*$^{Tom/+}$ (Madisen et al., 2010) mice pulsed with a single dose of tamoxifen at P5 demonstrated that the anagen follicle at P85 and the telogen bulge was labeled (Fig. 3H,I). This result indicates that during early hair follicle maturation, *Hopx*-expression marks cells that are destined to give rise to all lineages of the adult hair follicle.

Hopx^{+} cells in the mature anagen lower hair bulb escape catagen apoptosis and give rise to K6^{+} niche cells in the subsequent telogen

Unlike several other HFSC markers [e.g. *Sox9* (Nowak et al., 2008), *Lhx2* (Rhee et al., 2006), *Tcf3* (Nguyen et al., 2006) and *K15* (Lyle et al., 1998; Liu et al., 2003)], *Hopx* expression in the bulge of telogen follicles was restricted to the outer stem cell compartment and not present in the K6^{+} innermost layer. It has been postulated that K6^{+} inner cells arise from *Lgr5*$^{+}$ lower ORS cells in anagen (Hsu et al., 2011). Therefore, we investigated the *Hopx*$^{+}$ population of cells in late anagen and their derivatives.

Hopx was expressed in the matrix, companion layer, IRS and hair shaft of the mature anagen HF (anagen V-VI) (Müller-Röver et al., 2001; Fig. 4A-D). *Hopx* was expressed symmetrically and

