SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit

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ABSTRACT **Hair graying, an age-associated process of unknown etiology, is characterized by a reduced number and activity of hair follicle (HF) melanocytes. Stem cell factor (SCF) and its receptor c-kit are important for melanocyte survival during development, and mutations in these genes result in unpigmented hairs. Here we show that during cyclic HF regeneration in C57BL/6 mice, proliferating, differentiating, and melanin-producing melanocytes express c-kit, whereas presumptive melanocyte precursors do not. SCF overexpression in HF epithelium significantly increases the number and proliferative activity of melanocytes. During the induced hair cycle in C57BL/6 mice, administration of anti-c-kit antibody dose-dependently decreases hair pigmentation and leads to partially depigmented (gray) or fully depigmented (white) hairs, associated with significant decreases in melanocyte proliferation and differentiation, as determined by immunostaining and confocal microscopy. However, in the next hair cycle, the previously treated animals grow fully pigmented hairs with the normal number and distribution of melanocytes. This suggests that melanocyte stem cells are not dependent on SCF/c-kit and when appropriately stimulated can generate melanogenically active melanocytes. Therefore, the blockade of c-kit signaling offers a fully reversible model for hair depigmentation, which might be used for the studies of hair pigmentation disorders.—Botchkareva, N. V., Khlgatian, M., Longley, B. J., Botchkarev, V. A., and Gilchrest, B. A. SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit. *FASEB J.* 15, 645–658 (2001)**

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THE HAIR FOLLICLE (HF) represents a hair shaft-producing mini-organ that during postnatal life repeatedly undergoes tightly programmed transformations from resting (telogen) to active growth (anagen) and then to the regression (catagen) (1–3). HF regeneration is characterized by dramatic changes in its microanatomy and cellular activity. The pigmented hair shaft is produced only during anagen. Thereafter, the HF shortens in length up to 70% as a result of apoptosis of cells constituting the proximal HF epithelium (4, 5).

Within the HF, neural crest-derived melanocytes produce and transport melanin to the keratinocytes of the precortical zone that differentiates to form the pigmented hair shaft. During postnatal life, the HF pigmentary unit cyclically regenerates synchronously with the HF transition through distinct hair cycle stages (6). The melanogenic activity of the follicular melanocytes is strictly coupled to the anagen stage, decreases during late anagen and early catagen, and ceases during late catagen and telogen (7, 8). Melanogenically active melanocytes are located in the hair bulb, adjacent to the upper part of the HF dermal papilla and contact neighboring keratinocytes and dermal papilla fibroblasts with their dendrites (6). Amelanotic melanocytes are located along the HF outer root sheath and in the HF bulge (9, 10), the region known to contain HF stem cells (11, 12).

Tyrosinase, an enzyme uniquely expressed in melanocytes, catalyzes the rate-limiting initial events of melanogenesis (13–17). The tyrosinase gene maps to the *albino* locus in mice, and tyrosinase mutations lead to loss of pigment (18). Tyrosinase-related proteins 1 and 2 (TRP1 and TRP2) share 40–45% amino acid identity with tyrosinase and are also critically important for melanogenesis, functioning as downstream enzymes in the melanin biosynthetic pathway (13, 19). TRP1 and TRP2 map to the *brown* locus and the *slaty* locus in mice, respectively, and mutations lead to a brown or gray rather than normal black coat color (20, 21).

The expression of tyrosinase, TRP1, TRP2, and tyrosinase activity are hair cycle dependent (7, 8, 22). By western and northern blotting, tyrosinase and TRP1 are undetectable in telogen HF (7). Their expression increases rapidly during early anagen and peaks in late anagen (7). During the anagen-catagen transition, tyrosinase, TRP1, and TRP2 expression rapidly decreases in skin homogenates (8), but the expression of these melanogenic proteins in the distinct populations of HF melanocytes during the hair cycle remains to be elucidated.

Hair pigmentation is tightly regulated by several factors (23, 24). Stem cell factor (SCF) and its tyrosine kinase receptor c-kit are critically important for the

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migration, proliferation, and differentiation of melanoblasts during embryogenesis (25, 26). In mice, melanocytes progressively disappear from the epidermis shortly after birth and are present only in the HF (27). SCF and c-kit map to the steel (*Sl*) and the dominant white spotting (*W*) loci, respectively, and mutations in these genes result in unpigmented hairs (25, 28, 29), suggesting that SCF/c-kit signaling is required for maintenance of HF melanocytes. Administration of anti-c-kit antibody (ACK2) during embryonic development leads to coat depigmentation (27, 30). In contrast, mice overexpressing SCF under the control of the K14 promoter display hyperpigmentation and melanocyte localization in atypical areas, including nose, mouth, ears, and footpads (31, 32).

