

Hair Follicle Stem Cells Provide a Functional Niche for Melanocyte Stem Cells

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SUMMARY

In most stem cell systems, the organization of the stem cell niche and the anchoring matrix required for stem cell maintenance are largely unknown. We report here that collagen XVII (COL17A1/BP180/BPAG2), a hemidesmosomal transmembrane collagen, is highly expressed in hair follicle stem cells (HFSCs) and is required for the maintenance not only of HFSCs but also of melanocyte stem cells (MSCs), which do not express *Col17a1* but directly adhere to HFSCs. Mice lacking *Col17a1* show premature hair graying and hair loss. Analysis of *Col17a1*-null mice revealed that COL17A1 is critical for the self-renewal of HFSCs through maintaining their quiescence and immaturity, potentially explaining the mechanism underlying hair loss in human COL17A1 deficiency. Moreover, forced expression of COL17A1 in basal keratinocytes, including HFSCs, in *Col17a1*-null mice rescues MSCs from premature differentiation and restores TGF- β signaling, demonstrating that HFSCs function as a critical regulatory component of the MSC niche.

INTRODUCTION

The stem cell microenvironment, or niche, is critical for stem cell maintenance (Li and Xie, 2005; Moore and Lemischka, 2006). Accumulating evidence has confirmed that cell-cell and cell-extracellular matrix adhesion within the niche is essential for the establishment and maintenance of niche architecture in different stem cell systems (Raymond et al., 2009). Adhesion to the underlying extracellular matrix has been suggested as an important factor in epidermal stem cell maintenance (Green,

1977; Watt, 2002), but a specific stem-cell anchoring matrix for stem cell maintenance has not yet been identified. Hair follicle stem cells (HFSCs) are found in the hair follicle bulge, a distinct area of the outer root sheath that overlies the basement membrane at the lower permanent portion in mammalian hair follicles (Blanpain and Fuchs, 2006; Cotsarelis, 2006). The HFSC population is composed of multipotent keratinocyte stem cells and is responsible for the cyclic regeneration of hair follicles as well as a transient supply of progeny to the interfollicular epidermis (IFE) and to sebaceous glands after wounding (Blanpain and Fuchs, 2006; Cotsarelis, 2006; Oshima et al., 2001). The HFSC population in the bulge area normally supplies a short-term reservoir to the secondary hair germ (subbulge area), which is located just below the bulge area but above the dermal papilla and corresponds to the lowermost portion of resting hair follicles (Figure S1A available online; Greco et al., 2009). Melanocyte stem cells (MSCs), which are originally derived from the neural crest, also reside in the follicular bulge-subbulge area (Figure S1A). MSCs supply pigment-producing melanocytes to the hair matrix during each hair cycle to maintain hair pigmentation (Nishimura et al., 2002). Therefore, the bulge-subbulge area houses at least two distinct stem cell populations with different origins. However, it is still unclear to what extent these two different stem cells interact to promote each other's maintenance.

Hemidesmosomes are multiprotein adhesion complexes that promote stable epidermal-dermal attachments. The transmembrane protein collagen XVII (COL17A1/BP180/BPAG2) is a structural component of the outer hemidesmosomal plaque, which projects beneath hemidesmosomes in epidermal basal keratinocytes into the underlying basement membrane to mediate anchorage (Masunaga et al., 1997; Nishizawa et al., 1993; Powell et al., 2005). In patients with COL17A1 deficiency, a subtype of congenital junctional epidermolysis bullosa blistering disease, hemidesmosomes are poorly formed (McGrath et al., 1995; Nishie et al., 2007) and there is a characteristic premature hair loss (alopecia) with hair follicle atrophy (Darling et al.,

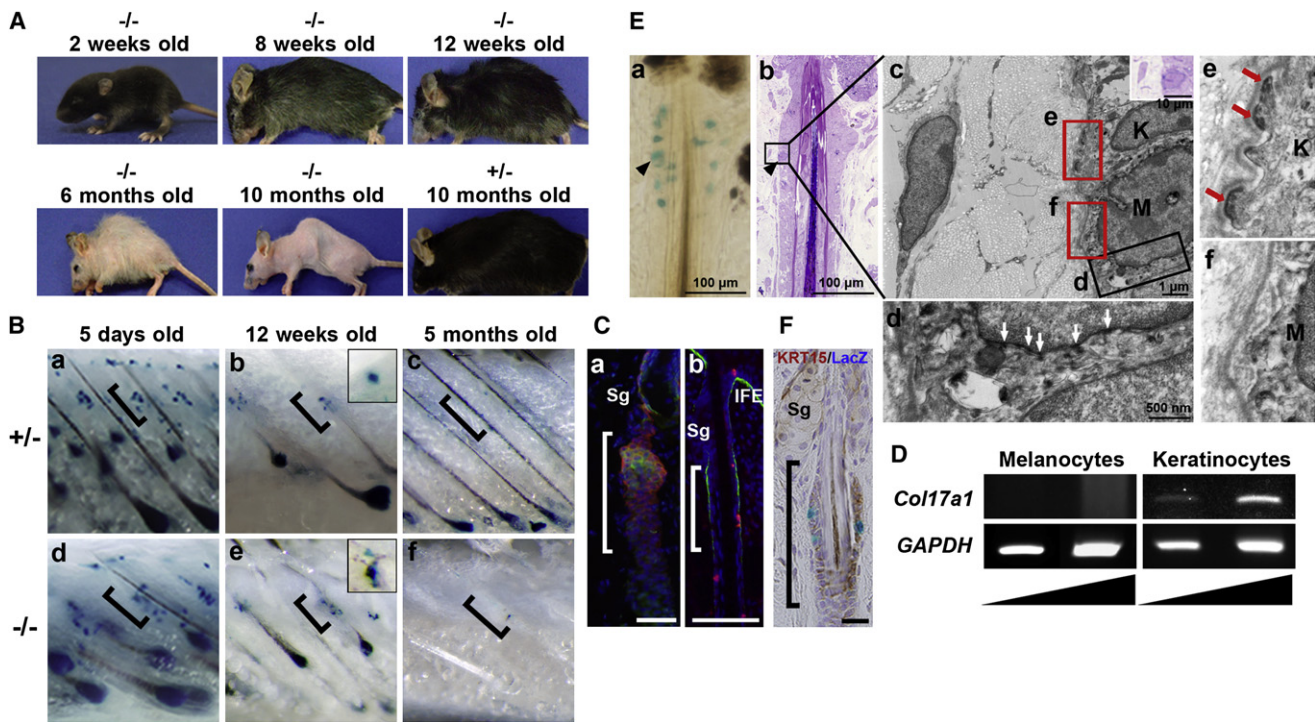


Figure 1. Hair Graying and Hair Loss Are Preceded by Depletion of MSCs in *Col17a1* Deficiency

(A) Macroscopic phenotype of *Col17a1*^{-/-} mice at different times as noted and of *Col17a1*^{+/-} littermates at 10 months of age.

(B) Deletion of *Col17a1* affects the maintenance of MSCs in the bulge-subbulge area. The distribution and morphology of *Dct-lacZ*-expressing melanoblasts is normal in the bulge-subbulge area of 5-day-old *Col17a1*^{-/-} mice (a and d). At 12 weeks of age, abnormal melanocytes with dendritic morphology were found in the bulge area of *Col17a1*^{-/-} anagen follicles (e). Inset in (e) shows magnified view of ectopically pigmented melanocytes in the hair follicle bulge-subbulge of *Col17a1*^{-/-} mice. By 5 months of age, *Dct-lacZ*-expressing cells were lost both in the bulge-subbulge area and in the hair bulb (f). Bulge-subbulge areas are demarcated by brackets.

