A Dual Role for SOX10 in the Maintenance of the Postnatal Melanocyte Lineage and the Differentiation of Melanocyte Stem Cell Progenitors

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Abstract

During embryogenesis, the transcription factor, Sox10, drives the survival and differentiation of the melanocyte lineage. However, the role that Sox10 plays in postnatal melanocytes is not established. We show in vivo that melanocyte stem cells (McSCs) and more differentiated melanocytes express SOX10 but that McSCs remain undifferentiated. Sox10 knockout (Sox10⁻/⁻; Tg(Tyr::CreER)) results in loss of both McSCs and differentiated melanocytes, while overexpression of Sox10 (Tg(DctSox10)) causes premature differentiation and loss of McSCs, leading to hair graying. This suggests that levels of Sox10 are key to normal McSC function and Sox10 must be downregulated for McSC establishment and maintenance. We examined whether the mechanism of Tg(DctSox10) hair graying is through increased expression of Mitf, a target of SOX10, by asking if haploinsufficiency for Mitf (Mitf⁻⁰.⁵) can rescue hair graying in Tg(DctSox10) animals. Surprisingly, Mitf⁻⁰.⁵ does not mitigate but exacerbates Tg(DctSox10) hair graying suggesting that MITF participates in the negative regulation of Sox10 in McSCs. These observations demonstrate that while SOX10 is necessary to maintain the postnatal melanocyte lineage it is simultaneously prevented from driving differentiation in the McSCs. This data illustrates how tissue-specific stem cells can arise from lineage-specific precursors through the regulation of the very transcription factors important in defining that lineage.

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Introduction

In the adult animal, tissue-specific stem cells exist in a number of organs and function to sustain these tissues during normal homeostasis. However, our understanding of the origin and establishment of tissue-specific stem cells during organogenesis is incomplete. Using melanocytes as a model, we investigated the process of lineage-specific stem cell fate acquisition by examining the role of the transcription factor SOX10 in the formation of the melanocyte stem cell (McSC) within the mouse hair follicle.

Melanocytes of the hair follicle have gained increasing attention for studying cell-specific contributions to organ development and maintenance. Individual hair follicles act as "mini-organs" [1], and each contains melanocytes that provide pigment to the hair shaft concomitantly with hair cycling. Two primary subpopulations of follicular melanocytes exist and are defined by their anatomical location—McSCs remain in hair bulge, whereas the terminally-differentiated and pigmented melanocytes reside in the transient hair bulb region [2,3]. Identification of each of these subpopulations has been defined molecularly, in part through the use of immunohistochemistry [4–7]. Disruption of McSC function results in hair graying, a non-lethal and visible phenotype, and gray-haired mouse models have been used successfully to study adult stem cell establishment and maintenance [7–11].

The most critical time point for establishing McSCs appears to be during hair morphogenesis. Studies using the KIT-blocking antibody, ACK, to deplete melanocyte populations perinatally show that McSCs inhabit hair follicles around P4, demonstrated by the fact that they survive independent of KIT-signaling and are sufficient to restore coat color pigmentation [2,6]. Many of the melanogenic genes expressed by melanoblasts or bulb melanocytes exist at low/absent levels in McSCs. This distinction arises between stages 6 and 8 of hair follicle morphogenesis (~P4–P8) and is indicated by the loss of ki67 expression and the downregulation of MITF, TRP1, TYR, and SOX10 within presumptive McSCs [4,7,12]. Although McSCs are not responsible for pigmenting the first morphogenetic hair [3], they are retained within the hair bulge while melanocytes of the hair bulb undergo apoptosis during hair regression [13,14]. These McSCs then function to regenerate bulb melanocytes during subsequent hair cycles [8].
**Author Summary**

The melanocyte stem cells (McSCs) that reside in the hair follicle are critical for generating the melanocytes that will differentiate and produce pigment for the hair during successive rounds of hair growth. The inappropriate maintenance of McSCs results in hair graying. Thus, our understanding of McSC biology is enhanced through the study of hair graying mouse models. We have discovered that sustained expression of the transcription factor, Sox10, in the melanocyte lineage results in loss of McSCs and consequently leads to premature hair graying. Through the use of mouse transgenics, we demonstrate that by changing Sox10 levels, melanocytes of the hair can preserve their ability to survive and produce normally pigmented hairs while also allowing a portion of them to fulfill the role of an undifferentiated McSC. We also discovered that Mitf, a downstream target of SOX10 and the master regulatory transcription factor for melanogenesis, appears to participate in McSC maintenance, perhaps by the negative regulation of SOX10-dependent processes. These observations raise the idea that adult stem cells, like McSCs, may rely on cell type specific transcription factors for their specification and survival, but that these transcriptions factors also have to be carefully regulated to maintain a stem cell fate.

The subpopulation-specific expression of the transcription factor Sox10, where it is expressed in melanoblasts of the skin and melanocytes of the hair bulb but absent from McSCs, suggests that transcriptionally downregulating Sox10 is the mechanism by which melanoblasts acquire a McSC fate. This hypothesis fits well with the known function of Sox10 as a transcription factor that participates in melanocyte differentiation by upregulating Mitf, the master regulatory gene for melanogenesis. The loss of melanin synthesis proteins, TRP1 and TYR, within presumptive McSCs further supports this idea since SOX10 transcriptionally activates these genes, and that TYR is required by mouse melanocytes to generate pigment [15–18]. In the mouse, Sox10 is expressed during neural crest development and its loss embryonically results in several neurocristopathies, including congenital hypopigmentation [19–24]. However, perinatal lethality in Sox10 null mice has precluded functional analysis of Sox10 in adult melanocytes. Using conditional transgenics we can now explore the role of Sox10 postnatally in the melanocytes of the mouse hair follicle.

Here we report that postnatal mouse melanocytes both express and require Sox10 for normal hair pigmentation. However, constitutive expression of Sox10 by McSCs disrupts their maintenance by driving their premature differentiation. We also demonstrate that Mitf contributes to this regulation, likely through a negative feedback mechanism. Together, these data support the theory that transcription factors responsible for the specification of lineage-defined precursors can later participate in the specification and maintenance of stem cells derived from those precursors.