Postnatally, SCF is expressed in dermal papilla fibroblasts of human HF and in the hair matrix of murine HF (33, 34), and c-kit is expressed in murine hair matrix keratinocytes (34). However, whether HF melanocytes express c-kit during the hair cycle and whether expression correlates with melanocyte proliferation, differentiation, and apoptosis are unknown. Although a possible requirement of SCF for the HF melanocytes was demonstrated by using ACK2 antibody during postnatal development (30), the precise role for SCF/c-kit signaling in the control of cyclic regeneration of the hair pigmentation unit remains to be elucidated.

To study the regeneration of the hair pigmentary unit and its dependence on SCF/c-kit during the hair cycle, we have analyzed the expression of TRP1, TRP2, tyrosinase, and c-kit in proliferating, differentiating, and melanogenically active melanocytes during depilation-induced HF telogen–anagen transition in normal C57BL/6 mice, in SCF overexpressing mice (promoter: K14), and after administration of anti-c-kit antibody to C57BL/6 mice. Our study provides evidence that SCF/c-kit signaling is indeed required for the generation and migration of functional melanocytes during each new hair cycle and that different populations of the HF melanocytes show differential dependence on SCF during cyclic regeneration of the hair pigmentary unit.

MATERIALS AND METHODS

Animals and tissue collection

C57BL/6 female mice (8-wk-old) were purchased from Charles River (Boston, Mass.) and housed in community cages at the animal facilities of the Boston University School of Medicine. SCF-overexpressing mice generated using K14 promoter in C57BL/6 background (32) were housed in the animal facility of Columbia University (New York, N.Y.). All mice were fed water and murine chow *ad libitum* and were kept under 12-h light/dark cycles. To induce hair cycle, depilation of the back skin was performed, as described previously (35).

In C57BL/6 mice, the next hair cycle stages were studied, using at least five mice per time point: telogen (unmanipulated skin), anagen II (3 days after depilation), anagen IV (5 days), and anagen VI (8–12 days). In SCF transgenic mice, only anagen IV stage was studied, and three transgenic mice and three wild-type animals were used for analyses 5 days after depilation. For the immunohistochemical analyses, back skin was harvested parallel to the vertebral line, embedded quickly, and frozen in liquid nitrogen, using a special technique for obtaining longitudinal cryosections through the HF from one defined site (36).

Immunohistochemistry, TUNEL, and multicolor confocal microscopy

For immunohistochemical analyses, acetone-fixed cryostat sections (8 μm) of adolescent C57BL/6 mouse back skin were used. To study the expression of tyrosinase, TRP1, or TRP2, we performed immunofluorescence protocol using the corresponding primary rabbit antisera (**Table 1**) and secondary tetramethylrhodamine-isothiocyanate (TRITC)-conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa.), as described previously (37). Briefly, cryosections were incubated with primary antisera overnight at room temperature, followed by application of secondary antibody (diluted 1:200) for 45 min at 37°C. Incubation steps were interspersed by four washes with tris buffer-saline (TBS, 5 min each).

For double immuno-visualization of melanogenic proteins and the proliferative marker *Ki*-67, cryosections were first incubated with rat monoclonal antibody against murine *Ki*-67 (Table 1) overnight at room temperature, followed by incubation with TRITC-labeled goat ant-rat IgG (Jackson ImmunoResearch; 45 min, 37°C). After subsequent washing in TBS, sections were incubated with primary rabbit antiserum against one of the melanogenic proteins (TRP1, TRP2, or tyrosinase) overnight at room temperature followed by incubation with fluorescein-isothiocyanate (FITC)-conjugated secondary goat-anti-rabbit rat IgG (Jackson ImmunoResearch; 45 min, 37°C). Finally, sections were washed three times with Tris buffer and then counterstained by TO-PRO3-iodide (Molecular Probes, Eugene, Oreg.) for visualization of cell nuclei (38).