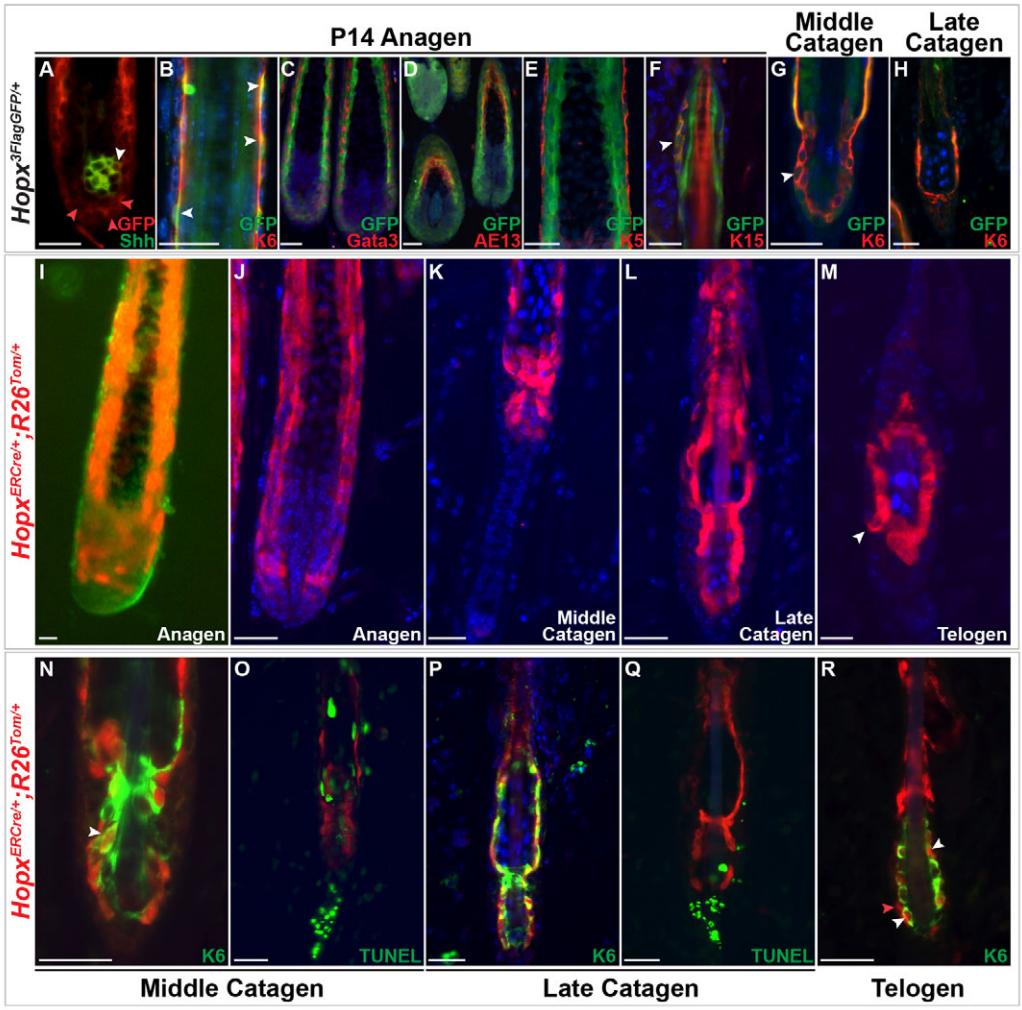

Fig. 4. *Hopx*$^{+}$ cells in the lower hair bulb give rise to K6^{+} cells in telogen. (**A**) *Hopx* is expressed in both *Shh*$^{+}$ and *Shh*$^{-}$ cells (white and red arrowheads, respectively). (**B-E**) *Hopx* is also expressed in the companion layer (B, arrowheads), IRS (C) and hair shaft (D), but is not expressed in the lower ORS (E) at late anagen, P14. (**F**) Hopx is expressed in K15^{+} bulge cells (F, arrowhead). (**G**,**H**) In middle catagen, *Hopx* is expressed in K6^{+} cells surrounding the club hair (G, arrowhead), but the expression there is absent in late catagen (H). (**I-M**) *Hopx*$^{ERCre/+}$;*R26*$^{Tom/+}$ mice were pulsed with tamoxifen once at P14. Samples were harvested during anagen (P15; I,J), middle catagen (P17, K), late catagen (P19, L) and telogen (P21, M). The IRS, matrix, companion layer and lower hair bulb cells are labeled in anagen (I,J). (I) Whole-mount image and the green background is a pseudocolored bright-field image. (K,L) Cells surrounding the club hair are labeled in catagen, whereas the epithelial strand is not derived from *Hopx*$^{+}$ cells. (M) Derivatives were found mostly in the innermost layer, whereas isolated cells were found in the outer bulge layer in telogen (white arrowhead). (**N-Q**) *Hopx* derivatives were found in the K6^{+} layer surrounding the club hair in middle and late catagen. The outermost *Hopx*-derived cells surrounding the club hair in middle catagen as well as the *Hopx* derivatives in late catagen were TUNEL negative. (**R**) *Hopx*-derived cells are found in the basal bulge (red arrowhead) but mostly in the innermost layer of cells (white arrowheads) in the following telogen. Scale bars: 25 μm.

broadly in the anagen hair bulb, whereas *Shh* expression was more restricted and asymmetric (supplementary material Fig. S2). *Hopx* was also expressed in the *Shh*$^{-}$/non-ORS lower hair bulb cells (Fig. 4A, red arrowheads). The K5-expressing lower ORS was devoid of *Hopx* expression, but K15^{+} upper ORS cells expressed *Hopx* (Fig. 4E,F). By middle catagen (catagen V) (Müller-Röver et al., 2001), low levels of *Hopx* were detected in the K6^{+} cells surrounding the club hair; however, expression in this region was extinguished by late catagen (catagen VII-VIII; Fig. 4G,H) (Müller-Röver et al., 2001).