(C) COL17A1 expression in the bulge area (demarcated by brackets). (a) COL17A1 (a: red) is expressed in KRT15 (a: green)-expressing bulge keratinocytes and in basal cells of the IFE (b: green) in wild-type skin. (b) *Dct-lacZ*-expressing melanoblasts (b: red) are located close to COL17A1⁺ basal cells (b: green) in wild-type follicles. Scale bars represent 40 μ m.

(D) RT-PCR analysis; the level of *Col17a1* mRNA is below the detection limit in flow cytometry-sorted GFP-tagged melanoblasts.

(E) Light and electron micrographs of *Dct-lacZ*-expressing melanoblasts in the bulge area (MSCs). The arrowheads in (a) and (b) point to *Dct-lacZ*-expressing melanocytes in the bulge areas in semithin sections of the skin. The ultrastructural high-power view is of the boxed areas shown in (b) and (c). X-gal reaction products accumulated in association with the nuclear membrane (d: white arrows). *Dct-lacZ*-expressing melanocytes lack hemidesmosome formation in the basement membrane zone (f), whereas adjacent keratinocytes form mature hemidesmosomes in the bulge area (e: red arrows).

(F) *Dct-lacZ*-expressing melanoblasts (blue) are in direct contact with keratin 15 (KRT15)-expressing keratinocytes in the bulge area (yellow brown). Scale bar represents 20 μ m.

M, melanocytes in bulge; K, keratinocytes in bulge; Sg, sebaceous gland; IFE, interfollicular epidermis. See also Figure S1.

1997; Hintner and Wolff, 1982) that suggests that COL17A1 plays a role in hair follicle homeostasis. We previously reported premature hair loss in *Col17a1*-deficient mice (Nishie et al., 2007), although the precise underlying mechanism is unknown. In this study, we used *Col17a1* knockout mice and COL17A1-expressing transgenic mice to show that *Col17a1* plays essential roles in the maintenance of HFSCs, which provide a functional niche for MSCs.

RESULTS

Defective MSC Maintenance and Resultant Hair Graying in *Col17a1*-Null Mice

To understand the role of collagen XVII in hair follicle homeostasis, we performed a careful chronological analysis of *Col17a1*-deficient mice. As shown in Figure 1A, *Col17a1* null mice showed

premature hair loss generally preceded by extensive hair graying. Conversely, heterozygous mice displayed a normal phenotype. The hair coats in *Col17a1*-null mice were indistinguishable from control littermates for 8 weeks after birth. However, progressive hair graying started from the snout at around 12 weeks of age and then became pronounced on their backs at around 4–6 months of age and was associated with a sparser hair distribution that was subsequently followed by progressive and more extensive hair loss (Figure 1A). It is notable that these hair changes were not accompanied or preceded by any apparent changes in the skin. Skin friction, such as attempting to artificially peel neonatal skin, can induce skin erosions in *Col17a1*-null mice (Nishie et al., 2007) but did not significantly accelerate hair graying or hair loss. Thus, it is unlikely that the hair changes are a secondary outcome of skin detachment but is more likely that the hair graying and hair loss are programmed through the

Col17a1 deficiency in the hair follicles. Our previous studies demonstrated that defective maintenance of MSCs in the hair bulge causes hair graying (Nishimura et al., 2005). Thus, we first examined the distribution and morphology of MSCs in *Col17a1*-null mice by using a melanocyte-targeted *Dct-lacZ* transgene (Mackenzie et al., 1997). As shown in Figures 1Ba and 1Bd, *Dct-lacZ*-expressing cells showed a normal morphology and distribution in the bulge area during hair follicle morphogenesis until initiation of the hair regeneration cycle both in *Col17a1*^{+/-} and in *Col17a1*^{-/-} mice. At around 12 weeks after birth, pigmented melanocytes with a dendritic morphology that expressed melanocyte markers appeared in the hair follicle bulge of *Col17a1*^{-/-} mice (Figure 1Be; Figure S1Be). At 5 months of age, *Dct-lacZ*-expressing cells were almost completely lost in the follicle bulge area as well as in the hair bulbs of *Col17a1*-null mice (Figure 1Bf; Figure S1Bf). These data demonstrate that MSC maintenance is defective in *Col17a1*-deficient mice and that this mechanism results in progressive hair graying.

Preferential Expression of COL17A1 in HFSCs but Not in MSCs

Collagen XVII is a hemidesmosomal transmembrane collagen expressed by basal keratinocytes of the IFE (McGrath et al., 1995). However, neither the expression of mouse *Col17a1* nor hemidesmosome assembly in melanocyte lineage cells and/or in bulge keratinocytes has been reported, so we first examined the expression of mouse COL17A1 protein in hair follicles by using immunohistochemistry. As shown in Figure 1Ca and Figures S1C and S1D, mouse COL17A1 was preferentially localized along the dermal-epidermal junction of bulge keratinocytes that express markers for HFSCs but not in follicular keratinocytes outside of the bulge area. However, the localization of COL17A1 in basal cell surface of MSCs could not be determined via normal immunohistochemical methods, because the attachment site of MSCs to the basement membrane is limited (Figure 1Cb). We therefore examined *Col17a1* expression by using RT-PCR in flow cytometry-sorted GFP⁺ cells from melanocyte lineage-tagged GFP transgenic mouse skin (Osawa et al., 2005). In sharp contrast to the significant expression of *Col17a1* in control keratinocytes, expression in GFP⁺ melanocytes was not detectable (Figure 1D). To support this finding, we used transmission electron microscopy (TEM) to check whether *Dct-lacZ*-expressing melanoblasts within the bulge area in wild-type animals have hemidesmosomes. As shown in Figure 1E, hemidesmosomes, which form regularly spaced electron-dense structures along the epidermal basement membrane zone (McMillan et al., 2003), were completely absent in *Dct-lacZ*-expressing melanoblasts in the bulge (Figures 1Ed and 1Ef), whereas typical hemidesmosomes were seen overlying the basal plasma membrane in surrounding bulge keratinocytes (Figure 1Ee). Because these bulge keratinocytes adjacent to *Dct-lacZ*-expressing melanoblasts express HFSC markers (Figure 1F), these data indicate that HFSCs but not MSCs are anchored to the underlying basement membrane via hemidesmosomes. We also confirmed the localization of COL17A1 to hemidesmosomes in basal keratinocytes but not in melanocytes by immunogold electron microscopic analysis of human epidermis (Figure S1E). Therefore, we conclude that MSCs do not express COL17A1 and do not assemble any discernible hemidesmosomal structures at their

surface. These findings suggested that the depletion of MSCs in *Col17a1*-null mice is caused by defects in the HFSC population that forms the main supportive cells surrounding MSCs.

Abrogated Quiescence and Immaturity of HFSCs Result in Depletion of HFSCs in *Col17a1*-Null Mice

Previous studies on wild-type mouse skin reported that mature hemidesmosomes exist at the follicular-dermal junction just below the level of sebaceous glands (Hojiro, 1972) and in hair germs of telogen hair follicles (Greco et al., 2009). Consistently, we found mature hemidesmosomes at these junctions within the hair follicle bulge (Figure 1Ee). However, mature hemidesmosomes have not been found in the transient portion of hair follicles (Hojiro, 1972), where COL17A1 expression is undetectable. These data suggested that hemidesmosome formation is important for anchoring of HFSCs located in the bulge-subbulge area of hair follicles to the basal lamina.