For the double immunodetection of c-kit, and one of the melanogenic proteins (TRP1, TRP2, or tyrosinase), the tyramide-amplification method was used, as described before (39). Briefly, after blocking of endogenous peroxidase and nonspecific avidin/biotin binding, sections were incubated in TNB buffer (DuPont/NEN, Boston, Mass.) followed by application of a rat monoclonal antibody against murine c-kit (1:1000) overnight. Then the corresponding biotinylated goat anti-rat antiserum (1:200, 30 min), diluted in TNB blocking buffer (DuPont/NEN), was applied. The reaction product was developed using commercial tyramide-amplification kit (DuPont/NEN). Sections were incubated in streptavidin-horseradish peroxidase (HRP; 1:100 in TNB, 30 min), washed with TNT buffer (DuPont/NEN), followed by a 10-min application of TRITC-tyramide (1:50 in Amplification Diluent, DuPont/NEN). After blocking nonspecific binding by 10% normal goat serum, sections were incubated with rabbit antiserum against one of the melanogenic proteins (TRP1, TRP2, or tyrosinase), washed in TBS (3×5 min), followed by incubation with FITC-conjugated secondary goat-anti-rabbit IgG (45 min, 37°C). Finally, sections were washed three times with Tris buffer and then counterstained by TO-PRO3-iodide for visualization of cell nuclei.

For the double immunodetection of TRP1 and TRP2, a method of simultaneous detection of tissue antigens using antibodies raised from the same species was applied (40), as described before (39). Rabbit anti-TRP1 antibody was used in the dilution 1:10000 and then was visualized by the biotinylated goat anti-rabbit IgG/streptavidin-HRP, followed by the application of TRITC-tyramide, as described above. Then rabbit anti-TRP2 antibody was applied at a dilution of 1:1000, followed by incubation with FITC-conjugated goat-anti-rabbit

TABLE 1. *Primary antibodies*

Antibody against	Abbreviation	Characterization of antigen	Dilution	Species	Manufacturer
c-kit receptor	c-kit	Murine c-kit (30)	1:1000	rat	Pharmingen, San Diego, Calif.
Nuclear matrix-associated proliferation-related antigen	Ki-67	Murine Ki-67 (66)	1:100	rabbit	Dianova, Hamburg, Germany
Nuclear matrix-associated proliferation-related antigen	Ki-67 (TEC-3)	Murine Ki-67 (67)	1:20	rat	Dianova, Hamburg, Germany
Stem cell factor	SCF	Murine SCF, clone 40215.11	1:1000	rat	R&D Systems Minneapolis, Minn.
Tyrosinase	αPEP 7	Polyclonal antiserum against a synthetic peptide that corresponds to the carboxyl terminus of Tyr, the murine albino locus encoded protein (43)	1:1000	rabbit	Gift of Dr. V. J. Hearing
Tyrosinase-related protein 1	TRP1 (αPEP1)	Polyclonal antiserum against a synthetic peptide that corresponds to the carboxyl terminus of the murine brown locus encoded protein (68)	1:1000	rabbit	Gift of Dr. V. J. Hearing
Tyrosinase-related protein 2	TRP2 (αPEP8)	Polyclonal antiserum against a synthetic peptide that corresponds to the carboxyl terminus of the murine slaty locus encoded protein (69)	1:1000	rabbit	Gift of Dr. V. J. Hearing

IgG. Therefore, anti-TRP1 antibody used in a very low concentration (1:10000) was undetectable for the FITC-conjugated goat anti-rabbit secondary antibody and could only be visualized by highly sensitive tyramide amplification assay. FITC-labeled secondary antibody was used consecutively only for the visualizing rabbit anti-TRP2 antibody (diluted 1:1000).

For triple immuno-visualization of the c-kit, one of the melanogenic proteins (TRP1, TRP2, or tyrosinase), and *Ki*-67, a method of simultaneous detection of tissue antigens using antibodies raised from the same species was applied (40). Briefly, skin cryosections were first immunostained with rat monoclonal antibody against c-kit (diluted 1:1000; Table 1) followed by the application of biotinylated goat anti-rat IgG, streptavidin-HRP, and TRITC-tyramide, as described above. Then sections were processed for visualization of *Ki*-67 with the corresponding rat monoclonal antibody (diluted 1:50; Table 1) overnight at room temperature followed by the application of Cy-5-conjugated secondary goat-anti-rat IgG (Jackson ImmunoResearch; 1:50, 45 min at 37°C). According to the original protocols (40), rat anti-c-kit antibody used in the dilution 1:1000 could only be detected by the tyramide amplification assay. Therefore, anti-c-kit antibody was undetectable for the Cy5-conjugated goat anti-rat secondary antibody used consecutively for the visualizing rat anti-*Ki*-67 antibody (diluted 1:50). After visualization of *Ki*-67 antigen, rabbit antiserum against one of the melanogenic proteins (TRP1, TRP2, or tyrosinase) was applied followed by the incubation with FITC-labeled goat anti-rabbit IgG, as described above. Thus, this method allowed us to visualize in skin sections simultaneously c-kit receptor on cell membrane (TRITC-coupled red fluorescence), *Ki*-67 in cell nucleus (Cy5-coupled blue fluorescence), and one of the melanogenic proteins in cytoplasm (FITC-coupled green fluorescence).

