Identification of hair shaft progenitors that create a niche for hair pigmentation

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Hair differentiates from follicle stem cells through progenitor cells in the matrix. In contrast to stem cells in the bulge, the identities of the progenitors and the mechanisms by which they regulate hair shaft components are poorly understood. Hair is also pigmented by melanocytes in the follicle. However, the niche that regulates follicular melanocytes is not well characterized. Here, we report the identification of hair shaft progenitors in the matrix that are differentiated from follicular epithelial cells expressing transcription factor KROX20. Depletion of Krox20 lineage cells results in arrest of hair growth, confirming the critical role of KROX20+ cells as antecedents of structural cells found in hair. Expression of stem cell factor (SCF) by these cells is necessary for the maintenance of differentiated melanocytes and for hair pigmentation. Our findings reveal the identities of hair matrix progenitors that regulate hair growth and pigmentation, partly by creating an SCF-dependent niche for follicular melanocytes.

Keywords: stem cell factor (SCF); hair pigmentation; hair shaft progenitor cell; hair follicle stem cell; hair matrix; KROX20

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hair shafts toward the skin surface, and the companion layer [the innermost layer of the outer root sheath] (Hsu et al. 2011, 2014). In contrast to HF stem cells in the bulge, the identities of the committed progenitor cells that give rise directly to hair shafts [the medulla, cortex and cuticle] are currently not well characterized. In addition, the development of hair is also accompanied by pigmentation that is contributed to the hair shafts by melanocytes. The reciprocal interactions between melanocytes and hair progenitor cells as well as the source of SCF, which regulates hair pigmentation, are not well characterized.

KROX20 (also known as EGR2) is a zinc finger transcription factor that regulates hindbrain segmentation (Schneider-Maunoury et al. 1993), Schwann cell myelination (Topilko et al. 1994), and lymphocyte immune responses (Li et al. 2012). In addition, KROX20 is expressed in subpopulations of HF cells (Gambardella et al. 2000). However, the role of the KROX20-expressing cells in HF development is not known. We now report that the transcription factor KROX20 identifies a sublineage of HF epithelial cells toward the differentiation of hair shaft during HF morphogenesis. We observed that they are a necessary source of SCF for the maintenance of mature melanocytes in the hair matrix to produce hair pigmentation, as evidenced by the complete loss of pigment when Scf is deleted in Krox20 lineage cells. We also demonstrate that deletion of Krox20 lineage cells in vivo results in a complete arrest of new hair growth, demonstrating the critical role of Krox20 lineage cells in hair development.

Results

Mice lacking SCF in Krox20 lineage cells exhibit progressive hair graying

While studying the role of mast cells and SCF during the initiation of neurofibroma, a Schwann cell neoplasm, we conditionally deleted Scf in neurofibroma neoplastic cells using the Schwann cell lineage Krox20Cre mouse line. Serendipitously, we found that the mice without SCF in Krox20 lineage cells developed hair graying and, early in life, lost all hair pigmentation. Invariably, all Scflox/gfp, Krox20Cre mice (n = 20) displayed progressive hair graying. The first round of hair graying occurred homogeneously during postnatal days 30–40 (P30–P40). As the mice aged, the hairs underwent further depigmentation in waves. This change converted all black pigmented hairs to white within 9 mo (Fig. 1A;Supplemental Fig. S1A). We believe that this pattern of hair color change is associated with the mouse hair cycle when old hairs are replaced by newly generated hairs (Plikus and Chuong 2008; Shimomura and Christiano 2010).