To test whether the abnormalities observed in *Col17a1* deficiency are specifically caused by any functional defects of HFSCs or by their detachment from the basal lamina, we first carefully examined the junctions of hair follicles in the dorsal skin of *Col17a1*-null mice and their controls by TEM. A significant number of hemidesmosomes are poorly formed in the bulge keratinocytes of *Col17a1*-deficient mice (Figure S2B), as seen in epidermal keratinocytes of those mice (Nishie et al., 2007). However, we did not find any significant microscopic separation at the follicular-dermal junction in sections of trunk skin from *Col17a1*-null mice (Figure 2; Figures S2A and S2B). Furthermore, we did not find significant inflammatory cell infiltrates or any signs of cell death, such as the appearance of eosinophilic cell bodies or TUNEL-positive or cleaved caspase 3-positive cells, at the follicular-dermal junction area of *Col17a1*-null mouse skin (Figure S2C and data not shown). Basement membrane thickening/reduplication, a sign of repeated regeneration of the epidermal and dermal junction, was also not found. These findings suggested that the hair graying and hair loss phenotypes in *Col17a1*-null mice cannot be explained simply by HFSC detachment from the basal lamina but instead may result from dysregulation or altered cell properties of HFSCs caused by *Col17a1* deficiency.

To examine whether HFSCs show any dysregulation caused by *Col17a1* deficiency, we carefully examined the hair follicle cycle progression, which alternates phases of growth (anagen), regression (catagen), and rest (telogen) in synchronization with the activation status of HFSCs, in *Col17a1*-null mice. While the first short telogen phase was transiently seen around 22 days after birth both in *Col17a1*-null mice and in control littermates, the second telogen phase was significantly shortened in *Col17a1*-null mice (Figure 2, summarized on the right side). At 6 weeks of age, just before normal hair follicles on the dorsal skin enter the second telogen phase, most hair follicles in *Col17a1*^{-/-} mice were not distinguishable from those in *Col17a1*^{+/-} mice either in morphology or in hair cycle progression. The second telogen phase is normally seen at around 7 weeks after birth and lasts about 4–5 weeks over the entire skin surface of wild-type mice (Paus and Cotsarelis, 1999; Paus et al., 1999). This phase was shortened to less than 2 weeks in all *Col17a1*^{-/-} mice examined at 8–12 weeks of age, whereas such an aberrant pattern was seen in only 14.3% of *Col17a1*^{+/-} mice. The subsequent

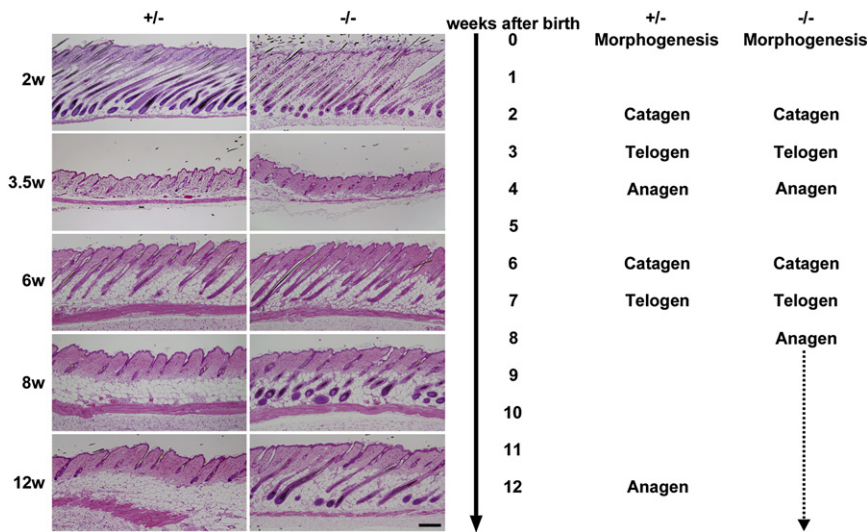


Figure 2. Loss or Shortening of the Resting State of Hair Follicles in *Col17a1*-Deficient Skin

Representative H&E images of dorsal skin sections (left) and time-scale for the hair cycle (right) from *Col17a1*^{-/-} mice and from *Col17a1*^{+/+} littermates during the first 12 weeks after birth. In *Col17a1*^{-/-} follicles, the second telogen was significantly shortened, and the second anagen lasted longer. Scale bar represents 200 μ m. See also Figure S2.

anagen phase was rather prolonged in *Col17a1*^{-/-} mice compared to their control littermates. These findings suggest that HFSCs are unable to remain quiescent for a sufficient time from the second telogen phase and thereafter in the absence of *Col17a1*.

To search for early events or changes in HFSCs in *Col17a1*-null mice, we performed immunohistochemical analysis with four different markers for HFSCs, keratin 15 (KRT15), CD34, α 6-integrin, and S100A6, at different stages (Figure 3A; Figure S3; Morris et al., 2004; Tumber et al., 2004). At 5 weeks of age, there was no difference in the expression of HFSC markers or the number of HFSC marker-positive cells between control and *Col17a1*-null mice. However, at around 8 weeks of age, HFSC marker-expressing cells were absent in the bulge area in selected null mouse hair follicles (Figures 3A and 3B; Figure S3A), and the number of these marker-deficient follicles increased over time. By 6 months of age, the HFSC population had been lost in most hair follicles of *Col17a1*-null mice (Figure S3B). Flow cytometric analysis also confirmed that the α 6-integrin^{high} CD34⁺ population (Blanpain and Fuchs, 2006), which represents basal HFSCs in the bulge area, was diminished (Figure 3C). Hair follicle atrophy with the loss of hair follicle structures were also observed once the HFSC population was diminished (Figure 3D). These data indicate that *Col17a1*-null HFSCs fail to maintain their stem cell characteristics, including their quiescence and immaturity, after the second telogen phase, resulting in hair follicle atrophy. Conversely, epidermal hyperplasia was also transiently found in some focal areas of the *Col17a1*-null skin at around 6 months of age (Figure 3D, arrowheads) but was normalized and subsequently became atrophic at later stages, which suggests that the epidermal stem cell population might also be gradually losing its self-renewing potential with age in *Col17a1* deficiency compared to controls.

To examine whether HFSC maintenance fails because the cells lose their immaturity or quiescence in the absence of *Col17a1*, we analyzed the expression of markers for keratinocyte differentiation and proliferation in *Col17a1*-null hair follicles. Interestingly, keratin 1 (KRT1), a differentiation marker for the IFE, was ectopically expressed in the bulge area of *Col17a1*-

present in the bulge areas of *Col17a1*-null mice at 8 weeks of age (Figure S4A). Furthermore, Ki67-positive cells were located in the bulge area of *Col17a1*-null mice, and those Ki67-positive cells showed an absent or reduced level of KRT15 expression (Figure 4A).

The maintenance of quiescence and immaturity of somatic stem cells in tissues is a prerequisite for sustained stem cell self-renewal, and which can be assessed for HFSCs by means of a colony-formation assay in vitro (Barrandon and Green, 1987; Oshima et al., 2001). We therefore took advantage of the type of assay by using neonatal epidermal keratinocytes, which contain the presumptive HFSC population (Nowak et al., 2008), to assess the self-renewal potential of that population in *Col17a1*-null mice. As shown in Figures 4Ba and 4Bb, *Col17a1* homozygous null keratinocytes showed defects in colony-forming ability on 3T3-J2 feeder cells compared to keratinocytes from control mice. Colonies larger than 0.5 mm in diameter were significantly decreased in number with *Col17a1*-null keratinocytes (Figure 4Bc). Although *Col17a1*-null keratinocytes showed defective binding ability to collagen I-coated dishes (Figure S4B), they showed no detectable defects in their ability to directly adhere to 3T3-J2 feeder cells (Figure 4Bd). These data strongly suggest that *Col17a1*-null keratinocytes have a much lower renewal capability than control keratinocytes. Taken together with the in vivo findings, we conclude that COL17A1 is critical for the self-renewal of HFSCs by maintaining their immaturity and quiescence.