Pulse labeling of *Hopx*$^{ERCre/+}$;*R26*$^{Tom/+}$ mice with a single dose of tamoxifen in late anagen (P14) allowed for the identification of *Hopx* derivatives. As expected, 1 day after a single pulse of tamoxifen, tdTomato-positive cells were identified in the lower hair bulb, matrix, companion layer, IRS and upper ORS (Fig. 4I,J). We did not detect labeled cells in the lower ORS (Fig. 4I). During catagen, derivatives were found in the layers surrounding the club hair, but not in the epithelial strand or lower ORS (Fig. 4K,L). The lack of derivatives in the epithelial strand is consistent with the absence of *Hopx* expression in the lower ORS (Hsu et al., 2011). Examination of HFs in the subsequent telogen stage revealed numerous fated cells in the innermost layer with rare derivatives in the outer layer (Fig. 4M, white arrowhead). A subset of *Hopx*-fate mapped cells in middle catagen surrounding the newly formed club hair expressed K6 (Fig. 4N). The outermost layer of K6^{+} cells surrounding the hair shaft, derived from *Hopx*$^{+}$ precursors, was resistant to apoptosis (TUNEL-negative, Fig. 4O). In late catagen, a single layer of tdTomato^{+}, K6^{+}, TUNEL^{-} cells surrounded the