**Results**

SOX10 is retained postnatally by McSCs and differentiated melanocytes

The expression of SOX10 within the postnatal McSCs and differentiated melanocytes of the hair bulb was compared to the expression of the melanocyte marker, dopachrome tautomerase (DCT). In this study, we define the McSC population by several characteristics: cells that exist within the hair bulge, are capable of self-renewal, and can give rise to melanocyte progenitors that colonize the newly developing hair bulb. Previously, we have shown that the transgenic line, Tyr::CreERT2, can target cells with these properties when induced either during postnatal development or within adults [25]. To specifically demonstrate that DCT marks this McSC population, we performed a similar lineage mapping analysis here (Fig. S1). Tyr::CreERT2; Rosa26tm1sor pups were given a pulse of tamoxifen (TAM) on postnatal days 2 and 3 (P2–3), and assessed for recombined cells by β-galactosidase staining. Melanocytes in Tyr::CreERT2 mice express CRE at P2, and within the same hair cycle as TAM treatment (P14); we observed LacZ+ cells in the hair bulge and bulb (Fig. S1A–B). This suggests by anatomical position that we have targeted both McSCs and differentiated melanocytes. We further confirmed that these LacZ+ bulge cells are McSCs by challenging them to repopulate new hairs after hair plucking. Hair plucking eliminates all differentiated melanocytes leaving only the McSCs to replenish newly generated hairs. Indeed, seven days after initiating a new hair cycle (7 days post plucking, 7dpp) we observed the retention of LacZ+ cells in the hair bulge and LacZ+ progeny in the hair bulb (Fig. S1C). This confirms that these LacZ+ bulge cells are indeed McSCs. We further analyzed these LacZ+ McSCs with immunolabeling (Fig. S1D) and confirm that nearly all (97%) express DCT. Thus for the remainder of our analysis we refer to DCT+ cells within the hair bulge as the McSC population.

Next we examined SOX10 expression in postnatal melanocytes during several key stages of hair morphogenesis and hair cycling (Fig. 1A; P2, P6, P14, adult anagen III/IV (7dpp) and adult catagen VII (21 days post plucking, 21dpp). Despite the availability of a number of useful fluorescent Sox10 reporter mice [26–28], we opted to characterize protein expression with immunolabeling as this method is applicable to non-transgenic mouse studies. Using antibodies, we observed that nearly all DCT+ cells within hairs co-express SOX10 regardless of time point, location or differentiation status (Fig. S2).

We expanded our results for the McSC population by quantifying the percentage of DCT+/SOX10+ cells within the lower permanent portion (LPP) of the hair (hair bulge) and the upper transitory portion (UTP) of the hair since these regions contained the majority of DCT+ melanocytes that exist along the hair shaft (Fig. 1B, Table S1). As defined previously, the LPP extends from the opening of the sebaceous gland to the junction between the dermis and subcutis, and the UTP sits between the same junction and the hair bulb (Fig. 1A, [29]). In late catagen hairs (21dpp), the entire follicle exists within the dermis and is divided into the hair bulge and the secondary hair germ (SHG), with the SHG visible as a small cluster of cells between the hair bulge and dermal papilla (Fig. 1A, [30]). Between P2 and P14, SOX10 is detected in 94–99% of LPP+UTP melanocytes (Fig. 1B). SOX10+/DCT+ cells comprise 87% of the LPP+UTP melanocytes at anagen (7dpp), and 96% of bulge+SHG melanocytes at catagen (21dpp, Fig. 1B). This result contradicts previous reports showing that Sox10 mRNA and protein are downregulated by the melanocytes that have colonized the hair bulge beginning at P2, and is absent in melanocytes found in catagen stage hairs [4,31]. However, we suspect that higher sensitivity of our SOX10 antibody may limit our ability to distinguish melanocytes with variable levels of SOX10 expression, thus explaining our observation that DCT and SOX10 co-label more bulge melanocytes than previously reported.

In contrast to SOX10, the expression patterns of other melanocyte markers, MITF, KIT, TRP1 and TYR, within postnatal hairs is more variable (Fig. S3, S4, S5, S6). Beginning at P2, the majority of DCT+ LPP melanocytes in stage 4 hairs double-label with MITF and KIT whereas only 56% and 38%
express the melanogenic enzymes TRP1 and TYR, respectively (Fig. 1B, Table S1). As hairs progress to stage 6 of their morphogenesis, nearly all LPP+UTP melanocytes continue to express MITF while the percentage of LPP+UTP melanocytes expressing TRP1 and TYR decreases. Previously, the intensity of KIT expression within the McSC niche was observed to be bipolar, either KIT\textsuperscript{high/+} or KIT\textsuperscript{low/-}, with the KIT\textsuperscript{low/-} melanocytes corresponding to the McSC population [3,32]. Although we rarely saw KIT\textsuperscript{-} melanocytes at any stage, we did observe an emergence of DCT\textsuperscript{+} KIT\textsuperscript{low} LPP+UTP cells in stage 6 hairs of P2 skins. By P14, LPP+UTP melanocytes have downregulated their differentiation markers and exist predominately in a SOX10\textsuperscript{+}/MITF\textsuperscript{+}/KIT\textsuperscript{low} state. Strikingly, the initiation of anagen (7dpp) corresponds with a dramatic escalation of the percentage KIT\textsuperscript{high/+} cells and a moderate increase of TRP1\textsuperscript{+} cells amongst LPP+UTP melanocytes. In contrast, entry into catagen (21dpp) is associated with LPP+UTP melanocytes downregulating MITF while still retaining TRP1, KIT and SOX10.

Across all hairs that contain bulbs (excluding catagen hairs), DCT\textsuperscript{+} melanocytes within the hair bulb also double-label with SOX10, MITF, TRP1 and TYR (Fig. S2, S3, S4, S5). KIT, on the other hand is robustly detected amongst melanocytes colonizing the bulbs of stage 4 morphogenetic hairs, but expressed with varying intensity in the matrix of older hairs (Fig. S6).