To characterize the cause of hair graying in the Scflox/gfp; Krox20Cre mice, we first analyzed the pelage hair shafts plucked from P20 dorsal skin. Interestingly, the amount of pigment at the distal and proximal ends of each hair shaft was quite different; the amount of pigment at the distal appeared normal, but the pigment was mostly absent from the proximal end [Fig. 1B]. Fontana-Masson staining confirmed that this hypopigmentation was caused by the absence of melanin in the hair shaft cortex and medulla [the compartments housing the most melanin in pigmented hair shaft] [Fig. 1C]. We then determined the time of onset of decreased pigmentation. The amount of pigment in individual hairs appeared normal at P9 [Fig. 1D, with the loss of hair pigmentation becoming noticeable at P11; it progressed quickly after that [Fig. 1D,E]. Analysis of melanin density in the HF revealed significant reductions at P11 and P13 (P < 0.0001) [Fig. 1F], explaining the early loss of hair pigmentation in Scflox/gfp; Krox20Cre mice (Fig. 1B). Scflox/gfp; Krox20Cre mice exhibited progressive hair graying; their coat color underwent a dynamic change from black to white within 9 mo [Fig. 1A, Supplemental Fig. S1A]. However, their newly synthesized hairs were already largely depigmented at P20 [Fig. 1B]. These results suggested that the long period of hair graying is due to the mixture of partly pigmented [old] and unpigmented [new] hair shafts [Supplemental Fig. S1B,C] rather than the continuous production of new gray hairs. To confirm this, gray Scflox/gfp; Krox20Cre mice were depilated at 2 mo to remove all hairs, including old hairs, and stimulate new hair regeneration, as expected, their new hairs showed a complete loss of pigmentation [Fig. 1G]. Most importantly, the fact that Scflox/gfp; Krox20Cre mice underwent a complete loss of hair pigmentation suggested that Krox20 lineage cells are the main source of SCF for follicular melanocytes to produce hair pigment.

Hair pigmentation is dependent on SCF expression by epithelial keratinocytes

In order to identify the cell type that produces SCF in the skin, thereby regulating hair pigmentation and being responsible for the hypopigmentation in Scflox/gfp; Krox20Cre mice, we examined the hair color phenotype of SCF ablation in different cell lineages in the skin by using several cell type-specific Cre mouse lines, including DhhCre [Schwann cell], PLPCreERT2 [Schwann cell], TyrCreERT2 [melanocyte], and K14Cre [keratinocyte]. In addition, CMVCreERT2 [all cells] was included as a control for the inducible Cre systems. The R26-LacZ reporter was introduced into all of the above conditional knockouts to validate Cre expression specificity and efficiency.

In mice lacking SCF in Schwann cells {Scflox/gfp; DhhCre}, we did not observe any hair color changes for up to 6 mo [Fig. 2A]. LacZ staining confirmed the specific DhhCre expression in Schwann cells in cutaneous nerves [Fig. 2B]. Similar results were observed in Scflox/gfp; PLPCreERT2 mice [Supplemental Fig. S2A]. These results revealed that hair pigmentation does not depend on SCF expression in cells of Schwann cell lineages. Likewise, mice with SCF depletion in melanocytes {Scflox/gfp; TyrCreERT2} had no phenotype in hair color [Fig. 2C] after validation of Cre expression in the hair matrix melanocytes [Fig. 2D]. Importantly, this result ruled out the possibility of melanocyte cell-autonomous SCF contributions to hair pigmentation.

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In Scfflox/gfp, PLPCreERT2 and Scfflox/gfp, TyrCreERT2 mice, we activated Cre expression by injecting 4-hydroxy-tamoxifen at P0. To be certain that this timing was suitable for examining the requirement for SCF in hair pigmentation, we generated Scfflox/flox; CMVCreERT mice as a control to ablate Scf ubiquitously by the same 4-hydroxytamoxifen induction condition. These mice lost their hair pigmentation nearly completely after the use of 4-hydroxytamoxifen (Fig. 2E), validating the Cre induction conditions. Similar results were also observed when these Cre expressions were induced in adult mice (Supplemental Fig. S2D–G).

Finally, we generated Scfflox/gfp, K14Cre mice to examine the phenotype after SCF ablation in keratinocytes. Strikingly, depletion of SCF in keratinocytes resulted in mice displaying a complete absence of hair pigmentation, beginning at birth (Fig. 2F), and this phenotype was limited to hair pigmentation without affecting the development or growth of hair and the epidermis (Fig. 2G). This result demonstrated the critical contribution of SCF from epithelial keratinocytes to hair pigmentation. This finding also supports the non-cell-autonomous SCF signaling model for hair pigmentation.

In addition, albeit with different timing of onset, aged Scfflox/gfp, Krox20Cre mice phenocopied Scfflox/gfp, K14Cre mice by having a complete absence of hair pigmentation (Figs. 1A, 2F), suggesting that KROX20-expressing cells originate from K14 lineage epithelial keratinocytes and that KROX20 expression in the HF starts later in development.