For the double immuno-visualization of one of the melanogenic proteins and TUNEL, cryostat sections were processed for the detection of TUNEL-positive (TUNEL+) apoptotic cells using commercially available Apo-Tag kit (Intergen, Purchase, N.Y.). Reaction product was visualized by FITC-labeled anti-digoxigenin antibody. Then, one of the melanogenic proteins was visualized using the corresponding primary antisera and TRITC-conjugated goat anti-rabbit IgG, as described above (4), and nuclei were counterstained by TO-PRO3.

For the analysis of SCF expression, the rat monoclonal antibody against murine SCF was used (Table 1), and the tyramide-amplification assay was applied, as described above. Sections were counterstained by TO-PRO3 for visualization of cell nuclei. Negative controls of immunostaining were performed by omitting primary antibodies. Skin cryosections of the SCF-overexpressing transgenic mice, which showed ectopic expression of SCF in the basal epidermal keratinocytes and abundantly contained melanocytes in the epidermis and mast cells in the dermis (32), were used as positive controls for antibodies against SCF, melanogenic proteins, and c-kit, respectively. HF in late anagen phase displaying a high number of proliferating cells in the proximal hair matrix were used as an internal positive control for the antibody against *Ki*-67. All slides were analyzed using a Zeiss confocal microscope and were photo-documented using a digital image analysis system (Pixera, San Diego, Calif.).

Western blot analysis

Total tissue proteins were obtained from extracts of full thickness C57BL/6 back skin and collected in a buffer consisting of 0.25 M Tris HCl (pH-7.5), 0.375 M NaCl, 2.5% sodium deoxycholate, 1% Triton X-100, 25 mM $MgCl_2$, 1 mM phenylmethyl sulfonyl fluoride, and 0.1 mg/ml aprotinin, as described previously (41, 42). Protein concentrations were determined by the Bradford method, and 100 μg of proteins was processed for Western blot analysis as described (41). Antibody reaction was performed with rabbit polyclonal anti-tyrosinase antibody (1:200) obtained from Dr. V. J. Hearing (43). Horseradish peroxidase-tagged donkey anti-rabbit IgG was used as secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.; 1:2000). The ECL detection kit (Amer-

sham) followed by autoradiography (Kodak X-Omatic AR) detected antibody binding.

Pharmacological manipulations *in vivo*

The rat monoclonal antibody ACK45 against mouse c-kit (Pharmingen, San Diego, Calif.) was administered intracutaneously to back skin of the 8-wk-old C57BL/6 mice in concentration 6 mg/kg at the various time points after the depilation. The specificity and activity of this antibody is similar to that of the published antibody ACK2 and was reported to block c-kit signaling *in vitro* and *in vivo* (27, 30). Intradermal administration of the same concentrations of mouse normal serum was used as a vehicle control. Three different experiments were done. In the first experiment, ACK45 treatment was performed on days 1, 3, and 5 after depilation. In the second experiment, ACK45 was administrated on days 1, 3, 5, 7, 9, 11, and 13 after depilation. In both experiments, skin samples from 12 control mice and 12 ACK45-treated mice were analyzed in mid- and late anagen (days 5–14 after depilation). In the third experiment, ACK45 antibody or vehicle control was administered intracutaneously to six 8-wk-old C57BL/6 mice (n=3 for each group) on days 1, 3, 5, 7, 9, 11, and 13 after depilation. After the completion of HF anagen-catagen-telogen transition (day 30 after depilation), hair cycle in these mice was induced again in the selected areas of their back skin, and skin was harvested on days 12–14 after the second depilation (days 42–44 after the beginning of the experiment).