Loss of TGF- β Expression by HFSCs and the Associated Differentiation of Adjacent MSCs

To examine whether the early changes in HFSC in *Col17a1* mutant mice affects the maintenance of MSCs in the hair follicle bulge, we carefully examined MSCs in hair follicle bulge areas in *Col17a1*-null mice beginning to show HFSC defects. At 8 weeks of age, when HFSCs in *Col17a1*-null mice are prematurely activated, KIT⁺ melanoblasts within the bulge area prematurely coexpressed TYRP1, a melanocyte differentiation marker, in *Col17a1*-null mice but not in control mice (Figure S5A). At around 12 weeks of age, pigmented melanocytes with a mature

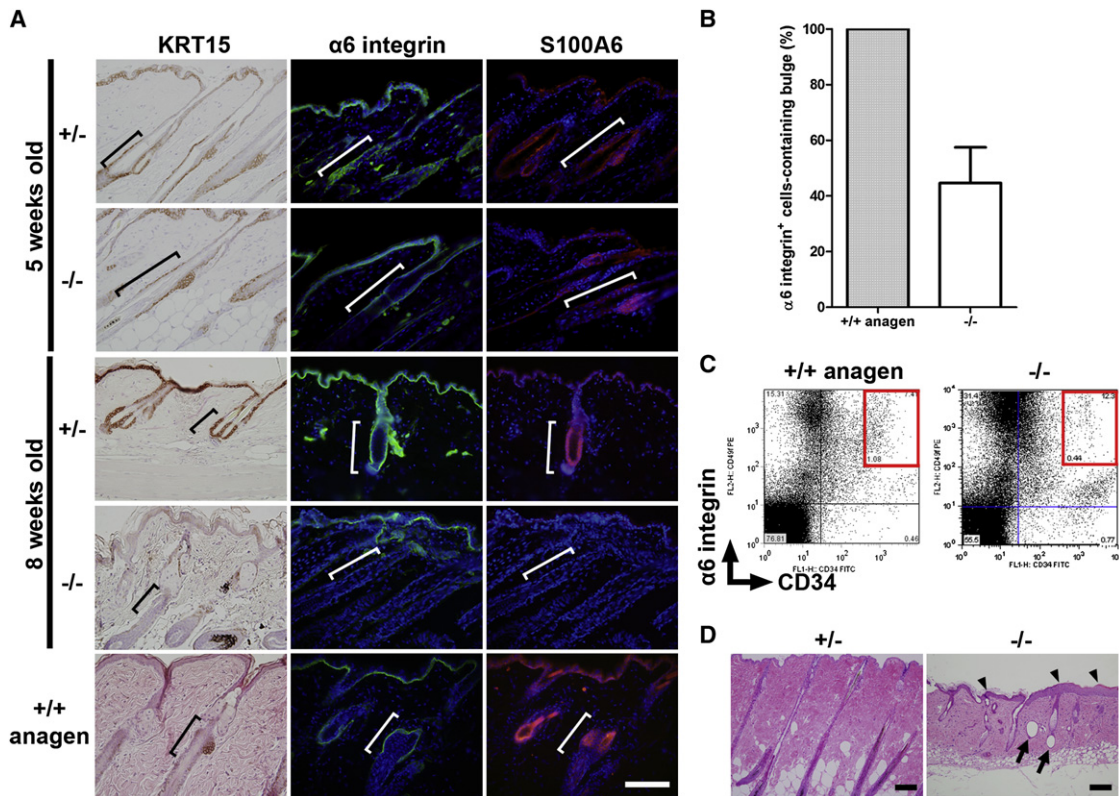


Figure 3. HFSC Depletion in COL17A1-Deficient Mice

(A) Immunostaining of the dorsal skin from *Col17a1*^{-/-} and from *Col17a1*^{+/-} mice with HFSC markers. The bulge areas are demarcated by brackets. HFSC marker (KRT15, $\alpha 6$ -integrin, and S100A6)-expressing cells were still maintained at 5 weeks of age in *Col17a1*^{-/-} mice, whereas follicles without HFSC marker-positive cells appeared at 8 weeks of age.

(B) Ratio of hair follicles with $\alpha 6$ -integrin⁺ cells in the bulge areas of skin from control mice and from 8- to 10-week-old *Col17a1*^{-/-} mice. In *Col17a1*^{-/-} mice, many hair follicles without $\alpha 6$ -integrin⁺ cells in the bulge areas were found (n = 3).

(C) Flow cytometric analysis of $\alpha 6$ -integrin and CD34 double-labeled keratinocytes. $\alpha 6$ -integrin⁺ CD34⁺ cells are almost completely lost in the skin of 9-month-old *Col17a1*^{-/-} mice.

(D) H&E-stained histological sections of *Col17a1*^{-/-} and of *Col17a1*^{+/-} mouse skin. At 6 months of age, there was a diminution of hair follicle bulbs, degeneration of the hair follicles (arrows), and epidermal hyperplasia (arrowheads) in *Col17a1*^{-/-} skin. As a control for the anagen phase in (A), (B), and (C), dorsal skin at 5 days after hair-plucking of telogen follicles was used.

Scale bars represent 100 μ m. See also Figure S3.

dendritic morphology and expressing TYRP1 in addition to *Dct-lacZ* and KIT were aberrantly found within the bulge area in mid-anagen hair follicles (*Dct-lacZ*-expressing cells in Figure 1B, Figure S1B, arrow in Figure 5A; KIT⁺/TYRP1⁺ cells in Figures 5B and 5C and Figure S5B). Conversely, only nonpigmented melanoblasts expressing *Dct-lacZ* and KIT but not TYRP1 and with small cell bodies (MSCs) were found in control littermates (Figure 1B; Figures S1B and S5B). Similar morphological changes were previously described as ectopic MSC differentiation within the niche (Inomata et al., 2009; Nishimura et al., 2005). These ectopically differentiated melanocytes were found in the bulge area at 12–13 weeks of age, prior to the hair graying seen in *Col17a1*-null mice (Figure 5D). Furthermore, it is notable that the ectopically differentiated melanocytes in *Col17a1*-deficient mice were typically found in association with early changes in bulge keratinocytes including the enlarged morphology of surrounding bulge keratinocytes (Figure 5A, arrowheads) and an increased number of Ki67-expressing bulge keratinocytes in midanagen follicles (Figures 4A and 5E and data

not shown). The appearance of ectopically differentiated melanocytes within the bulge area was followed by progressive hair graying in *Col17a1*-null mice (Figures 1A and 1B; Figure S1B).