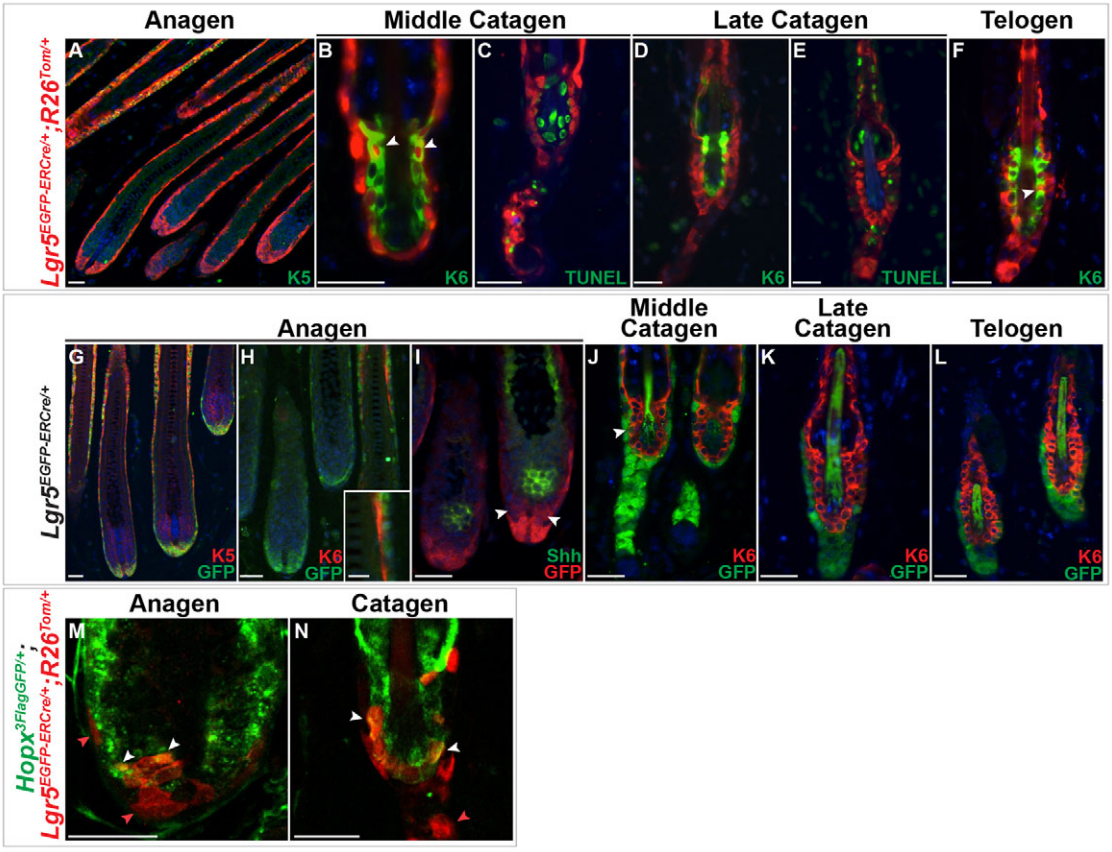

Fig. 5. *Lgr5* expression in the lower hair bulb and contribution to the telogen bulge. (**A-F**) $Lgr5^{EGFP\text{-}ERCre/+}$;$R26^{Tom/+}$ were pulsed with tamoxifen once at P13 and sacrificed at anagen (P15; A), middle catagen (P17; B,C), late catagen (P19; D,E) and the ensuing telogen (P21; F). (A) Cells were labeled in the lower hair bulb and ORS in late anagen. (B-E) In catagen, $K6^+$, *Lgr5*-derived cells surrounding the club hair were TUNEL resistant (in contrast to derivatives in the epithelial strand). (F) In telogen, derivatives were found in the all layers of the bulge and the secondary HG. (**G**,**H**) *Lgr5* expression was found in the ORS ($K5^+$) and not in the companion layer ($K6^+$) in late anagen, P14. Inset in H shows high magnification of companion layer adjacent to ORS. (**I-K**) *Lgr5* is expressed in non-ORS, Shh^- cells in the lower hair bulb (white arrowheads, I) and in the outermost $K6^+$ layer surrounding the club hair in middle catagen (P17, J), whereas expression is absent from late catagen in $K6^+$ cells (P19, K). (**L**) *Lgr5* is expressed in the secondary HG and lower basal bulge in telogen, P21. (**M**) $Hopx^{3FlagGFP/+}$;$Lgr5^{EGFP\text{-}ERCre/+}$;$R26^{Tom/+}$ were pulsed with one dose of tamoxifen at P14, sacrificed at P15. TdTomato$^+$ cells were found in the lower hair bulb (white arrowheads), as well as the ORS (red arrowheads). White arrowheads demonstrate examples of a Flag$^+$, tdTomato$^+$ cell in the lower hair bulb. (**N**) $Hopx^{3FlagGFP/+}$;$Lgr5^{EGFP\text{-}ERCre/+}$;$R26^{Tom/+}$ were pulsed with one dose of tamoxifen at P17 and sacrificed at P18, middle catagen. TdTomato$^+$ cells were found in the epithelial strand (red arrowhead), whereas Flag$^+$, tdTomato$^+$ cells were found in the outermost layer of cells surrounding the club hair (white arrowheads). Confocal microscopy was used to image M and N. Scale bars: 25 μm; 10 μm in H (inset).

newly formed club hair (Fig. 4P,Q). During the following telogen (P21), tdTomato$^+$ cells were found in the $K6^+$ innermost layer (Fig. 4R, white arrowheads), as well as in the outer layer (Fig. 4R, red arrowhead). Similar results were obtained with lineage-tracing experiments conducted with adult $Hopx^{ERCre/+}$;$R26^{Tom/+}$ mice (tamoxifen pulse P35-36, sacrificed at P50; data not shown). It is unlikely that the late anagen bulge cells are responsible for the contributions to the $K6^+$ innermost layer in telogen, as they have been shown to give rise to only the outer bulge and HG in the subsequent telogen (Ito et al., 2004; Greco et al., 2009; Hsu et al., 2011).

Previous studies have suggested that $K6^+$ innermost telogen cells derive from lower ORS cells expressing *Lgr5* and *K14* in late anagen (Hsu et al., 2011). However, as noted above, *Hopx* is not expressed in the lower ORS in mature anagen. Therefore, we re-examined the fate of late anagen $Lgr5^+$ cells using $Lgr5^{EGFP\text{-}ERCre/+}$;$R26^{Tom/+}$ mice (Barker et al., 2007) and an identical labeling protocol, as described for the analysis of *Hopx* derivatives (single pulse of tamoxifen at late anagen, P13; Fig. 5A). Consistent with previous reports (Hsu et al., 2011), we identified tdTomato$^+$ derivatives in middle catagen in the outermost $K6^+$ layer surrounding the club hair that were TUNEL negative (Fig. 5B,C). In contrast to *Hopx* lineage tracing, *Lgr5* derivatives were also found in the ORS and epithelial strand (consistent with expression in the ORS), and, as expected, the derivatives were TUNEL positive (Fig. 5C). During late catagen, $K6^+$ *Lgr5*-derived cells were resistant to apoptosis (Fig. 5D,E). In telogen, $K6^+$ cells derived from *Lgr5*-expressing precursors were clearly evident, as previously reported (Hsu et al., 2011), as were *Lgr5*-derived lower basal layer and HG cells (Fig. 5F). Similar results were obtained when lineage-tracing experiments were conducted with $K14^{ERCre/+}$;$R26^{Tom/+}$ mice (Vasioukhin et al., 1999) (supplementary material Fig. S3). Taken together, these data suggest two possible interpretations for the source of $K6^+$ innermost telogen cells. First, they may arise from at least two distinct sources in late anagen (either $Lgr5^+$ or $Hopx^+$ cells). Alternatively, there may be a population of cells that express