**Dynamic localization of Krox20 lineage cells during HF morphogenesis**

To characterize the late onset of hair hypopigmentation in Scfflox/gfp, Krox20Cre mice, we explored Krox20 lineage...
we found that LacZ+ cells were detected only as a small portion of the HF at P0. At P2, LacZ+ cells occupied the entire population of cells located at the infundibulum and bulge.

Krox20Cre

ing the cells in skin at different HF developmental stages by cross-analyzed at least three mice from each age and observed a consistent recombination pattern [Fig. 3A]. At the final stage of differentiation, hair matrix keratinocytes undergo a unique type of programmed cell death that is marked by systematic destruction of the nucleus. These keratinized anucleate cells become enveloped by specialized and highly cross-linked membranes that eventually form the building blocks of the hair shaft. Strikingly, in P12 skin, we also observed LacZ activity within the hair shafts [Fig. 3A]. This is direct evidence demonstrating that Krox20 lineage labels hair progenitor cells that give rise directly to the hair shafts. Importantly, considering the timing of the first appearance of LacZ+ cells in the hair matrix, Krox20 lineage appears to mark only a subsequent wave of hair progenitors postnatally. This observation corresponds with the late onset of hair hypopigmentation in the Scfflox/gfp; Krox20Cre mice [Fig. 1A, Supplemental Fig. S1A]. It also revealed distinct developmental waves of hair shaft progenitor cells and, importantly, suggested that hair pigmentation is regulated by SCF expression in hair shaft progenitor cells.

We therefore examined SCF expression in Krox20 lineage cells by Scf promoter-driven GFP (ScfβTr). Our data showed that SCF-expressing and Krox20 lineage cells were colocalized at the hair matrix at P6 [the time at which Krox20 lineage cells begin to colonize in the matrix] [Fig. 3B] and at the hair shaft at P12 [the time at which Krox20 lineage cells build the hair shaft] [Fig. 3C]. These results support the model in which SCF expression in hair shaft progenitor cells controls hair pigmentation.

Our lineage tracing of Krox20 revealed a new identity of epithelial cells giving rise to the hair shaft [Fig. 3A]. To determine the developmental origin of KROX20-expressing cells in the HFs, we generated K14Cre; R26-YFP mice to genetically label all K14 lineage epithelial keratinocytes early in development with YFP. We subsequently performed immunofluorescence staining against KROX20 and observed that KROX20 is expressed in a subpopulation of the of K14 lineage cells in the HFs [Fig. 3D], indicating that that HF KROX20+ cells are differentially derived from a K14+ keratinocyte lineage. The detection of KROX20 protein in the outer root sheath and matrix demonstrates that KROX20 is expressed actively during hair development. Taken together with the findings that Scf depletion in Schwann cells does not cause hair hypopigmentation [Fig. 2A; Supplemental Fig. S2A,D], these results distinguish the origin of KROX20+ HF cells (epithelial derivative) from KROX20+ Schwann cells (neural crest derivative) during skin development.

Because hair shaft progenitor cells are differentiated from HF stem cells in the bulge and because their activation is associated with hair cycle, we therefore tested the hypothesis that depletion of SCF in HF stem cells will result in hair hypopigmentation in a hair cycle-dependent manner. To address this hypothesis, we used the inducible K14CreERT mouse line (Vasioukhin et al. 1999) to examine the effects of Scf gene ablation in HF stem cells. The inductions of SCF ablation in Scfflox/gfp; K14CreERT mice were performed at different HF growth stages. Scfflox/gfp; K14CreERT mice with induction of SCF ablation at P0 [in anagen, when some hair shaft progenitor cells have already formed] showed normal hair pigmentation initially; however, their coat color turned gray after subsequent anagen [Supplemental Fig. S3A], phenocopying the Scfflox/gfp; Krox20Cre mice. On the other hand, Scfflox/gfp; K14CreERT mice with induction of SCF loss and depilation together at P90 [in telogen, when hair shaft progenitor cells have not
The hair matrix is the SCF-dependent niche for hair pigmentation

In Scfflox/gfp; K14Cre mice, the complete loss of hair pigmentation is caused by Scf deletion in K14 lineage cells. We therefore hypothesized that this depigmentation could be rescued by ectopic Scf expression under a K14 promoter. To test this hypothesis, we used a mouse transgene with a K14 promoter-driven membrane-bound form of Scf [K14-Scf], which produces a dominant phenotype of epidermal hyperpigmentation [Kunisada et al. 1998a]. Given that a mouse mutant expressing only a soluble form of SCF [SFd/SFd] has no skin or hair pigmentation [Copeland et al. 1990; Brannan et al. 1991], we reasoned that hair pigmentation is dependent on membrane-bound SCF and thus that the K14-Scf transgene should be a suitable tool to address the requirement of SCF for hair pigmentation.