TGF- β signaling is activated in the hair follicle bulge and is involved in but is not essential for the maintenance of HFSCs (Guasch et al., 2007; Qiao et al., 2006; Yang et al., 2005, 2009). Our recent study showed that the signal is required for the maintenance of MSCs through promoting MSC immaturity and quiescence (Nishimura et al., 2010), but it was not clear whether the signal is derived from HFSCs or MSCs. As similar changes in MSCs, such as the appearance of ectopically differentiated melanocytes in the niche and the subsequent depletion of MSCs seen in *Col17a1*-null mice, were found in *TGF β RII* conditional knockout mice (Nishimura et al., 2010), we hypothesized that the defective renewal of MSCs in *Col17a1*-null mice might be mediated by defective TGF- β signaling from the surrounding HFSCs. To test this model, we examined the involvement of TGF- β signaling in the defects of MSCs in *Col17a1*-null mice and their controls. We found that KRT15-expressing

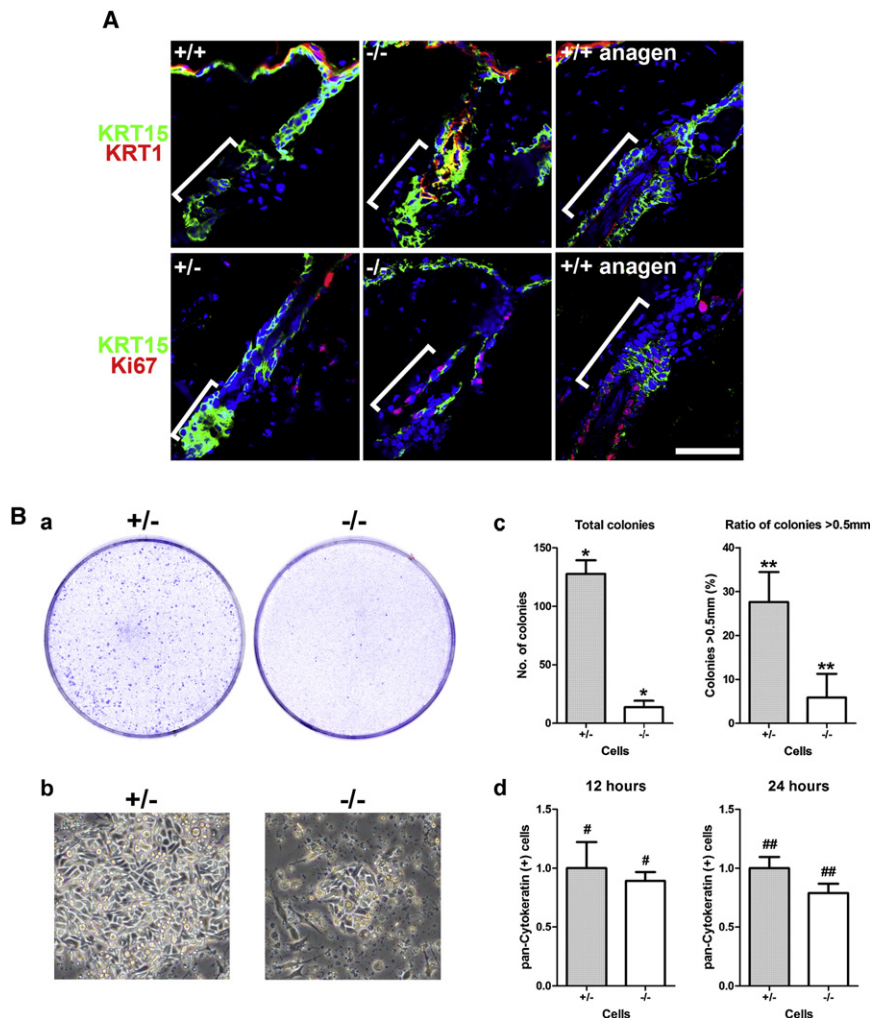


Figure 4. Deficient Stemness of HFSCs in *Col17a1*-Null Skin

(A) Immunostaining of the dorsal skin from 8-week-old *Col17a1*^{-/-} and from control mice with the IFE differentiation marker keratin 1 (KRT1) and Ki67. The bulge areas are demarcated by brackets. Top: In *Col17a1*^{-/-} mice, cells coexpressing KRT15 (green) and KRT1 (red) appeared within the bulge area. Bottom: Cells in the bulge area of *Col17a1*^{-/-} mice proliferated abnormally. As a control for anagen phase, dorsal skin at 5 days after hair-plucking of telogen follicles was used. Scale bar represents 50 μm.

(B) Loss of keratinocyte clonal growth potential resulting from *Col17a1* deficiency. (a) Clonal growth assays of keratinocytes from *Col17a1*^{-/-} and from *Col17a1*^{+/-} mice; representative dishes are shown. (b) *Col17a1*^{-/-} keratinocytes formed only small colonies. (c) Colonies from *Col17a1*^{-/-} skin were significantly fewer and smaller than those from *Col17a1*^{+/-} control mice. *, **p < 0.05. (d) Keratinocytes from *Col17a1*^{-/-} skin did not show decreased binding to 3T3-J2 feeder cells. #p = 0.6653, ##p = 0.162. See also Figure S4.

in MSCs resulting from the loss of TGF-β production from HFSCs affects MSC maintenance in *Col17a1* mutant mice and that HFSC-derived TGF-β signaling mediates the niche function of HFSCs for MSC maintenance.

Human COL17A1-Mediated Rescue of HFSCs Normalizes Maintenance of MSCs in *Col17a1*-Null Mice

Finally, to address whether the defects in *Col17a1*-null HFSCs induce the ectopic

keratinocytes coexpress TGF-β1/2 in wild-type hair follicles (Figure 5F), demonstrating that HFSCs produce TGF-β1/2 in the bulge area. At 6 weeks of age, the expression of TGF-β1/2 was similar in bulge keratinocytes in the control and in *Col17a1*-null mice (Figure 5G). At 8 weeks of age or later, however, the hair follicle bulge exhibited significantly downregulated expression of TGF-β1/2 in *Col17a1*-null mice, although the hair follicle bulge in control mice showed a normal expression pattern (Figure 5G). Furthermore, phospho-Smad2 signals were not found either in bulge keratinocytes or in melanocytes of *Col17a1*-null mice but were present in control mice (Figure 5H). These findings demonstrate that niche features, including the loss of TGF-β1/2 production, are defective in *Col17a1*-null HFSCs. We reported previously that *Tgfb2* (TGF-β receptor II) conditional knockout in mice via a bitransgenic system causes mild hair graying with incomplete penetrance (73.3% within 10 months after birth) possibly because of incomplete CRE-mediated recombination (Nishimura et al., 2010). In this study, we found that *Tgfb2* straight knockout mice (with a *Rag2*-null background for the inhibition of multiorgan autoimmunity) show a severe hair graying phenotype with 100% penetrance within 5–6 weeks of age (Figure 5I). Thus, these data suggest that defective TGF-β signaling

differentiation and eventual depletion of MSCs in the bulge area, leading to hair graying, we studied the impact of the transgenic rescue of *Col17a1*-null mice and in particular the HFSC phenotype resulting from forced expression of human COL17A1 under control of the *Keratin 14* (*Krt14*) promoter (Olasz et al., 2007). In these rescued mice, human COL17A1 expression was restricted to basal keratinocytes and not to the melanocyte lineage (Figure S6A). As shown in Figure 6A, the hair coat of these mice was quite similar to that of *Col17a1*^{+/-} mice and did not show progressive hair depigmentation or hair loss at 6 months of age, or even at 1 year of age (data not shown), whereas control *Col17a1*-null mice demonstrated the hair graying and other typical changes described above. Interestingly, both the distribution and morphology of *Dct-lacZ*-expressing melanoblasts in the bulge area were normal in the *Col17a1*^{-/-}; *Krt14-hCOL17A1* rescued mice (Figure 6B). Furthermore, the aberrant expression of Ki67 and KRT1, downregulation of TGF-β1/2 expression, and inactivation of TGF-β signaling in bulge keratinocytes were also normalized (Figures 6C and 6D; Figure S6B). These findings demonstrate the dual critical roles of COL17A1 in HFSCs for their maintenance and for providing a niche for MSC maintenance through HFSC-derived TGF-β signaling (Figure 7).