This analysis demonstrates that while the differentiation status of melanocytes that exists within the hair bulge fluctuates in concert with hair morphogenesis and adult hair cycling, SOX10 expression remains static amongst LPP and UTP melanocytes.
Sox10 is required for the retention of McSCs and differentiated melanocytes and for pigment production

In light of our discovery that SOX10 is retained by both McSCs and differentiated bulb melanocytes within the hair follicle, paired with previous in vitro experiments indicating that TYR expression in mouse is Sox10-dependent [15], we anticipate Sox10 plays an important role in postnatal melanocyte biology. We tested this by generating Sox10<sup>fl/fl</sup>; Tyr::CreERT2 animals to conditionally knockout Sox10 in mouse melanocytes postnatally [33,34]. Previously, we confirmed that Tyr::CreERT2 is effective at inducing recombination of floxed alleles in a significant number of McSCs as well as the more differentiated melanocytes when TAM is administered transiently during perinatal growth or during adult anagen [25]. Using the same approach, we administered TAM in a pulse-like fashion to both pups and adult animals. Pups were given TAM just prior to their initial hair growth by receiving breastmilk from lactating mothers injected intraperitoneally (IP) with TAM on P0–P3. Adults, at approximately eight weeks of age, were plucked on their lower backs to induce anagen, and TAM was administered by IP injection on the same day as plucking and for three additional days (0–3dpp). In both cases, we observed hypopigmentation in a subset of the hairs in the Sox10<sup>fl/fl</sup>; Tyr::CreERT2 mice in regions of the skin where newly grown hairs were emerging. This loss of pigmentation was not observed in similarly-treated Sox10<sup>0/0</sup> and Sox10<sup>0/+</sup>; Tyr::CreERT2 or Sox10<sup>0/+</sup>; Tyr::CreERT2 mice that were not treated with tamoxifen (Fig. 2A–B, D–E; Fig. 7A–B). Using PAX3 as a marker for melanocytes, we found that this lack of pigmentation is associated with an overall reduction in the number of differentiated melanocytes per hair bulb, with a significant percentage of hair bulbs lacking melanocytes altogether (Fig. 2C, F). This indicates that Sox10 is required for the retention of differentiated melanocytes in the hair.

In Sox10<sup>0/0</sup>; Tyr::CreERT2 animals, we also observed a population of PAX3<sup>+</sup>/SOX10<sup>−</sup> cells within the melanocytic region of the hair matrix whose presence correlates with hairs that contained little or no pigmentation (Fig. 2G–I). This was particularly noticeable in TAM-treated Sox10<sup>0/0</sup>; Tyr::CreERT2 adults (Fig. 2I). This indicates that Sox10 is also required by bulb melanocytes to differentiate, or produce pigment. These PAX3<sup>+</sup>/SOX10<sup>−</sup> cells also do not express MITF, a SOX10 target gene, suggesting that the reduced pigment seen in SOX10<sup>−</sup> bulb melanocytes is likely a result of aberrant melanocytic transcriptional regulation (Fig. S7C–D). The fate of melanocytes lacking Sox10 remains unclear as positive staining for the apoptosis

Sox10<sup>fl/fl</sup> (A–B) Sox10<sup>0/0</sup> (fl/fl; +/+ and Sox10<sup>0/0</sup>; Tyr::CreERT2 (fl/fl; Cre/+)) pups treated with TAM by IP injection to the lactating mother on P0–3 display variegated hypopigmentation on the belly and back and exhibit a white head spot upon the emergence of the morphogenetic coat (P10 shown here, n>5). (C) Number of PAX3<sup>+</sup> melanocytes per hair bulb in skins harvested from mice at P10 are significantly decreased in Sox10<sup>0/0</sup>; Tyr::CreERT2 animals compared to similarly-treated Sox10<sup>0/0</sup> animals (*p = 0.002). (D–E) Adult Sox10<sup>0/0</sup>; Tyr::CreERT2 mice treated with TAM by IP injection on 0–3dpp exhibit white hairs within the plucked region upon hair regrowth that is not visible in similarly treated Sox10<sup>0/0</sup> mice (brackets indicate plucked region, lower image is a magnification of plucked region). (F) Number of PAX3<sup>+</sup> melanocytes per hair bulb in skins harvested from similarly-treated mice at 7dpp are significantly decreased in Sox10<sup>0/0</sup>; Tyr::CreERT2 animals compared to Sox10<sup>0/0</sup> animals (*p = 0.001). (G–H) Fluorescent and corresponding brightfield images of hair bulbs from mice described in D–E. Arrows and arrowheads indicate PAX3<sup>−</sup>/SOX10<sup>10/10</sup> and PAX3<sup>−</sup>/SOX10<sup>0/0</sup> melanocytes, respectively. (I) Distribution of melanocytes double-labeled for PAX3 and SOX10 within pigmented (gray) and non-pigmented (white) hair bulbs in skins from Sox10<sup>0/0</sup> (n = 3) and Sox10<sup>0/0</sup>; Tyr::CreERT2 (n = 4) harvested on 7dpp from mice treated with TAM on 0–3dpp (*p<0.0006).

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References

1. Role of Sox10 in Postnatal Melanocytes

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July 2013 | Volume 9 | Issue 7 | e1003644
markers CG3 and/or TUNEL is not correlated with PAX3+/SOX10+ bulb cells or non-pigmented hairs in tamoxifen-treated Sox10fl/fl; Tyr::CreERT2 pups or adult animals (not shown).

Nevertheless, these data demonstrate that the hypopigmentation observed with Sox10 knockout is due to an overall loss of bulb melanocytes and a deficiency in their ability to produce pigment. We have shown previously that the Tyr::CreERT2 transgene is effective at inducing recombination in McSCs [25], and thus we also analyzed the effects of Sox10 knockout on LPP (bulge) melanocytes. Using KIT as our marker for melanocytes, we discovered that LPP melanocytes are decreased in Sox10fl/fl; Tyr::CreERT2 mice that were treated with TAM by IP injection on 0–3dpp, allowed for complete hair regeneration, replucked and allowed for a second round of hair regrowth (brackets indicate plucked/replucked region, lower image is a magnification of plucked region; mouse in 2E and 3B are the same, imaged prior to and after replucking). Figure 3.

Figure 3. Sox10 is required by LPP melanocytes postnatally. (A) Number of KIT+ LPP melanocytes within hairs from Sox100/0 (fl/fl; +/+ ) and Sox100/0; Tyr::CreERT2 (fl/fl; Cre/+ ) mice. P0–3/P10 indicates skins harvested from pups on P10 that were maintained by lactating mothers that were IP injected with TAM on P0–3. 0–3dpp/7dpp indicates skins harvested from adult mice on 7dpp after IP injections of TAM on 0–3dpp. (B) White hairs remain visible in adult Sox100/0; Tyr::CreERT2 mice that were treated with TAM by IP injection on 0–3dpp, allowed for complete hair regeneration, replucked and allowed for a second round of hair regrowth (brackets indicate plucked/replucked region, lower image is a magnification of plucked region; mouse in 2E and 3B are the same, imaged prior to and after replucking). (C) Number of PAX3+ bulb melanocytes within hairs from Sox100/0 and Sox100/0; Tyr::CreERT2 mice treated as described in B but harvested on 7dpp after replucking (0–3dpp/7dpp repluck). (D) Distribution of melanocytes double-labeled for PAX3 and SOX10 within pigmented (gray) and non-pigmented (white) hair bulbs in skins from Sox100/0 (n = 3) and Sox100/0; Tyr::CreERT2 (n = 3) mice treated as described in B but harvested on 7dpp after replucking (*p<0.0002). (E) Persistent hair graying is visible in Sox100/0; Tyr::CreERT2 mice treated with IP TAM for pulse of five days beginning at five weeks old and imaged at one and two years old.