Surprisingly, the K14-Scf; Scfflox/gfp; K14Cre mice displayed white hair with dark skin [Fig. 4A,B], indicating that even though Scf is expressed under a K14 promoter, K14-Scf was unable to rescue the hair depigmentation in Scfflox/gfp; K14Cre mice. Likewise, we observed that K14-Scf did not rescue the hair-graying progress in Scfflox/gfp; Krox20Cre mice. The K14-Scf; Scfflox/gfp; Krox20Cre mice underwent hair graying identical to that of Scfflox/gfp; Krox20Cre control mice [Fig. 4C], although the K14-Scf transgene appeared to remain active over time [Fig. 4D]. These findings are similar to earlier experiments in which the K14-Scf transgene was unable to rescue hair hypopigmentation in SFd/SFd mice [Yoshida et al. 2001] or KIT-neutralizing antibody ACK2-treated mice [Nishimura et al. 2002].

To explore the underlying mechanism by which ectopic expression of K14-Scf failed to rescue the Scf loss-induced hair hypopigmentation, we reasoned that perhaps the K14 promoter activity is turned off in the KROX20+ hair shaft progenitor cells in the matrix, and therefore the lack of SCF expression had regulated hair pigmentation. To address this question, we lineage-traced the cells with "Scf ablation" by R26-YFP [as a marker for K14Cre lineage]...
and identified the cells with “Scf reconstitution” by K14 immunostaining [as a marker for K14 promoter] in the skin from K14Cre; R26-YFP mice. Intriguingly, YFP was detected in the interfollicular epithelium and the entire HF (except dermal papilla), whereas K14 was detected only in the interfollicular epithelium and only the top portion of anagen HF [Fig. 4E]; similar results were obtained from K14Cre; R26-LacZ mice [Fig. 4F]. This expression pattern matches previous studies on K14Cre lineage tracing [Huelsken et al. 2001] and K14 expression [Coulombe et al. 1989]. Most importantly, these data demonstrate that hair pigmentation is tightly dependent on SCF from the Krox20 lineage cells in the hair matrix of HFs that are differentiated from K14 lineage but no longer express K14.

To better characterize the expression of SCF during HF development, we then took advantage of another marker, GFP, to locate the SCF-expressing cells by using the Scflox/gfp mice. We found that GFP+ cells are predominantly localized in the upper hair matrix and hair shaft [but not the bulge or outer root sheath area] throughout the anagen stage [Fig. 5A], pointing to the hair shaft progenitor cells as a unique cell type expressing SCF during the HF growth stage. This observation was supported by the inclusion of melanin pigment [Fig. 5B] and the expression of P-cadherin [Fig. 5C] in these SCF-expressing cells. P-cadherin is an epithelial cell–cell adhesion molecule and, in HFs, marks the outer root sheath, inner matrix, and hair shaft [Samuelov et al. 2012]. As the hair matrix is also colonized by melanocytes, we therefore determined the location of mature melanocytes by immunostaining of dopachrome tautomerase (DCT). DCT is an enzyme participating in melanin biogenesis and, in HFs, marks mature melanocytes in the hair matrix predominantly and a small number of melanocyte stem cells in the bulge [Nishimura et al. 2005]. DCT+ melanocytes are tightly adjacent to the SCF-expressing cells in the matrix [Fig. 5D]; this result is in agreement with the identification of matrix SCF-expressing cells as the hair shaft progenitor cells. Taken together, we concluded that hair pigmentation is controlled exclusively by SCF on hair shaft progenitor cells, which regulates melanocytes in the HF matrix in a non-cell-autonomous fashion.

SCF in the matrix is crucial for the maintenance of mature melanocytes

We showed that depletion of SCF in the hair shaft progenitor cells results in loss of hair pigmentation. In addition, melanocytes are the specialized melanin-producing cells as well as the predominant KIT-expressing cells in the HF [Lin and Fisher 2007]. Therefore, melanocytes mediate SCF signaling to hair pigmentation, and SCF is necessary for melanocytic activity and hair pigmentation. Thus, we next addressed the fate of follicular melanocytes in response to the loss of SCF in the hair shaft progenitor cells.