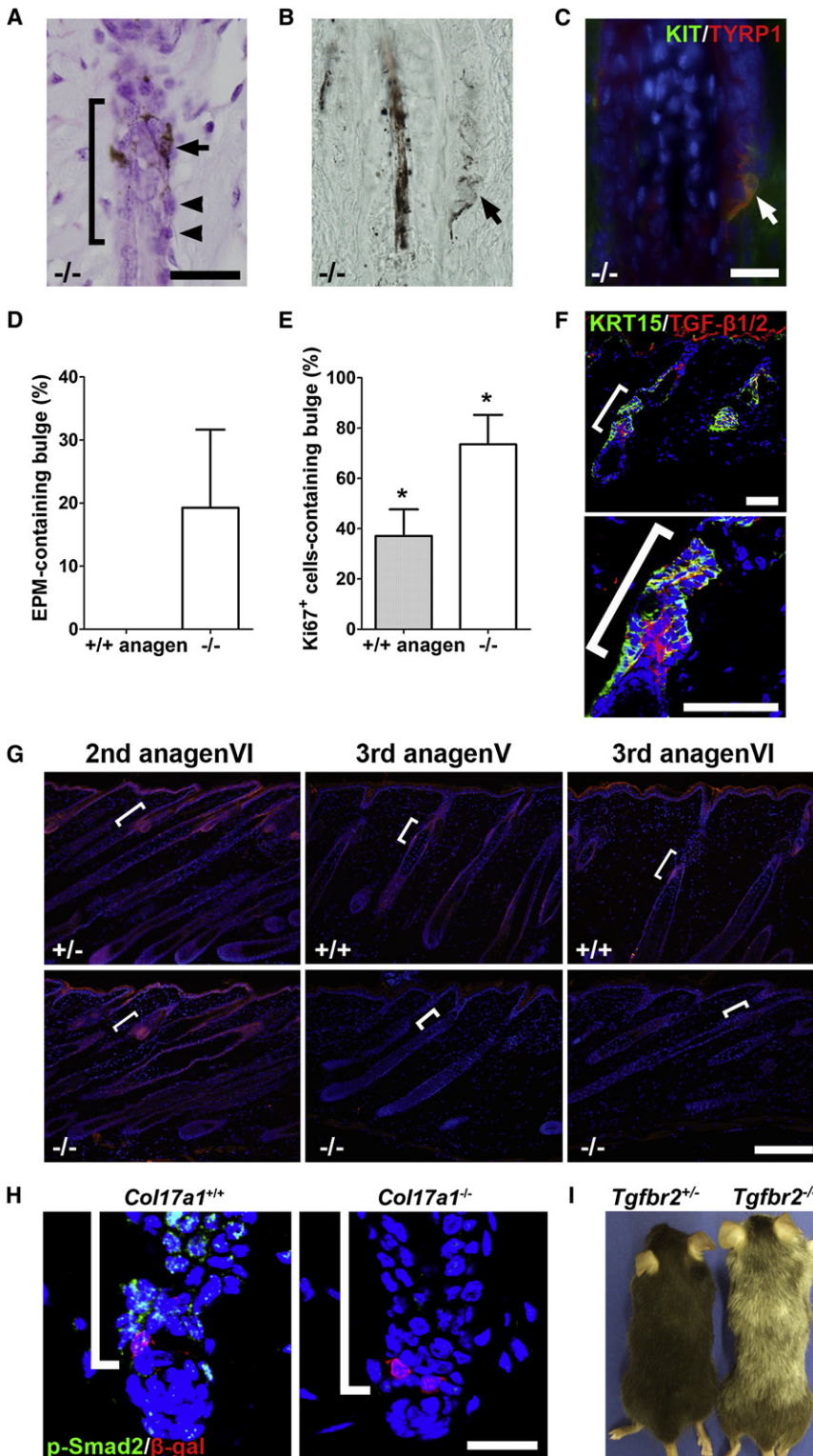


Figure 5. Ectopic Differentiation of MSCs in the Bulge Area with Diminished TGF- β Signaling Resulting from *Col17a1* Deficiency

(A–E) Ectopic differentiation of MSCs and surrounding keratinocytes in the bulge areas of *Col17a1*^{-/-} follicles at 12 weeks of age. The bulge areas are demarcated by brackets. Ectopically pigmented melanocytes (A; arrow) are in direct contact with enlarged keratinocytes with large nuclei (A; arrowheads) in an anagen VI follicle; these ectopically pigmented melanocytes (arrow) are KIT⁺TYRP1⁺ cells with a dendritic morphology (B and C). Ectopically pigmented melanocytes were detected only in the bulge-subbulge area of *Col17a1*^{-/-} follicles (D), and the proliferation of *Col17a1*^{-/-} bulge keratinocytes at 12–13 weeks of age was abnormally accelerated compared with that of control anagen V follicles (E). **p* < 0.05. Scale bars represent 30 μ m in (A) and 20 μ m in (B) and (C).

(F) Localization of TGF- β 1/2 expression (red) in *Col17a1*^{+/+} hair follicles. Plucked dorsal skins (4 days after hair plucking in telogen skin from 7-week-old *Col17a1*^{+/+} mice) were used. KRT15-expressing keratinocytes (shown in green) express TGF- β 1/2 (red). Scale bars represent 50 μ m.

(G) *Col17a1*^{-/-} mouse hair follicles from 5-week-old mice showed normal TGF- β 1/2 expression patterns (left). However, at 8 weeks of age or later in *Col17a1*^{-/-} mice, the TGF- β 1/2 expression was downregulated (right and middle). Scale bar represents 200 μ m.

(H) Phosphorylated Smad2 (shown in green) was not detected at 8 weeks in the *Col17a1*^{-/-} hair follicle bulge. Dct-lacZ-expressing melanocytes in the bulge area are shown in red. Bulge areas are demarcated by brackets. Scale bar represents 20 μ m.

(I) *Tgfb2* straight knockout mice (*Tgfb2*^{-/-}) (right) show severe hair graying phenotype at 6 weeks of age.

See also Figure S5.

maintenance of stem cell properties (Li and Xie, 2005; Moore and Lemischka, 2006). Although previous in vitro studies suggested some correlation in keratinocytes between integrin-mediated extracellular matrix adhesion and proliferation potential, in vivo ablation studies of major integrins in basal keratinocytes have not provided data on stem cell-specific depletion phenotypes (Dowling et al., 1996; Georges-Labouesse et al., 1996; Raghavan et al., 2000; van der Neut et al., 1996; Watt, 2002). In the present study, we demonstrated that COL17A1, a hemidesmosomal transmembrane collagen, is

DISCUSSION

Interactions between somatic stem cells and their surrounding niche microenvironment are critical for the establishment and

highly expressed in HFSCs within hair follicles and is required for the self-renewal of HFSCs. We found that *Col17a1* ablation in mice results in premature hair loss almost homogeneously over the entire body surface without showing any specific association