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Figure 4. Tg(DctSox10) results in congenital white spotting and premature hair graying. (A, B) Ventral and dorsal views demonstrating variable hypopigmentation in Tg(DctSox10)+/+ and Tg(DctSox10)/Tg(DctSox10) mice during hair morphogenesis and adult hair cycling. (C) Frequency of pigmented (pig+) and non-pigmented (pig−) anagen III/IV (7dpp) hairs that contain (DCT+ LPP cells) or do not contain (no LPP cells) LPP melanocytes within Tg(DctSox10) or +/+ mice. The ages of mice analyzed ranged between 9–22 weeks at harvest. Significance determined by chi-square analysis (p<0.0001) and evaluation of standardized residuals (*, z = −8.84; **, z = 12.24).

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Role of Sox10 in Postnatal Melanocytes

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CreERT2 adults were likely a consequence of recombining the premature hair graying observed in Sox10\textsuperscript{fl/fl}; Tyr:: (Fig. 3B–C). However, the PAX3 treated in this manner still exhibit white hairs and reduced bulb melanocytes (Fig. 3D). This suggests that the PAX3\textsuperscript{+} replucked animals almost completely lack bulb melanocytes observed after the initial TAM treatment of Sox10\textsuperscript{fl/fl}; Tyr:: pigmentation and melanocytes. The region, and assessed this subsequent round of hair growth for hairs to regrow (similar to Fig. 2E), then replucked in the same on their lower back, administered TAM on 0–3dpp, allowed these replucked animals almost completely lack bulb melanocytes (Fig. 3D). Together these observations reveal a postnatal requirement for Sox10 in mouse melanocytes. This extends to both the McSC and differentiated melanocyte populations and demonstrates that Sox10 is necessary during the establishment of melanocytes within the hair follicle during hair morphogenesis as well as during the regeneration of melanocytes during adult hair cycling.

Overexpression of Sox10 induces McSC loss and premature hair graying

Expression of SOX10 by McSCs of the hair, a subpopulation that by their nature is inhibited from differentiation, suggests that McSCs uniquely regulate Sox10 in order to maintain their stem cell properties. To determine whether changing the threshold of Sox10 levels in the melanocyte lineage affects the ability of melanocytes to become established in the hair or maintained as McSCs, we examined mice that overexpress Sox10 in melanocytes under the control of the Dct promoter (Tg(DctSox10), line CF1-10; [35]). This transgene expresses a 2.4-fold increase in Sox10 expression in skins obtained from Tg(DctSox10)/+ animals compared to wild type (Fig. S8A).

The increase in Sox10 expression manifests in two ways: congenital hypopigmentation (white spotting) and hair graying (Fig. 4A, B). At P8, Tg(DctSox10)/+ mice exhibit hypopigmentation that is evident as small, ventral belly spots that are highly penetrant (97% in adults, n = 29/30 with belly spots; Fig. S8B). Tg(DctSox10)/+ mice at P8 have more extensive hypopigmentation with large white ventral spots that encompass the majority of the belly, dorsal spotting and occipital head spots. The white spotting observed with Tg(DctSox10) suggests that overexpression of Sox10 affects the embryonic melanoblast population. Tg(DctSox10)/+ mice also display variable loss of hair pigmentation (premature hair graying) after the onset of the first adult hair cycle (first adult anagen is ~28, Fig. 4B).

Hair graying in Tg(DctSox10)/Tg(DctSox10) mice continues to increase progressively as these animals age (not shown). Tg(DctSox10)/+ mice also exhibit hair graying but with a reduction in severity and with a later onset, beginning after the second adult hair cycle (second adult anagen is ~12 weeks, 4/11 animals exhibit sparse gray hairs at ~16 weeks). Hair graying in the Tg(DctSox10) line was first examined histologically in mice after the first adult hair cycle (between 9–22 weeks in age) and after hair cycle synchronization by plucking (Fig. 4C). Analysis at anagen (7dpp) demonstrated that in wild type and Tg(DctSox10)/+ mice, the majority of hairs were both pigmented and contained LPP melanocytes (92.4 ± 6.8% and 90.3 ± 6.9%, respectively). In contrast, Tg(DctSox10)/Tg(DctSox10) mice exhibited primarily non-pigmented hairs that lacked LPP melanocytes (75.6 ± 4.3%). From these observations we conclude that Tg(DctSox10)-induced hair graying is a direct consequence of McSC deficiency.
Overexpression of Sox10 disrupts McSC establishment

The fact that Tg(DctSox10)/Tg(DctSox10) animals exhibit premature hair graying at the first adult hair cycle suggests that the loss of McSCs observed in these animals occurs during hair morphogenesis when melanocytes colonize the hair. Melanocytes within the morphogenetic hair bulge and bulb are thought to become molecularly and anatomically distinct around P4 [4]. If Sox10 overexpression affects McSC establishment then we would expect that the LPP melanocytes in Tg(DctSox10)/+ LPP regions, this pigmentation often appeared in cells that were highly dendritic. (C, D) Brightfield and corresponding fluorescent images of anagen III/IV hair follicles double labeled for DCT and TRP1 (C) or KIT (D) in wild type and Tg(DctSox10)+/ mice. The intensity of KIT fluorescence expression was variable, and categorized as KITlow (arrows) or KIThigh (arrowheads), and did not appear to correlate with the presence or absence of pigmentation. (E,F) Comparison of the number LPP melanocytes per anagen III/IV hair follicle in+/+ and Tg(DctSox10)+/ mice that express DCT, and TRP1 or KIT, and produce ectopic pigmentation (*p<0.008).