both *Lgr5* and *Hopx* during late anagen and give rise to K6$^+$ niche cells in the subsequent telogen. Therefore, we investigated the relative expression of these factors in late anagen HFs, with particular attention to the ORS and the inner matrix where *Shh* is also expressed.

In accordance with previous reports (Jaks et al., 2008; Hsu et al., 2011), *Lgr5*-expressing cells were present in the ORS (K5$^+$) and not in the companion layer (K6$^+$) in late anagen (Fig. 5G,H). Surprisingly, however, we also identified *Lgr5* expression in *Shh*$^-$, lower hair bulb cells medial to the ORS (Fig. 5I). In middle catagen, the outermost K6$^+$ layer of cells surrounding the club hair was *Lgr5*$^+$ (Fig. 5J). However, by late catagen *Lgr5* expression was present only in the ORS and epithelial strand (Fig. 5K). *Lgr5*$^+$ cells in late catagen fate-mapped to the HG and basal layer of the stem cell compartment, but did not give rise to the K6$^+$ innermost layer in telogen (supplementary material Fig. S4A). In telogen, *Lgr5* was not expressed in the K6$^+$ layer (Fig. 5L and supplementary material Fig. S4B).

To determine whether *Lgr5* and *Hopx* are co-expressed in the lower hair bulb, we pulsed *Hopx*$^{3FlagGFP/+}$;*Lgr5*$^{EGFP\text{-}ERCre/+}$;*R26*$^{Tom/+}$ mice with a single dose of tamoxifen at mature anagen and sacrificed the animals 24 hours later. (We chose this approach because, in our hands, available antibodies to Hopx, Lgr5 and Cre are unreliable in the skin, and available targeted alleles of both genes express GFP.) TdTomato expression was present in the ORS (Fig. 5M, red arrowheads) and closely resembled *Lgr5* expression (supplementary material Fig. S4C,D), suggesting that most or all of the tdTomato$^+$ cells were actively expressing *Lgr5*. Flag immunohistochemistry was specific to *Hopx*-expressing cells in the IRS and matrix (Fig. 5M). In the lower hair bulb, Flag and tdTomato double-positive cells were identified but not in the middle hair follicle (Fig. 5M; supplementary material Fig. S4E). We repeated this experiment in middle catagen; *Hopx*$^{3FlagGFP/+}$;*Lgr5*$^{EGFP\text{-}ERCre/+}$;*R26*$^{Tom/+}$ mice were pulsed once with tamoxifen at P17 and sacrificed at P18. Our model predicts that double-positive cells will be found in the K6$^+$ outermost layer surrounding the hair shaft in middle catagen, cells that are destined to become the K6$^+$ niche cells in telogen. Indeed, we found multiple tdTomato cells co-expressing *Hopx* in the outermost layer of cells surrounding the club hair (Fig. 5N, white arrowheads). Taken together, these data suggest that *Lgr5*$^+$, *Hopx*$^+$ cells found in the lower hair bulb give rise to cells in catagen that are destined for the K6$^+$ innermost layer in telogen.