Morphometry and statistical analysis

In the back skin, >50 HF at the distinct stages of hair cycle, derived from three to five different mice, were studied for each antigen analyzed. Only every 10th cryosection was used for analysis to exclude the repetitive evaluation of the same HF, and two to three cryosections were assessed from each animal. For the analysis of melanocyte proliferation, cryosections of the early and mid-anagen skin harvested on days 3 and 5 after depilation were used. At least 60–70 HF at anagen stages II and IV were analyzed, and the percentage of *Ki*-67—positive (*Ki*-67+) melanocytes per total number of the HF melanocytes was assessed. All sections were analyzed at ×200–400 magnification, and means and SE were calculated from pooled data. Differences were judged as significant if the P value was <0.05 as determined by the independent Student's t test for unpaired samples.

RESULTS

Proliferating, differentiating, and actively pigment-producing HF melanocytes express c-kit receptor

To study the distribution of proliferating, differentiating, and actively melanogenic melanocytes during HF anagen development, tissue sections were stained with TRP2, understood to be the earliest indicator of non-differentiated melanocytes, as well as with TRP1 and tyrosinase, markers for more differentiated and fully differentiated (melanogenic) melanocytes, respectively (44). We performed double immunostainings for these melanogenic proteins (TRP1, TRP2, or tyrosinase) and the proliferative marker *Ki*-67 at distinct hair cycle stages in C57BL/6 mice to determine the relation between differentiation and proliferation in these cells. To visualize whether different subpopulations of the HF melanocytes express the c-kit receptor during defined hair cycle stages, triple immunofluorescence for c-kit, one of the melanogenic proteins, and *Ki*-67 was used.

In resting (telogen) HF, melanocytes were found in the secondary hair germs adjacent to the dermal papilla. These cells did not express TRP1 and could only be visualized by TRP2, known to be expressed by non-differentiated melanocytes (**Fig. 1*A, B***). In telogen HF, melanocytes were also tyrosinase negative (tyrosinase−) (not shown) and nonproliferating, as assessed by the absence of *Ki*-67 immunostaining (Fig. 1*A*, *B*; **Fig. 2*A***). However, single proliferating keratinocytes were seen in the epidermis and in the HF infundibulum (Fig. 1*A*, *B*), consistent with the accepted patterns of cell proliferation in murine telogen skin (5, 45). Double immunostaining for c-kit and TRP2 revealed that most of the TRP2-positive (TRP2+) follicular melanocytes also express c-kit, whereas some of the TRP2 immunoreactive melanocytes were c-kit negative (c-kit−) (Fig. 1*C*).

During early anagen (anagen II), TRP2+ melanocytes located in the proximal part of the secondary hair germ also expressed TRP1, whereas single cells located in distal part of the secondary hair germ (bulge) remained only TRP2+ (Fig. 1*D*). TRP1-positive (TRP1+) and TRP2+ cells located in the proximal part of the secondary hair germ of anagen II HF were also c-kit–positive (c-kit+) (Fig. 1*E, F*). However, TRP2+ melanocytes located in the HF bulge were c-kit− (Fig. 1*F*). The latter also were *Ki*-67 negative (*Ki*-67−), whereas 56.7 ± 7.3% of TRP1/c-kit double-positive cells and 40.7 ± 5.4% of TRP2/c-kit double-positive cells located in the proximal part of the secondary hair germ also showed *Ki*-67 immunostaining in their nuclei, indicating that they were proliferative (Fig. 1*E, F*; Fig. 2*B*). In addition, c-kit was also expressed in single cells of the secondary hair germ that were TRP1 negative (TRP1−) and TRP2− (Fig. 1*E*). Because c-kit expression was found on HF keratinocytes previously (34, 46), we interpret these cells to represent a subset of c-kit+ keratinocytes of the HF.

During mid-anagen (anagen IV), HF bulge melanocytes expressed only TRP2 (Fig. 1*G*, Fig. 2*C*), and did not expressed TRP1, c-kit, or *Ki*-67. Melanocytes in the elongating outer root sheath were TRP2+ and c-kit+, showed relatively low levels of TRP1, and did not express tyrosinase (Fig. 1*H–L*, Fig. 2*C*). Quantitative immunohistomorphometry showed that 35.6 ± 4.8% of TRP2+ cells located in the outer root sheath of anagen IV HF were proliferative. Melanocytes located in the forming hair bulb above the dermal papilla expressed all three melanogenic proteins (TRP1, TRP2, and tyrosinase) and were c-kit+, and 64.5 ± 7.1% of them were proliferative, as indicated by *Ki*-67 expression (Fig. 1*G–L*, Fig. 2*C*).