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Overexpression of Sox10 results in premature differentiation of LPP melanocytes in anagen hairs. (A) Number of DCT+ LPP melanocytes per anagen III/IV hair follicle (independent of the presence or absence of hair pigmentation) is significantly reduced in Tg(DctSox10)/Tg(DctSox10) mice when compared to wild type and Tg(DctSox10)+/ mice (*p<0.0003). The ages of mice analyzed ranged between 9–22 weeks at harvest. (B) Eosin-stained skin sections of these hairs demonstrate the presence of ectopic pigmentation in the LPP of Tg(DctSox10)+/ and Tg(DctSox10)+/Tg(DctSox10) hairs (arrows) that is not seen in wild type hairs. In Tg(DctSox10)+/ LPP regions, this pigmentation often appeared in cells that were highly dendritic. (C, D) Brightfield and corresponding fluorescent images of anagen III/IV hair follicles double labeled for DCT and TRP1 (C) or KIT (D) in wild type and Tg(DctSox10)+/ animals. The intensity of KIT fluorescence expression was variable, and categorized as KITlow (arrows) or KIThigh (arrowheads), and did not appear to correlate with the presence or absence of pigmentation. (E,F) Comparison of the number LPP melanocytes per anagen III/IV hair follicle in+/+ and Tg(DctSox10)+/ mice that express DCT, and TRP1 or KIT, and produce ectopic pigmentation (*p<0.008).
consequent hair graying observed in Tg(DctSox10)/Tg(DctSox10) mice. Together these experiments demonstrate that although increased Sox10 expression does not affect the ability of melanocytes to produce normally pigmented morphogenetic hairs, it does result in changes in the expression status of the LPP melanocytes that may affect their establishment as McSCs.

Overexpression of Sox10 disrupts McSC maintenance

The absence of McSCs in the adult hairs of Tg(DctSox10) homozygotes precludes our ability to phenotypically assess them at this age, however, the fact that Tg(DctSox10)/+ mice exhibit changes in LPP expression profiles at P2 suggests that a closer look at Tg(DctSox10)/+ skins is warranted. First as expected, immunolabeling validates the presence of SOX10 in both LPP and bulb...
melanocytes of anagen (7dpp) hairs in Tg(DctSox10)/+ and wild type adult mice (Fig. S0C). Second, in contrast to the dramatic loss of LPP melanocytes observed in adult Tg(DctSox10) homozygotes, no change in the total number of melanocytes per LPP was detected within anagen (7dpp) hairs of Tg(DctSox10)/+/+ animals in comparison to wild type (Fig. 6A) when assayed in mice ranging from 9–22 weeks of age.

Closely inspection of Tg(DctSox10)/+/+ hairs revealed the presence of pigmented, often dendritic, cells within the McSC compartment. Ectopic LP pigmentaation was also detected in Tg(DctSox10)/+/+ hairs that remained pigmented into the adult hair cycle, but was rarely present in wild type hairs (Fig. 6B). LPP melanocytes of Tg(DctSox10)/+/+ adult mice also exhibit changes in the expression pattern of TRP1 and KIT at anagen (Fig. 6C–F). In wild type animals, LPP melanocytes are mostly unpigmented and fall evenly into two categories, either anagen (Fig. 6C–F). In the second to third adult anagen (induced by plucking at 6–10 weeks of age), we observed significantly more non-pigmented hair bulbs in Tg(DctSox10)/+; Mitf<sup>−/−</sup> mice in comparison to either single heterozygote or +/+ animals (Fig. 7E). Hairs from Tg(DctSox10)/+; Mitf<sup>−/−</sup>/+ mice also display a noticeable expansion in the amount of ectopic pigmentation within the LPP in comparison to Tg/+ animals (Fig. 7E). By immunofluorescent analysis we discovered that at this time point (6–10 weeks of age), nearly all melanocytes present in the LPPs of Tg(DctSox10)/+; Mitf<sup>−/−</sup>/+ mice express TRP1 and are pigmented. This phenomenon is less pronounced in Tg(DctSox10)/+/+ mice and is not observed in Mitf<sup>−/−</sup>/+ or +/+ mice (Fig. 7G, 6E). The fact that loss of Mitf exacerbates rather than alleviates the premature differentiation of LPP melanocytes and hair graying seen in this Tg(DctSox10) line, suggests that MITF participates in the negative regulation of Sox10 or Sox10-dependent processes within the McSC.

### Discussion

In this study we show that Sox10 is critical postnatally for the establishment and maintenance of cells in the melanocyte lineage. The role of Sox10 is twofold—first, it is necessary for the retention of mature bulb melanocytes and undifferentiated McSCs, and second, it is required for the production of normal follicular pigmentation. The apparently contradictory requirements for Sox10 by undifferentiated McSCs and in the differentiation of melanocyte progenitors can be explained through our evidence that the McSC is maintained through the modulation of Sox10 levels itself. Accordingly, while SOX10 is expressed at all stages of the melanocyte lineage in mouse, increased Sox10 levels results in premature differentiation of the McSC population eliminating their capacity for self-renewal. This observation supports the idea that Sox10 activity within the McSC is normally decreased, and we provide evidence suggesting that this may occur through a MITF-mediated negative feedback loop. From these observations we propose a model for McSC establishment during early postnatal development whereby melanocytes migrating into the morphogenetic hair assume either a stem or differentiated cell fate depending on the environment they colonize. In this model, the hair follicle bulge would remain high, and these melanocytes would undergo hypertrophy because of the survival of non-skeletogenic neural crest sublineages, particularly, is credited for defining successive stages of neural crest development during embryogenesis; beginning with the maintenance of multipotency within the neural crest stem cell lineages, including one involving MITF-mediated negative feedback. Subsequent downregulation of Sox10 would establish the McSC. Melanocytes that colonize the hair bulb would not be subject to these repressive signals, Sox10 activity would remain high, and these melanocytes would undergo differentiation. This mechanism is also applicable to the maintenance of the McSC and production of pigmented melanocytes during adult hair cycling.

The idea that Sox10 can contribute to the preservation of the undifferentiated McSC population while also driving melanogenesis is in agreement with current views on the ability of SOX proteins to confer different states of cellular maturity. Sox10, in particular, is credited for defining successive stages of neural crest development during embryogenesis; beginning with the maintenance of multipotency within the neural crest stem cell [37,38], and later for its participation in cell fate specification and survival of non-skeletogenic neural crest sublineages, particularly the melanocytes and glial cells (reviewed in [39]).