Interestingly, our data suggest that the region of *Hopx* and *Lgr5* co-expression in the lower hair bulb is immediately adjacent to, but non-overlapping with, *Shh*-expressing cells. Recent studies have shown that *Shh*$^+$ cells in late anagen do not give rise to K6$^+$ innermost cells in telogen (Greco et al., 2009; White et al., 2011), and our own fate-mapping studies using *Shh*$^{ERCre/+}$ (Harfe et al., 2004) mice confirmed this finding (supplementary material Fig. S5). Therefore, although we cannot rule out the possibility that multiple distinct populations of progenitor cells give rise to K6$^+$ innermost cells or migration of *Lgr5*$^+$ ORS cells to the lower hair bulb, the most parsimonious interpretation of these data is that *Hopx*$^+$, *Lgr5*$^+$, *Shh*$^-$ cells in the lower hair bulb give rise to these K6$^+$ niche cells (Fig. 6A,B).

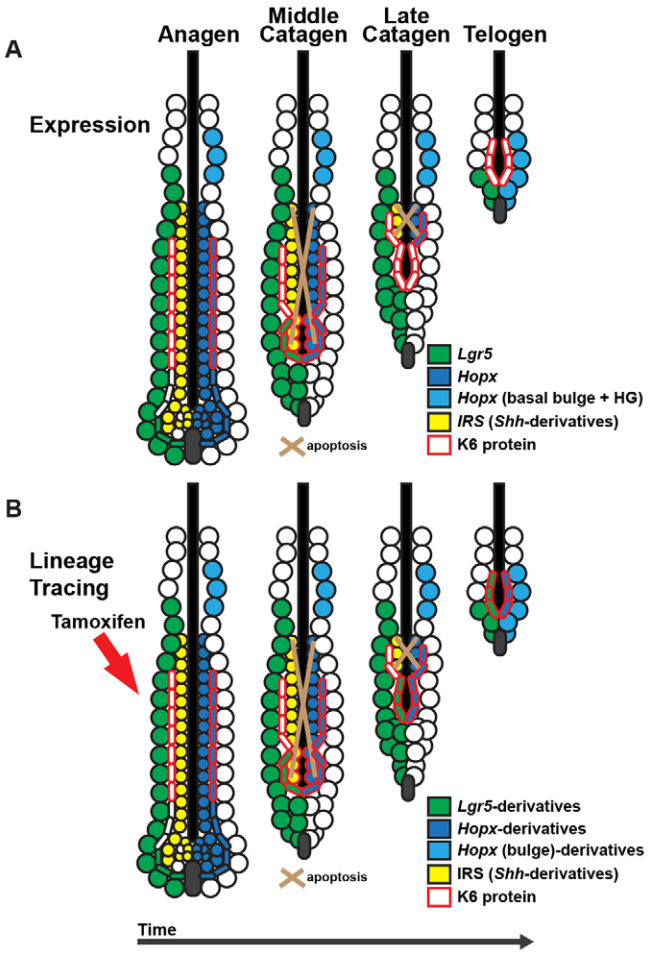

Fig. 6. Contribution of anagen *Hopx*$^+$ and *Lgr5*$^+$ cells to the telogen bulge. Model depicting *Hopx* and *Lgr5* expression (A) and their relative contributions to the hair follicle (B). (For clarity and comparison purposes, *Hopx* and *Lgr5* expression and their relative contributions are depicted on opposite sides of the same hair follicle.) (**A**) *Hopx* is expressed in companion layer, IRS, matrix, lower hair bulb and basal bulge. It is absent from the lower ORS. Its expression is extinguished by late catagen and is limited to the basal bulge in telogen. *Lgr5* is expressed in the ORS and lower hair bulb in late anagen and in the outermost K6$^+$ layer in middle catagen. Its expression is limited to the ORS and epithelial strand in late catagen and to the secondary HG and lower basal bulge in telogen. (**B**) *Hopx*$^+$ cells in late anagen will eventually give rise to the K6$^+$ cells surrounding the club hair in catagen, which transition to the niche cells in telogen. *Lgr5*$^+$ cells in late anagen will also give rise to the outermost K6$^+$ layer surrounding the club hair in catagen and niche cells in telogen.