The Scflox/gfp; Krox20Cre mice began to lose hair pigmentation at P11–P13 due to SCF loss in their hair shaft progenitor cells [Fig. 1D–F]. We took advantage of this model to address the fate of melanocytes during SCF loss. As hair shaft progenitor cells are tightly adjacent to melanogenic melanocytes in the upper HF matrix [Fig. 5D], we thus hypothesized these mature melanocytes to be the first affected cells. Indeed, DCT immunostaining revealed a significant loss of DCT+ differentiated mature melanocytes in the Scflox/gfp; Krox20Cre mouse HF at
P10 ($P < 0.05$) and P11 ($P < 0.0001$) (Fig. 6A–C). This result demonstrated a critical role of SCF in the maintenance of mature melanocytes. Furthermore, we explored the fate of DCT$^+$ melanocytes in another mouse model: Scfflox/gfp; K14Cre. These mice had a complete loss of hair pigmentation throughout their lifetimes (Fig. 2F). Therefore, it was not surprising that DCT$^+$ melanocytes were not detected in their hair matrices (Fig. 6D). The HF melanocytes have been characterized previously to express KIT, which is the receptor for SCF (Lennartsson and Ronnstrand 2012). Melanocytes in the upper and lower follicular matrix compartments can be separated by the Line of Auber (an imaginary line drawn across the widest region of the hair bulb) (Peters et al. 2002). Melanocytes in the lower matrix are relatively undifferentiated and nonmelanogenic. However, differentiated mature melanocytes reside in the upper HF matrix and produce melanin. We further characterized the extent to which SCF can affect these two melanocyte populations by utilization of this other melanocyte marker: KIT. KIT$^+$ melanocytes were present in both lower and upper HF matrices in the control skin. However, in the skin from Scfflox/gfp; K14Cre mice, KIT$^+$ melanocytes were detected only in the lower matrix but were completely absent in the upper HF matrix (Fig. 6D). This finding demonstrates that loss of SCF in the hair shaft progenitor cells influences only the mature melanocytes, highlighting the upper HF matrix as a critical niche where non-cell-autonomous SCF/KIT signaling regulates hair pigmentation. It also points to the critical role of follicular epithelial SCF in controlling the fate of melanocytes in their final maturation without affecting their previous differentiation and migration.

**HF Krox20 lineage cells are critical for hair development**

Based on our work, we concluded that the transcription factor KROX20 marks a lineage of resident hair shaft progenitor cells in the follicular matrix. Therefore, we hypothesized that Krox20 lineage cells are indispensable for hair growth. To test this hypothesis, we generated a doxycycline-inducible mouse model (Krox20Cre; R26-rtTA; tetO-DTA) to deplete Krox20 lineage cells in vivo. The R26-rtTA expresses reverse tetracycline-controlled transactivator (rtTA) (Belteki et al. 2005) in the Krox20 lineage cells. The tetO-DTA expresses diphtheria toxin A (DTA) (Lee et al. 1998) to eliminate cells with rtTA expression (Krox20 lineage cells in this case) upon doxycycline induction (Fig. 7A). We used this model to examine the effects of Krox20 lineage cell depletion during the second anagen phase (starting at about P23–P24). Mice were fed with doxycycline water starting at P20. We observed signs of hair loss (rough coat) starting at P40, and they had significant hair loss by P50 (Fig. 7B). As expected, these mice also displayed weak muscle tone (data not shown), which is likely associated with the peripheral nerve demyelination caused by KROX20$^+$ cell depletion (Decker et al. 2006). To determine whether this hair loss, induced by Krox20 lineage cell depletion, was caused by impaired hair growth or increased hair