The mechanism by which Sox10 levels are so precisely regulated within the postnatal melanocyte remains unclear. Previously, Sox10 expression was reported to decrease as melanocytes colonized the hair bulb leading to the speculation that establishment of the McSC is dependent on downregulation of Sox10 [4]. Despite the fact that our SOX10 immunolabeling does not exhibit the same temporal pattern, our loss and gain of
function results do not contradict this theory. Basal levels of Sox10 may provide survival of the postnatal melanocyte lineage, McSCs included, while a higher threshold of Sox10 expression is required to drive melanocyte progenitor differentiation and pigment production. This idea is similar to the Mitf rheostat model proposed by Carreira et al. [40] to explain how varying levels of Mitf expression can produce a range of melanoma phenotypes from stem cell-like to proliferative to terminally differentiated. While the precise mechanisms regarding Sox10 regulation are not fully known, conserved regulatory regions have been identified for Sox10 and encompass binding sites for transcriptional activators including SOX9B, NOTCH, β-catenin, LEF1, MED1/PBP, ATF2, and TEAP2. [41–44]. WNT/β-catenin signaling in particular, is a candidate for controlling the switch in Sox10 expression—β-catenin remains in the cytoplasm of McSCs during telogen, but shuttles to the nucleus during anagen where it is sufficient to drive the melanocyte differentiation program [9]. Interestingly, constitutive activation of WNT signaling also results in ectopic pigmentation of McSCs and premature hair graying after several hair cycles.

The above observations do not discount the possibility that stem and progenitor fates in the melanocyte lineage may also be explained by a combinatorial mechanism where the availability of Sox10, regulatory regions of its targets, or partner transcription factors influence the cell state. For instance, Sox10 functions synergistically with a number of cofactors, namely PAX3, MITF, and CREB, during the activation of downstream genes [17,45–48]. In particular, Sox10 and MITF cooperate to promote the transcription of DCT [17,47], an interaction that is repressed by PAX3 and the corepressor GRG4. Displacement of PAX3 by activated β-catenin releases repression allowing Sox10/MITF-mediated upregulation of Dct expression [12]. Similar negative regulation of Sox10 function is observed in melanoblasts; Sox5 can both compete with Sox10 for binding while also recruit HDAC1 and CtBP2 corepressors to melanocyte gene promoters [20]. In other neural crest-derived cell types, the repression of Sox10 can also be achieved by direct sequestration. For example, in oligodendrocyte progenitors, an effector of Notch signaling, HES5, can bind SOX10 affecting its bioavailability [49]. This observation is intriguing in that Hes1 and Hes5 are expressed by melanoblasts, and that Notch signaling, is critical in their survival [50]. In particular, loss of Notch signaling in adult mice results in premature hair graying characterized by ectopically pigmented McSCs [29,51,52]. This suggests a possible link between the Notch pathway, Sox10 and McSC maintenance.

A number of observations support the idea that MITF may repress McSC differentiation. First, the possibility of a negative feedback mechanism for the regulation of Sox10 by MITF was shown using mathematical modeling to explain the dynamics of melanocyte differentiation within zebrafish [53]. Second, hypomorphic, Mif<sup>+/−</sup> mice exhibit similar ectopic pigmentation and hair graying defects as we observed with Tg(DctSox10)/+; Mif<sup>+/−</sup> mice [8]. Lastly, the fact that Mif<sup>−/−</sup> reduces congenital white spotting but exacerbates hair graying in with Tg(DctSox10) suggests a role for MITF within the McSC that is unique from its role within the melanoblast. In regards to the latter, we believe the white spotting phenotypes observed in Tg(DctSox10) may be explained by increased Mitf expression. MITF directly binds and upregulates genes required for melanin synthesis and melanosomal biogenesis, including Tyr, Tip1, Dct, MLV, MLANA, and GPRI [12,54–57]. MITF is also widely implicated in cell cycle regulation. In particular, MITF can positively control transcription of the cell cycle inhibitor genes, CDKN1A (p21) and CDKN2A (p16) [58,59]. Fittingly, loss of MITF results in increased proliferation of melanoblasts in vivo [60]. Studies of the Chx10 mutant mouse reveal that inappropriate maintenance of Mitf within the retinal progenitor cells leads to their reduced proliferation, transdifferentiation into pigmented cells, and consequent microphthalmia [61,62]. Anecdotally, we observe that Tg(DctSox10)/Tg(DctSox10) mice have small eyes that are rescued by haploinsufficiency for Mitf (Hakami, RM, Arnhiteer, H, and Ravan WJ; unpublished observation). Together these observations indirectly support the idea that the hypopigmentation observed in this Tg(DctSox10) line may be attributed to increased levels of MITF within melanoblasts inhibiting their proliferation and/or causing their inappropriate temporal differentiation.

The presence of ectopically pigmented cells within the hair bulges of Tg(DctSox10) mice fits with the assertion that overexpression of Sox10 drives the premature differentiation of McSCs. The increase in the percentage of LPP melanocytes that are TRP1<sup>+</sup>/pigment<sup>+</sup> in hairs of adult Tg(DctSox10) heterozygotes compared to wild type animals confirms this. However, we also observed an unexpected change in KIT receptor expression in LPP melanocytes with Sox10 overexpression. At adult anagen, the majority of bulge melanocytes in wild type mice exhibit high KIT immunofluorescence intensity (KIT<sup>hi</sup>) and those in Tg(DctSox10) mice appear KIT<sup>low</sup>. Previous reports show that McSC progenitors rely on KIT signaling for their appropriate proliferation and pigmentation during hair growth, and bulge melanocytes that retain a KIT<sup>low</sup>− status represent the McSC population. [2,6,32]. Together with our data, showing that overexpression of Sox10 produces numerous pigmented, KIT<sup>−/−</sup> bulge melanocytes, suggests that regulation of melanocyte lineage differentiation can also occur independent of high KIT expression. This idea is supported by the observation that KIT mutants, when treated with ionizing radiation, produce ectopic pigmentation within the hair bulge and exhibit hair graying [63]. No evidence to date has identified a role for Sox10 in the transcriptional control of KIT, and this is exemplified in recent microarray studies showing that Sox10 knockdown in melanoma cells results in no significant change in KIT expression (data analysis by GEO2R for datasets GSE37059, GSE25501; [64,65]). Further investigation into KIT regulation and how KIT signaling contributes to McSC maintenance during aging is warranted.