DISCUSSION

In this report, we demonstrate that *Hopx* is specifically expressed in the basal layer of the HFSC compartment of telogen phase hair follicles, and that *Hopx*-expressing cells can give rise to all the lineages of the hair follicle over multiple cycles of hair growth. Some *Hopx*$^+$ cells are label retaining and contribute to regenerating epidermis after wounding. Therefore, these and other data indicate that *Hopx* expression identifies a HFSC population. Our studies demonstrate that *Hopx*$^+$ cells at P5, during early follicle maturation, contribute to all differentiated lineages of the hair follicle in the adult. *Sox9*-expressing cells in the suprabasal layer of the hair germ during embryogenesis may also give rise to HFSCs, although lineage-tracing studies are necessary to confirm the contribution of these cells in the adult (Vidal et al., 2005; Nowak et al., 2008). Finally, our work also provides evidence for a previously unrecognized population of cells in the lower hair bulb (*Hopx*$^+$, *Lgr5*$^+$, *Shh*$^-$) that can give rise to K6$^+$ niche cells in telogen.

Hopx marks stem cell populations in multiple tissues

With the advent of inducible Cre-Lox technologies, the stem cell biology field has been able to identify a variety of markers for adult resident tissue-specific stem cell populations in many tissues (Simons and Clevers, 2011; Kretzschmar and Watt, 2012). *Hopx* appears to be one of only a few genes that identifies quiescent, self-renewing adult stem cell populations in multiple tissues. *Hopx* expression marks epithelial stem cells in the hair follicle and intestinal crypt (Takeda et al., 2011). *Hopx* is also expressed in neural progenitor cells of the adult brain (De Toni et al., 2008). Expression in the adult pancreas (Dorrell et al., 2011), lung (Yin et al., 2006), mammary gland and kidney (J.A.E., unpublished) has also been identified. Future investigations will address whether *Hopx* marks progenitor cells in these organs. Additional genes that mark multiple adult stem cell populations include *Sox9* (intestine and skin) (Vidal et al., 2005; Nowak et al., 2008; Gracz et al., 2010; Furuyama et al., 2011), *Bmi1* (pancreas and intestine) (Sangiorgi and Capecchi, 2008; Sangiorgi and Capecchi, 2009), *Lrig1* (intestine and skin) (Jensen and Watt, 2006; Jensen et al., 2009; Powell et al., 2012; Wong et al., 2012) and *Lgr5* (intestine and skin) (Barker et al., 2007; Jaks et al., 2008). This conservation of gene expression raises the intriguing possibility that there may be shared homeostatic mechanisms regulated by these genes among tissue-specific stem cell populations, which is beyond the scope of this manuscript and will be addressed in future studies.

Several markers have been identified to mark the skin stem cell compartment, including *CD34* (Albert et al., 2001; Trempus et al., 2003; Blanpain et al., 2004), *Sox9* (Nowak et al., 2008), *Tcf3* (Nguyen et al., 2006), *Lhx2* (Rhee et al., 2006), *K15* (Lyle et al., 1998; Liu et al., 2003), *Lrig1* (Jensen and Watt, 2006; Jensen et al., 2009), *Lgr5* (Jensen et al., 2009) and *Lgr6* (Snippert et al., 2010). Many of these have overlapping expression domains, but careful inspection identifies subtle differences. *Hopx* appears to be specific to the basal bulge layer and is absent from the $K6^+$ layer in telogen, in contrast to *Sox9*, *Lhx2*, *K15* and *Tcf3*, which mark both layers (Hsu et al., 2011). The secondary hair germ robustly expresses *Lgr5*, whereas *Hopx* may be weakly expressed there, although we cannot exclude the possibility of GFP perdurance. Therefore, combinations of markers may be useful to best identify subsets of stem cell populations and allow for the dissection of the heterogeneity of cells composing stem cell compartments in the skin. Ongoing studies are aimed at elucidating the function of *Hopx* in progenitor populations.