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**Figure 5.** SCF is expressed in hair shaft progenitor cells. [A] Scf promoter-driven GFP (Scfgfp) showed that SCF is expressed in the upper HF matrix and hair shaft throughout anagen. [B] SCF is expressed in melanin-containing cells [arrowheads] in the HF in P10 Scfflox/gfp skin. [C] SCF is expressed in a subpopulation of P-cadherin$^+$ HF matrix cells and the hair shaft in P9 Scfflox/gfp skin. [D] DCT$^+$ melanocytes are tightly adjacent to SCF-expressing cells in the upper HF matrix in P10 Scfflox/gfp skin. [Yellow dashed line] Line of Auber (an imaginary line drawn across the widest region of the hair bulb); [blue dashed line] dermal papilla. Nuclei were stained with DAPI. Bars: A, 100 µm; B–D, 50 µm.
sheding, we examined the effects of depilation on new hair growth. Mice were depilated to induce new hair growth at P20. At the same time, they were started on doxycycline water to deplete Krox20 lineage cells in vivo. Within 15 d, control mice (R26-rtTA; tetO-DTA) regrew their entire hair coat. On the other hand, Krox20Cre; R26-rtTA; tetO-DTA mice had a complete arrest of new hair generation (Fig. 7C), demonstrating the critical role of Krox20 lineage cells in hair development.

To characterize further the role of Krox20 lineage cells in HF development, we examined the skin of Krox20Cre; R26-rtTA; tetO-DTA mice after doxycycline treatment between P20 and P50 to assess the impact of Krox20 lineage cell depletion on hair regeneration. We found that Krox20 lineage cell-depleted HFs were arrested at anagen (Fig. 7D), whereas the control HFs completed the anagen phase and rested at telogen (Fig. 7F). Importantly, in contrast to normal anagen HFs, Krox20 lineage cell-depleted HFs had shrunken hair bulbs, which were located near to where the hair shaft progenitor cells had resided previously (Fig. 7E). This atypical anagen phase, with missing stereotypic bulb structure (the reservoir of matrix cells), explained their inability to grow new hair. We also noticed that the Krox20 lineage cell-depleted skin exhibited ectopic melanin deposition around the HFs (Fig. 7G); this is likely contributed by the death of Krox20 lineage cells after obtaining the melanin from melanocytes, as we started the doxycycline treatment on the same day of depilation, and therefore it will take some time after the doxycycline treatment for the diphtheria toxin A to delete the Krox20 lineage cells. The appropriateness of this system was validated by faithful depletion of KROX20 cells in vivo after doxycycline induction (Fig. 7H). Taken together, these analyses confirm the critical role of Krox20 lineage cells in hair development as the cells that produce hair directly.

Discussion

We identified that the transcription factor KROX20 marks a cell lineage differentiating toward the hair shaft and that SCF in these hair shaft progenitor cells acts as a critical intrinsic rheostat of hair pigmentation by managing mature melanocyte in the upper HF matrix. Ablation of Scf in Krox20 lineage cells consequently leads to a complete absence of hair pigmentation, showing an indispensible non-cell-autonomous SCF contribution to melanocytes and that Krox20 lineage cells are the main source of SCF for follicular mature melanocytes to produce hair pigment. The completion of hair regeneration is dependent on the presence of HF Krox20 lineage cells as the cells giving rise directly to hair shafts (Fig. 7I).
SCF production in keratinocytes appears solely to regulate the generation of hair pigmentation by melanocytes because no skin or hair developmental defect was observed in the Scfflox/gfp; Krox20Cre or Scfflox/gfp; K14Cre mice (Figs. 1D, 2G). The critical role of SCF/KIT signaling in hair pigmentation has long been recognized (Copeland et al. 1990; Reith et al. 1990; Okura et al. 1995); however, this is the first report to identify Krox20 lineage hair shaft progenitor cells definitively as the source of SCF to control this process. This discovery could lead to additional studies to characterize their contributions to other signaling events that are critical for melanocyte development, including the effects of Notch (Schouwey et al. 2007), TGFβ (Nishimura et al. 2010), and β-catenin (Rabbani et al. 2011). During the development of melanocytes, the neural crest stem cells generate melanoblasts that subsequently become melanocyte stem cells. Although melanocyte stem cells in the bulge do not require SCF, their differentiation into mature melanocytes is SCF-dependent [Nishimura et al. 2002], consistent with our finding that loss of SCF in hair shaft progenitor cells targets only the mature melanocytes in the matrix and not the undifferentiated melanocyte precursors.