The translational importance of Sox10 in melanocytic disease is highlighted in recent studies linking Sox10 with cell cycle regulation and reduction of Sox10 expression correlating with reduced tumor cell burden in a mouse melanoma model [64]. Our study on the role of Sox10 in the postnatal follicular melanocytes suggests a mechanism where Sox10 supports the maintenance of the melanocyte lineage while being inhibited from driving McSC differentiation. Our illustration of how tissue-specific stem cells might arise from lineage-specified precursors, and how this can occur through the regulation of the transcription factors critical in specifying this lineage may lead to further insights into how these processes can be disrupted or manipulated within disease.

Materials and Methods

Ethics statement

Animal care and experimental animal procedures were performed in accordance with the NIH IACUC.

Animals

Tg(CreER<sup>T2</sup> and Sox10<sup>LacZ</sup>) mice were rederived on and maintained by outcross to C57BL/6J [34,66]. Rosa26<sup>CreERT2</sup> mice were obtained as homozygotes, maintained by intercross and bred together with Tg(CreER<sup>T2</sup>) mice to generate compound
heterozygotes [67]. Sox10^+/− and Mtif^−/− mice were rederived on C57BL/6 and maintained by intercross [33,68]. The Tg(DctSox10) line (CF1-10, [35]) was maintained through a combination of outcrossing to C57BL/6 and by intercross.

Genotyping

Mice were genotyped using DNA isolated from tail tips and PCR analysis. Primers for the TIR:CreER(T2) allele, 5′-TCCGCGCGGA-TAACCAGTGA-3′ and 5′-CGGAAAATGGGTTCGCCAGA, were used to amplify the Cre recombinase sequence under standard PCR conditions (30 cycles of 45 s at 94°C, 45 s at 65°C and 60 s at 72°C). Mtif^−/− and Sox10^+/− alleles were detected using PCR primers for β-galactosidase, 5′-GATCCGGCTGCGC-TACCGGC-3′ and 5′-GGATACCTGAGCAGACGCTGCC-3′, using the same PCR conditions described above. Primers and cycling conditions for the Sox10^+/− allele was described previously [33]. Zygosity for the chromosome 1 that distinguish the original FVB donor strain from Tg(DctSox10)[33]. Zygosity for the Tg(DctSox10) transgene was determined by TaqMan analysis for two SNPs flanking the transgene on chromosome 1 that distinguish the original FVB donor strain from the C57BL/6 background strain (rs13475089 and rs13475987).

Induction of CRE activity

TAM (TS648, Sigma) was dissolved in corn oil or a combination of ethanol and sunflower oil. TAM treatment was performed by IP injection of lactating mothers or adults with 2 mg/animal for the number of days indicated.

Hair cycle staging and synchronization

Morphogenetic and adult hairs were staged according to [69,70]. Plucking was performed to synchronize adult hairs. Briefly, mice were anesthetized and hairs were removed by hand over a 1.5 cm x 2 cm region on the lower back. Hairs within this region were allowed to regenerate for 7 (7 days post plucking, 7dpp) or 21 days (21 days post plucking, 21dpp). At each stage, the regions of the hair follicle were strictly defined based on visible anatomical landmarks (as described in [29]).

Immunohistochemistry

After shaving, skin from the lower back was immersed in 2% formaldehyde, and irradiated in a 540W variable wattage microwave (BioWave, Pelco) three times in intervals of 30 s irradiation followed by 60 s on ice. After microwaving, samples remained in fixative for an additional 25 minutes on ice. Skins were cryoprotected in 10% sucrose overnight, embedded in NEG-50 (Thermo Scientific), frozen and sectioned with a cryostat microwave (BioWave, Pelco) three times in intervals of 30 s. Sections were then permeabilized by treating with 1% Triton X-100 for 15 minutes. Sections were blocked for two hours in 1% bovine serum albumin (Sigma) and incubated with primary antibody overnight at 4°C. Primary antibodies include those against DCT (1:300; TRP2, Santa Cruz Bio, sc-10451), SOX10 (1:75; Santa Cruz Bio, sc-17342), PAX3 (1:75, Developmental Studies Hybridoma Bank), MITF (1:1000; rabbit polyclonal, gift from Heinz Arnheiter, NINDS-NIHIH), c-KIT (1:100; AACK, Cedarlane, CL8936AP), TRP1 and TYP (1:300; PEP-1 and PEP-7, rabbit polyclonal, gift from Vince Hearing, NCI-NIHIH), Cre recombinase (1:1000; Novagen, #69050-3), β-galactosidase (1:32,000; MP Bio, #08559761), and cleaved Caspase-3 (1:100; Cell Signaling, #9661). After washing, sections were incubated in the appropriate secondary antibodies (1:5000; Alexafluor488 or 568, Invitrogen) for two hours at room temperature.

Sequential immunolabeling was performed for co-detection of DCT and SOX10 as these antibodies were both generated in goat. After labeling for SOX10 using the protocol described above, sections were blocked with rabbit secondary goat IgG FAB (1:10; Jackson Immuno, #05-007-003) for two hours, washed and then labeled for DCT as described above.

Brightfield and fluorescence microscopy was performed on a Zeiss Observer.D1 compound microscope. Images were obtained with an Axiscam Hrc camera using the Axiosvision 4.8.2 software and processed with Adobe Photoshop. Quantitation of hair and cell phenotypes of immunolabeled tissue was performed on every fourth section of sequentially obtained skin sections. Data is presented as the mean ± standard deviation. Student’s T-test with Bonferroni correction was used to determine statistical significance, unless stated otherwise.

β-galactosidase staining

Skin samples were fixed in 2% formaldehyde/0.2% glutaraldehyde for 30 min at room temperature. Samples were then washed with rinse buffer (2 mM MgCl2/0.1% NP40/PBS) and stained overnight in X-Gal solution consisting of 0.32 mg/ml X-Gal, 5 mM ferrothiocyanide, and 5 mM ferrithiocyanide in rinse buffer.

Quantitative PCR

RNA from E17.5 skins from wild type and Tg(DctSox10)/+ mice was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ABI). Quantitative PCR was performed using Taqman Fast Universal PCR Master Mix (ABI) and the following Taqman gene expression assays: Sox10 (Mm01300162_m1) and Pax3 (Mm00435493_m1). All experiments were performed with technical and biological replicates of ≥3.