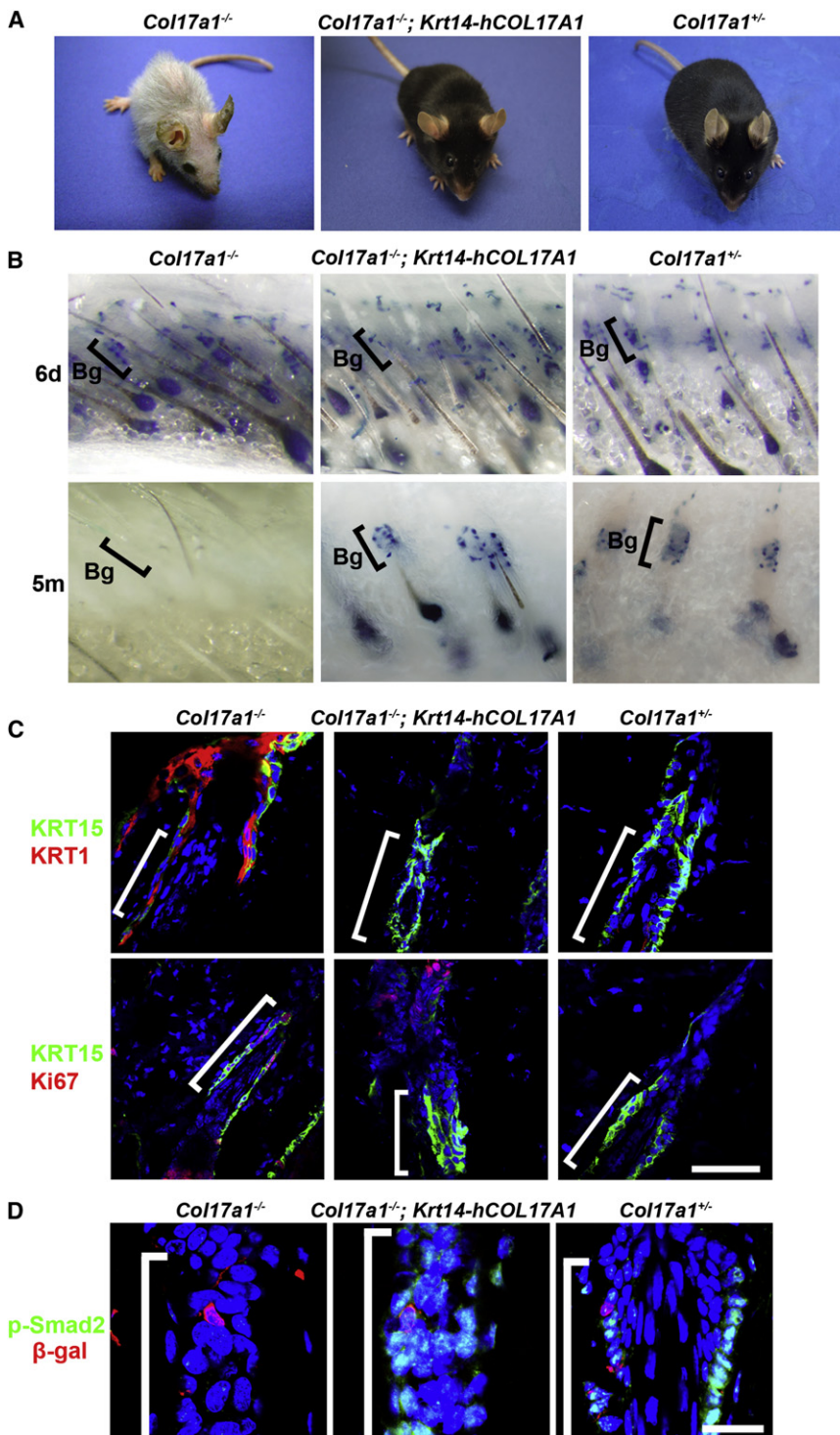


Figure 6. Transgene-Mediated Correction of COL17A1 Expression in *Col17a1^{-/-}* Basal Keratinocytes Rescues the Loss of MSCs

The *Krt14-hCOL17A1* transgene was introduced into *Col17a1^{-/-}* mice.

(A) Macroscopic phenotype of 7- to 9-month-old *Col17a1^{-/-}* mice with the *Krt14-hCOL17A1* transgene and *Col17a1^{+/-}* mice.

(B) Distribution and morphology of *Dct-lacZ*-expressing melanoblasts in the bulge area (Bg) are normalized by the *Krt14-hCOL17A1* transgene in *Col17a1^{-/-}* mice. Bulge-subbulge areas are demarcated by brackets.

(C) Ectopic KRT1 expression and abnormal proliferation of HFSCs in the bulge-subbulge area (brackets) are corrected by the *Krt14-hCOL17A1* transgene in *Col17a1^{-/-}* mice. These mice were observed at 13 weeks of age during the anagen phase. Scale bar represents 50 μ m.

(D) The downregulated expression of phospho-Smad2 (in green) in *Col17a1^{-/-}* HFSCs and MSCs within the bulge-subbulge areas (demarcated by brackets) was also normalized by forced expression of the *Krt14-hCOL17A1* transgene in *Col17a1^{-/-}* keratinocytes. *Dct-lacZ*-expressing melanocytes in the bulge area are shown in red. Scale bars represents 20 μ m.

See also Figure S6.

membrane in *Col17a1*-null mouse skin. Instead, we found that significant defects in HFSC quiescence and immaturity in *Col17a1*-null mice were the earliest events that could explain the defective maintenance of HFSCs over ensuing hair cycles. These findings underline a critical cell-autonomous role for COL17A1 in the maintenance of HFSCs under physiological conditions. Although we did not detect adhesion defects of *Col17a1*-null keratinocytes on feeder cells used for colony assay in this study, weakening of cell attachment has been found with human cultured keratinocytes treated with COL17A1 antibody under vibration conditions (Iwata et al., 2009). One adhesion-based explanation for the premature HFSC depletion in *Col17a1*-deficient mice is that COL17A1-dependent anchoring of HFSCs to the basal lamina might regulate the quiescence and differentiation of HFSCs by modifying their division frequency and properties.

with mechanical stress. Although mechanical stress, such as attempts to peel the neonatal mouse skin, can induce skin erosion or blistering in *Col17a1*-null mice (Nishie et al., 2007), it did not significantly accelerate hair graying or hair loss in these mice. Importantly, we did not find evidence of macroscopic/microscopic junctional separation, basal cell death, nor inflammatory cell infiltrates between the HFSCs and the basement

Regardless of the precise mechanism involved, our findings reveal a potential mechanism for the hair loss (alopecia) seen with human COL17A1 deficiency, which causes the nonlethal form of junctional epidermolysis bullosa, also known as generalized atrophic benign epidermolysis bullosa (GABEB) (McGrath et al., 1995; Nishie et al., 2007). It has been reported that the hair loss in GABEB patients is not always associated with

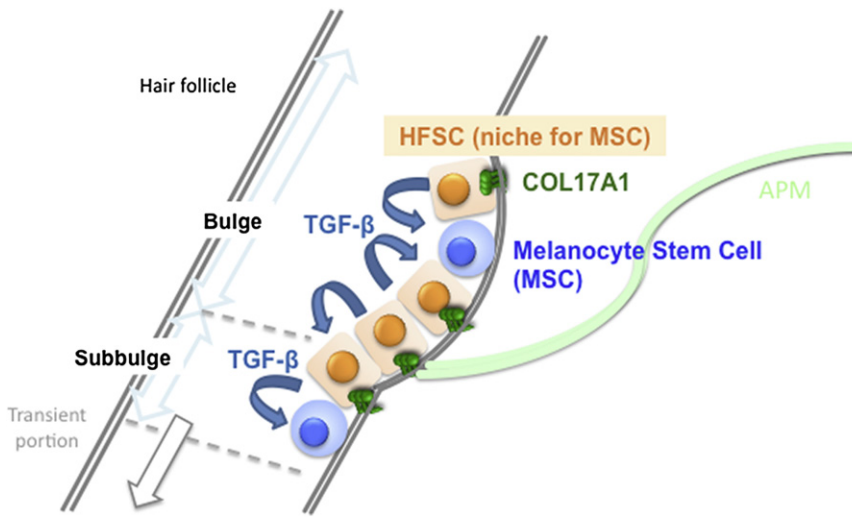


Figure 7. A Schematic Model for HFSCs and MSC Niche

HFSCs provide COL17A1-dependent niche for MSCs through TGF- β signaling. APM, arrector pili muscle.

surrounding skin surface changes but is associated with hair follicle atrophy or hair follicle loss (Hintner and Wolff, 1982). This finding is consistent with the late skin changes such as hair follicle atrophy seen in *Col17a1*-null mice. Therefore, we suggest that this mouse model may be a powerful tool for helping to understand the pathomechanisms of premature alopecia.