While dissecting the hair-graying phenotype of Scfflox/gfp; Krox20Cre mice, we surprisingly discovered that this hair color change is associated with distinct stages in the cells committed to hair production. Krox20 lineage marks the hair shaft progenitor cells only after P6 – P10 (Fig. 3A), which demonstrates the presence of a second wave of hair shaft progenitor cells and explains the late onset of hair hypopigmentation and eventual complete depigmentation in Scfflox/gfp; Krox20Cre mice. Nuclei were stained with DAPI. (I) Schematic illustration of the roles of SCF and Krox20 lineage cells in hair pigmentation and development. Lineage tracing of Krox20 lineage cells revealed a dynamic expansion toward the direction of hair-producing cells during HF morphogenesis in the upper HF matrix (red box). Hair progenitor cells sustain differentiated melanocytes through non-cell-autonomous SCF/KIT signaling. Depletion of SCF in hair progenitor cells results in loss of hair pigmentation. Depletion of Krox20 lineage cells, the cells that differentiate into hair-producing cells, results in impaired hair regeneration. Bar, 50 µm.
the hair graying in Bcl2−/− mice (McGill et al. 2002, Nishimura et al. 2005). On the other hand, β-catfllox−/−, K14Cre mice (Huelsken et al. 2001) display late-onset hair loss. They develop hairs after birth, but most of these hairs are lost as they grow into adulthood. Taken together, these late-onset phenotypes might be related to our two-wave hair growth model. Furthermore, this model could shed light on the classification of embryonic and adult stem cells that are committed to the same type of tissue generation.

KROX20 is best known as a transcription factor for Schwann cells during differentiation from promyelinating to myelinating states (Topilko et al. 1994). This phenomenon is similar to the place primarily during early postnatal development (Zorick et al. 1999). This phenomenon is similar to the expansion of HF (Zorick et al. 1999). This phenomenon is similar to the expansion of HF lineage cells during hair morphogenesis (Fig. 3A). A recent study has revealed that peripheral nerve KROX20 induction is regulated by the Lin28/peripheral nerve KROX20 induction is regulated by the axis (Gokbuget et al. 2015). Lin28 is a conserved RNA-binding protein that is expressed highly in embryonic stem cells. It is also diminished sharply after birth; this change results in the induction of Let-7 microRNA (Shyh-Chang and Daley 2013). Interestingly, Lin28a transgenic mice exhibit a thickened hair coat due to prolonged anagen (Shyh-Chang et al. 2013). These results suggest that KROX20 could be a downstream regulator for Lin28/Let-7-mediated perinatal development in both nerves and skin.

The bone morphogenetic protein [BMP] and Wnt/β-catenin signaling plays a central role in epidermal homeostasis and HF development [Blanpain and Fuchs 2009; Solanis and Benitah 2013; Plikus and Chuong 2014]. Mice with K5 promoter-driven BMP antagonist noggin (K5-noggin) are characterized by enlarged anagen HFs with thickened hair shafts; this feature is associated with hyperproliferative hair matrix cells with a sixfold decrease in Krox20 expression (Sharov et al. 2006). Similarly, K14-noggin mice have a faster response to hair regeneration [Plikus et al. 2008]. Moreover, Wnt activation leads to up-regulation of KROX20, and this induction can be synergized by BMP inhibition with noggin (Saint-Jeannet et al. 1997; Baker et al. 1999). Taken together with our findings, the functional prediction for KROX20 in HF development could be a downstream effector of Wnt/β-catenin and BMP signaling, thereby directing HF stem cell differentiation.

In conclusion, this study delineates the origin of SCF expression in the hair matrix progenitors as a niche for follicular mature melanocytes and that their SCF is indispensable for hair pigmentation. In addition, SCF expression in the matrix identifies immediate antecedents of hair shaft structural cells, and transcription factor KROX20 marks a sublineage of epithelial cells differentiating toward this hair shaft progenitor cells. Future studies to reconstitute SCF in the matrix niche will be interesting and will address whether hair hypopigmentation could be reversible. Furthermore, genetic ablation of Krox20 and global profiling of KROX20 transcriptional targets will help to elucidate its biological roles in epidermal differentiation and HF development.

Materials and methods

Mice

Animal care and experiments were approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center. Scflox/lox and Scf+/+ were generated in a previous report [Ding et al. 2012]. K14Cre [Dassule et al. 2000], K14CreERT [Vasioukhin et al. 1999], TyrCreERT2 [Bosenberg et al. 2006], R26-rtTA [Belteki et al. 2005], and tetO-DTA [Lee et al. 1998] mice were purchased from the Jackson Laboratory. K14-Scf [Kunisada et al. 1998a] was kindly provided by Dr. John Harris (University of Massachusetts Medical School). Krox20Cre [Voiculescu et al. 2000], DbhCre [Skucas et al. 2013], LplCreERT2 [Leone et al. 2003], CMVCmERT2 [Hayashi and McMahon 2002], R26-LacZ, and R26-YFP were described previously (Le et al. 2009; Chen et al. 2014).