Hopx expression defines a previously unrecognized progenitor pool in anagen

Hopx is expressed in the companion layer as well as in a Shh^-, lower hair bulb population of cells in anagen, which is marked by *K6* mRNA expression (Winter et al., 1998). Recent studies have suggested the existence of a distinct population of cells in the lower hair bulb in position 4-5 that can give rise to the companion layer (Sequeira and Nicolas, 2012), but the additional functions and potential of these cells, especially during hair follicle cycling, is unknown. Our lineage-tracing studies demonstrate that a $Hopx^+$, Shh^- population of lower hair bulb cells in anagen gives rise to the $K6^+$ innermost layer of the telogen bulge. Previous reports have shown that *Lgr5*-expressing cells in the lower ORS are one source of $K6^+$ niche cells in telogen (Hsu et al., 2011), but *Hopx* is not expressed in the lower ORS. It is possible that two distinct progenitor populations can give rise to $K6^+$ niche cells, but our data suggesting the existence of $Lgr5^+$, $Hopx^+$, Shh^- lower hair bulb cells provides an alternative explanation that reconciles our findings with prior observations. We suggest that $Hopx^+$, $Lgr5^+$ cells in the lower hair bulb can give rise to $K6^+$ niche cells. Alternatively, we cannot exclude the possibility that $Lgr5^+$ lower ORS cells migrate into the lower hair bulb and subsequently activate *Hopx* expression. However, we believe this is less likely in light of recent data indicating that the ORS and the lower hair bulb represent distinct lineages (Sequeira and Nicolas, 2012). As the lower hair bulb cells transition/differentiate into K6-protein expressing cells surrounding the club hair during catagen, *Hopx* and *Lgr5* expression is extinguished whereas *Sox9*, *Lhx2* and *Tcf3* expression commences (DasGupta and Fuchs, 1999; Vidal et al., 2005; Rhee et al., 2006; Hsu et al., 2011; supplementary material Fig. S6). These latter HFSC markers remain expressed into telogen as the innermost $K6^+$ layer fully matures. It should also be noted that *Shh* expression marks the matrix, and lineage tracing of *Hopx*- or *Shh*-expressing cells demonstrates that they give rise to the IRS. A subset of Shh^+ cells in anagen co-expresses *Hopx* (Fig. 4A, white arrowhead).

During anagen, the upper companion layer expresses K6 protein whereas the lower companion layer and hair bulb express *K6* mRNA exclusively (Winter et al., 1998). In telogen, the niche cells express K6 protein. The shared expression of K6 by the companion layer and the niche has led to the investigation of potential similarities and differences among these populations of cells and raised the issue of whether there is a shared lineage relationship (Higgins et al., 2009). In fact, K6-expressing cells in telogen and anagen, as well as the cells surrounding the club hair in catagen, share a common molecular signature [expression of K6 (Winter et al., 1998; Wang et al., 2003; Hsu et al., 2011), PAI-2 (Lavker et al., 1998), calretinin (Poblet et al., 2005) and S100A6 (Ito and Kizawa, 2001)]. The companion layer is composed of thin long cells adjacent to the IRS, whereas the $K6^+$ niche (and catagen $K6^+$) cells are cuboidal (Hsu et al., 2011). Although they exhibit morphological differences, both layers remain adjacent to the ORS and appear to serve anchorage functions (Hsu et al., 2011; Chapalain et al., 2002). However, although the companion layer and $K6^+$ niche cells share some gene expression characteristics, our data demonstrate that the K6 protein-expressing companion layer is not the source of the $K6^+$ niche cells in telogen.

In summary, our data demonstrate that *Hopx* is a unique marker of multiple stem cell populations and marks a subset of known stem cell populations in the telogen hair follicle. Our data in anagen demonstrate the presence of a previously unappreciated progenitor population residing in the anagen lower hair bulb that expresses both *Hopx* and *Lgr5*, and gives rise to K6-expressing cells in telogen that are responsible for stem cell homeostasis, providing another example in which the progeny of a lineage cycles back to regulate its stem cell compartment.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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