Human patients with GABEB also show epidermal atrophy with aging. *Col17a1*-deficient mice show transient epidermal hyperplasia in some focal areas at around 6 months of age (Figure 3D) but the entire skin becomes gradually more atrophic over time. Similar but more pronounced changes have been observed in the setting of stem cell depletion such as is seen in *Rac1* conditional knockout mice (Benitah et al., 2005) and in c-Myc transgenic mice (Arnold and Watt, 2001; Waikel et al., 2001). The late onset of epidermal atrophy seen in *Col17a1*-null mice might represent the eventual depletion or a decreased self-renewing potential of epidermal stem cells for the IFE.

More generally, *Col17a1*-null mice have provided evidence of an unexpected biological function for HFSCs. Although we have previously shown that the niche microenvironment plays a dominant role in fate determination for MSCs (Nishimura et al., 2002), the type of cell and/or the extracellular matrix in the bulge area that comprises the functionally essential component(s) of the niche has been unclear. Our current data indicate that HFSCs serve as a functional niche for MSCs and act through HFSC-derived TGF- β signaling, which is critical for MSC maintenance (Figure 7). It is notable that MSC immaturity was lost in *Col17a1*-deficient mice at a time when HFSCs were undergoing aberrant proliferation and differentiation in the bulge area with gradual loss of HFSC characteristics, including TGF- β production. There are a number of keratinocyte-specific gene-deficient mice that display a hair loss phenotype caused by HFSC depletion (Benitah et al., 2005; Zanet et al., 2005). However, as far as we know, characteristic premature hair graying has not been reported in those mice. It is also interesting that HFSCs nurture MSCs even though they are derived from a completely different developmental origin (Nishimura et al., 1999, 2002). A similar niche function provided by one type of stem cell for

another was reported in *Drosophila melanogaster* testis and mouse bone marrow during the revision of this paper (Leatherman and Dinardo, 2010; Méndez-Ferrer et al., 2010; Omatsu et al., 2010). The maintenance of somatic stem cell populations in a coherent cell mass with a specialized tissue organization such as in the hair follicle bulge might be a recurring strategy for somatic stem cell maintenance. COL17A1 in the basal cell population of HFSCs (the $\alpha 6$ -integrin^{high} population) (Blanpain et al., 2004) is critical not only for the maintenance of MSCs but also for the suprabasal HFSCs ($\alpha 6$ -integrin^{low} population), which suggests a common niche function for basal HFSCs for the maintenance of adjacent MSCs and HFSCs. Further studies to elucidate the precise niche properties of HFSCs may clarify additional fundamental mechanisms for the maintenance of stem cell pools as clustered stem cell populations.

EXPERIMENTAL PROCEDURES

Animals

Dct-lacZ transgenic mice (Mackenzie et al., 1997) (a gift from I. Jackson), *Col17a1*-knockout mice (Nishie et al., 2007), and *Krt14*-human *COL17A1* transgenic mice (Olasz et al., 2007) have been described previously. *Col17a1*^{+/+} and *Col17a1*^{+/-} mice are referred to as control mice. CAG-CAT-EGFP mice (a gift from J. Miyazaki) were bred with *Dct*^{tm(Cre)Bee} mice (a gift from F. Beermann) to generate compound heterozygotes as described previously (Osawa et al., 2005). All mice were backcrossed to C57BL/6J. Animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation.

TGF- β RII straight knockout mice (a gift from M. Taketo) (Oshima et al., 1996) were bred with Rag2-deficient mice at the Animal Research Facility of the Institute of Medical Science, University of Tokyo. Animal care of the line was carried out in accordance with the guidance of Tokyo University for animal and recombinant DNA experiments.

Histology, Immunohistochemistry, and Flow Cytometry Analyses

Paraffin, frozen sections, and whole-mount β -galactosidase staining were performed as previously described (Nishimura et al., 2002, 2005). Additional details on the methods and antibodies used are provided in the Supplemental Information. Multicolor flow cytometry analysis for HFSCs was performed with a FACSCalibur (BD).

Electron Microscopy

For electron microscopy, 20 μ m cryostat sections were cut and stained in X-gal solution for 12 hr at 37°C. The sections were postfixed in 0.5% osmium tetroxide for 30 min, stained with 1% uranyl acetate for 20 min, dehydrated in a graded ethanol series, and then embedded in epoxy resin. Semithin sections (1 μ m thick) were examined after toluidine blue staining and were observed by light microscopy. Ultrathin sections were observed with a JEM-1210 transmission electron microscope (JEOL) at 80 KV.

Isolation of Melanocytes

Dorsal skin was harvested from 6-day-old *CAG-CAT-EGFP/+; Dc^{tm1(Cre)Bee1/tm1(Cre)Bee1}* mice. The skin specimens were incubated in PBS containing 300 U/ml dispase (Sanko Junyaku) overnight at 4°C, and then the dermis was removed from the epidermis with a stereomicroscope. The epidermis was further dissociated by treatment with 0.25% trypsin for 10 min at 37°C. After neutralization with fetal calf serum (FCS), GFP⁺ melanocytes were sorted with JSAN (Bay Bioscience).

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction

Total RNAs from mouse skin or sorted GFP⁺ melanocytes were isolated with TRIzol (GIBCO) according to the manufacturer's instructions. 3 μg total RNA was used for cDNA synthesis in THERMOSCRIPT RT-PCR System (GIBCO) according to the manufacturer's instructions. The following primers were used for the analysis: mouse *Col17a1* (forward primer 5'-actgcctcttctca acca, reverse primer 5'-gagcaggacgccatgtatt) and *GAPDH* (forward primer 5'-accacagtcctcatccatcac, reverse primer 5'-tccaccaccctgttctgta).

Colony-Formation and Adhesion Assays

For the colony-forming assay, keratinocytes from newborn mice were used. Dorsal skins were incubated in PBS containing 300 U/ml dispase (Sanko Junyaku) for 1 hr at 37°C, after which the dermis was removed from the epidermis with a stereomicroscope. The epidermis was further dissociated by treatment with TrypLE Select (GIBCO) for 10 min at 37°C. The isolated cells (10⁵ per 6 cm dish) were seeded on 3T3-J2 feeder cells treated with mitomycin C. The cells were grown in calcium-free medium (3:1 = calcium-free DMEM:CnT-57CF.S [Celltec]) supplemented with 1.8 × 10⁻⁴ M adenine, 1% antibiotic-antimycotic solution (Sigma), 2 mM L-glutamine, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10⁻¹⁰ M cholera enterotoxin, 10 ng/ml EGF, and 10% FCS treated with Chelex-100 resin (BioRad) at 32°C in a humidified atmosphere with 8% CO₂ for a total of 14 days. To visualize the keratinocyte colonies, the cells were washed with PBS and were then fixed in 4% formalin for 20 min at room temperature. After further washing in PBS, the cultures were stained for 5 min at room temperature with crystal violet.

For the adhesion assay, isolated keratinocytes (10⁵ per well in 6-well plates) were seeded on 3T3-J2 feeder cells treated with mitomycin C or on collagen I-coated 6-well plates. 12 or 24 hr later, keratinocytes was washed three times in PBS and were collected with 0.05% trypsin-EDTA. Collected cells were fixed with 2% formaldehyde for 10 min at 37°C, permeabilized by ice-cold 100% methanol for 30 min, and stained with an Alexa Fluor 488-conjugated pan-cytokeratin monoclonal antibody (EXBIO). Detection of adherent keratinocytes was performed with a FACSCanto II (BD).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at [doi:10.1016/j.stem.2010.11.029](https://doi.org/10.1016/j.stem.2010.11.029).

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