Supporting Information

Figure S1 Perinatal melanocytes give rise to the McSC population. (A) Anti-Cre recombinase (green) is present in the majority of DCT+ melanocytes (red) present in the skin at P2. (B, C) Skins from Tyr::CreER(T2); Rosa26αLSL-MerCreMer reporter mice treated at P2 and P3 with tamoxifen and harvested and analyzed for Bgal (blue) activity at P14 and 7 days post plucking (7dpp). Bgal+ cells are visible in the LPP (arrows) and bulge of the hair at both timepoints confirming that induction of Tyr::CreER(T2) perinatally successfully targets McSCs and their more differentiated progeny. (D) Double immunolabeling of these 7dpp skins reveals that 97% of perinatally lineage-marked Bgal+ cells (green) that exist with the LPP are DCT+ melanocytes (red, arrows; 115 LPP cells analyzed across 3 animals).

(TIF)

Figure S2 SOX10 expression in follicular melanocytes. Immunofluorescence staining of skins harvested at P2, P6, P14, 7dpp and 21dpp reveals that the majority of DCT+ melanocytes (melanosomal, red) located in the LPP and bulge of the hair follicle also express SOX10 (nuclear, green). Arrows indicate examples of double-labeled cells.

(TIF)

Figure S3 MITF expression in follicular melanocytes. Immunofluorescence staining of skins harvested at P2, P6, P14, 7dpp, and 21dpp for DCT (melanosomal, red) and MITF (nuclear,
green). Double-labeled cells are apparent in the LPP and bulb of the hair from P2 through 7dpp, but are not visible in melanocytes at 21dpp. Arrows indicate examples of double-labeled cells. (TIF)

**Figure S4** TRP1 expression in follicular melanocytes. Immunofluorescence staining of skins harvested at P2, P6, P14, 7dpp, and 21dpp for DCT (melanosomal, red) and TRP1 (melanosomal, green). TRP1 expression is visible in hair bulb melanocytes throughout hair cycling, but is variable in LPP melanocytes. At P6 very few LPP melanocytes express TRP1, but this number increases through P14 and 7dpp and then remains relatively static during 21dpp. Arrows indicate examples of double-labeled cells. (TIF)

**Figure S5** TYR expression in follicular melanocytes. Immunofluorescence staining of skins harvested at P2, P6, P14, 7dpp, and 21dpp for DCT (melanosomal, red) and TYR (melanosomal, green). In general TYR expression is detected most strongly in the melanocytes that exist in the hair bulb, and very rarely in LPP melanocytes. Few TYR+ melanocytes are detected at catagen, shown at 21dpp. Arrows indicate examples of double-labeled cells. (TIF)

**Figure S6** KIT expression in follicular melanocytes. Immunofluorescence staining of skins harvested at P2, P6, P14, 7dpp, and 21dpp for DCT (melanosomal, red) and KIT (membrane-bound, green). In the LPP of hairs at P2, P6, P14 and 7dpp nearly all melanocytes are KIT+, but with variable fluorescence signal intensity. At 7dpp, KIT highlights the dendrite of some LPP melanocytes. In the bulbs of P6, P14 and 7dpp hairs KIT expression is strongly localized to the keratinocytes at the bulb tip (previously reported, [71]), but is also apparent in a more diffuse, speckled pattern in the hair matrix where the differentiated melanocytes exist. KIT expression is also retained in nearly all melanocytes through catagen, shown at 21dpp. Arrows indicate examples of double-labeled cells. (TIF)

**Figure S7** Sox10 loss results in a corresponding loss in MITF expression. (A–B) Control animals do not exhibit a hypopigmentation phenotype; (A) *Sox10*+/–; *Tyr::CreERT2* animal treated with topical tamoxifen at P2–3 and imaged at P14, (B) untreated *Sox10*+/–; *Tyr::CreERT2* animal imaged at P43. (C) Triple-labeling of hair bulbs from *Sox10*+/–; *Tyr::CreERT2* (β/β; *Kit+/+; Sox10+/-) mice described in Fig. 2E. Arrows and arrowheads indicate *Pax3*+/MITF*/Sox10+* and *Pax3*+/MITF*/Sox10+* melanocytes, respectively. (D) Distribution of melanocytes double-labeled for *Pax3* and MITF within pigmented (gray) and non-pigmented (white) hair bulbs in skins from *Sox10*+/–; *Tyr::CreERT2* (β/β; *Kit+/+; Sox10+/-) and *Sox10*+/–; *Tyr::CreERT2* (β/β; *Kit+/+; Sox10+/-) mice described in Fig. 2E. Arrows and arrowheads indicate *Pax3*+/MITF*/Sox10+* and *Pax3*+/MITF*/Sox10+* melanocytes, respectively. (E) Comparison of the number LPP melanocytes per anagen III/IV hair follicle in *Tyr::CreERT2* and *Kit+/+* animals. (F) Quantitation of melanocyte immunolabeling during hair morphogenesis and hair cycling. Percentage of DCT+ melanocytes doubled labeled with the indicated marker per LPP+UTP or Bulge+SHG (~25–50 hairs analyzed/animal, n = 3 animals per timepoint, data reported as mean ± S.D.). Grayed cells indicate the combination with the highest percentage of cells doublelabeled. *SOX10* expression in melanocytes at 21dpp is present, but weak, and normal staining protocols for DCT/SOX10 double labeling diminished visible SOX10 signal. Thus melanocytes at this timepoint were identified using KIT and then double labeled for SOX10. LPP, lower permanent portion of the hair; UTP, upper transitory portion of the hair; dpp, days post plucking; SHG, secondary hair germ of the hair. (PDF)

**Table S1** Quantitation of melanocyte immunolabeling during hair morphogenesis and hair cycling. Percentage of DCT+ melanocytes doubled labeled with the indicated marker per LPP+UTP or Bulge+SHG (~25–50 hairs analyzed/animal, n = 3 animals per timepoint, data reported as mean ± S.D.). Grayed cells indicate the combination with the highest percentage of cells doublelabeled. *SOX10* expression in melanocytes at 21dpp is present, but weak, and normal staining protocols for DCT/SOX10 double labeling diminished visible SOX10 signal. Thus melanocytes at this timepoint were identified using KIT and then double labeled for SOX10. LPP, lower permanent portion of the hair; UTP, upper transitory portion of the hair; dpp, days post plucking; SHG, secondary hair germ of the hair.

**Author Contributions**

Conceived and designed the experiments: MLH KB OS RMH LS WJP. Performed the experiments: MLH KB OS RMH LS WJP. Analyzed the data: MLH OS LS WJP. Contributed reagents/materials/analysis tools: RMH MW. Wrote the paper: MLH.

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