Tamoxifen and 4-hydroxytamoxifen induction

Tamoxifen or 4-hydroxytamoxifen (Sigma-Aldrich) was administered to mice to induce CreERT or CreERT2 expression. For induction in a newborn mouse, a single-dose treatment of 40 µg of 4-hydroxytamoxifen was injected subcutaneously. For induction in adult mice, 3 mg of tamoxifen was oral-gavaged daily for five consecutive days.

Doxycycline induction

For the induction of tetO-DTA, mice were fed with water containing 2 mg/mL doxycycline [Sigma-Aldrich] and 5% sucrose.

Mouse hair depilation

Mice were anesthetized by intraperitoneal injection of 100 µL [for a 25-g mouse] of a mixture of 30 mg/mL ketamine and 4 mg/mL xylazine solution. Mouse hair was trimmed by an electrical clipper. Depilatory cream [Nair] was applied gently on the area to be depilated and then wiped off with water-moistened gauzes to remove the hair.

Histology analysis

For histology analysis, skin tissue was harvested and fixed in 10% formalin overnight followed by paraffin embedding. The tissue was then sectioned at 5-µm thickness. Hematoxylin and eosin staining was performed according to the manufacturer’s protocol (StatLab).

LacZ staining

For LacZ staining, mice were anesthetized by intraperitoneal injection of ketamine and xylazine as described above. Mice were then subjected to total body perfusion with 4% paraformaldehyde. Skin was harvested and fixed in 4% paraformaldehyde for 30 min followed by PBS rinses. LacZ staining was performed in 4-chloro-5-bromo-3-indolyl-β-galactoside [X-gal] solution [1 mg/mL X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride in PBS] overnight at 37°C. X-gal-stained tissue was fixed in 10% formalin overnight and then subjected to paraffin embedding and tissue sectioning. Nuclei were counterstained with nuclear Fast Red.

Fontana-Masson staining

Paraffin-embedded tissue sections were deparaffinized and rehydrated. Melanin staining was performed by microwaving tissue...
sections in ammoniacal silver working solution for 2 min. The ammoniacal silver stock solution was prepared by adding ammonium hydroxide to 10% silver nitrate until the solution precipitated and cleared again; the working solution was freshly prepared by mixing the stock solution with water at 1:3 ratio followed by filtering. Slides were then incubated with 0.1% gold chloride for 10 min and 5% sodium thiosulfate for 5 min with water rinses between each step. Nuclei were counterstained with nuclear Fast Red.

**Immunostaining**

For immunostaining, frozen sections or paraffin sections after deparaffinization, rehydration, and antigen retrieval were used. The primary antibodies used in this study were P-cadherin (R&D Systems, AF761), DCT (PEF89; gift from Dr. Vincent Hearing, National Institutes of Health) [Virador et al. 2001], K14 [biotin-labeled, Thermo, clone LL002], LacZ [Abcam, ab9361], GFP/YPF [Aves, 1020], Krox20 [Covance, PRB-236P], and KIT [Cell Signaling, National Institutes of Health] (Virador et al. 2001), K14 (bioconjugated with Cy3 or Alexa fluor 488 (Jackson Immunoresearch), and nuclei were counterstained with DAPI (Vector Laboratories). For immunofluorescent staining, the primary antibodies were detected by secondary antibodies or streptavidin conjugated with Cy3 or Alexa fluor 488 (Jackson Immunoresearch, 3074). For immunofluorescent staining, the primary antibodies were detected by secondary antibodies or streptavidin conjugated with 3,3’-diaminobenzidine (Vector Laboratories).

**Statistical analysis**

Data statistical analyses were performed by unpaired two-tailed Student’s t-test (Prism7, GraphPad). Data are mean ± SEM. Significant differences were noted by asterisks (P < 0.05 [*], P < 0.01 [**], P < 0.001 [***], and P < 0.0001 [****]).

**Densitometry analysis**

The quantification of HF melanin density and DCT immunofluorescence staining intensity was performed with ImageJ software (National Institutes of Health) in arbitrary units.

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