In late anagen HF (anagen VI), melanocytes located in the HF bulge, outer root sheath, and proximal hair

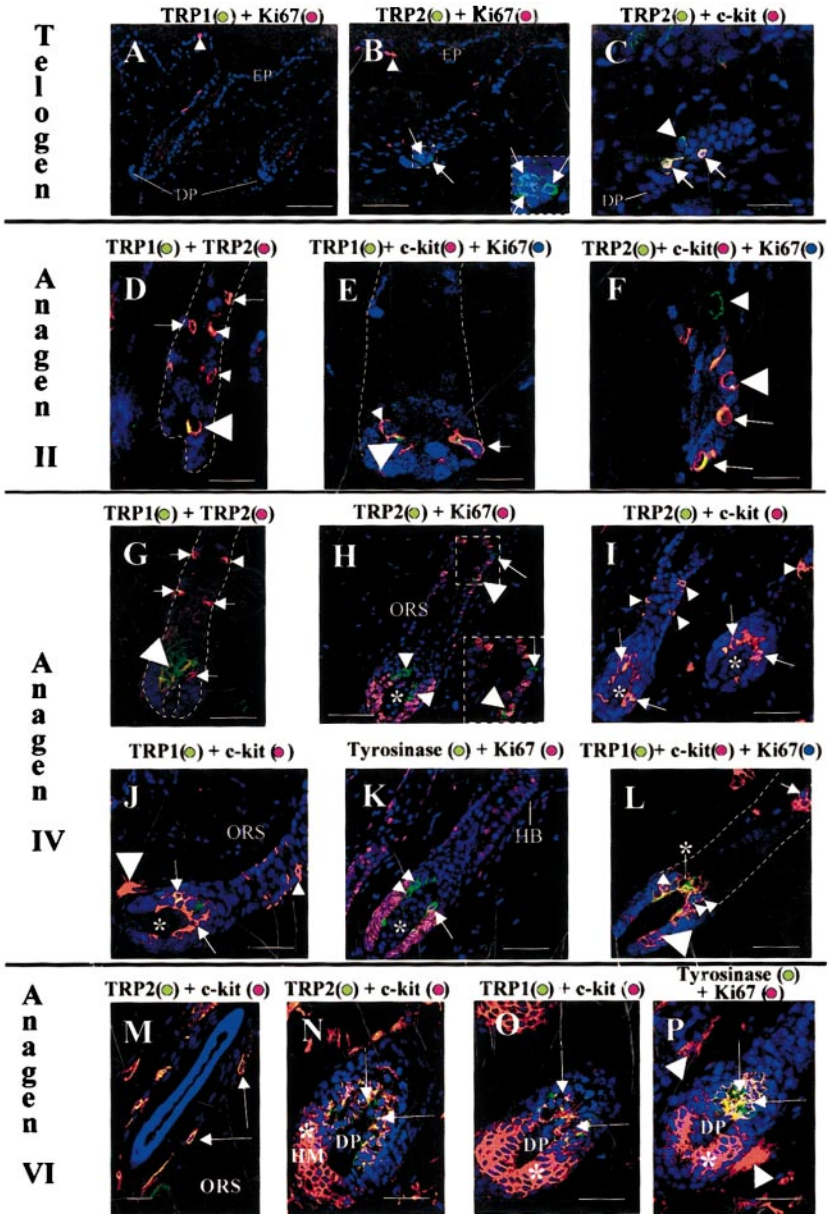

Figure 1. Expression of melanogenic proteins, c-kit, and *Ki*-67 in the HF melanocytes during cyclic regeneration of hair pigmentation unit. Back skin cryostat sections (8-μm thickness) of postnatal C57BL/6 mice in defined hair cycle stages (telogen, unmanipulated skin; anagen II, 3 days after anagen induction by depilation; anagen IV, 5 days after depilation; anagen VI, 8–12 days after anagen induction) were processed for the double and triple immunovisualization of TRP1, TRP2, tyrosinase, c-kit, and *Ki*-67. *A–C*) Telogen skin. *A*) TRP1 (green fluorescence) + *Ki*-67 (pink fluorescence). Absence of TRP1 in the telogen HF and expression of *Ki*-67 in single keratinocytes of the epidermis and HF outer root sheath (arrowhead). *B*) TRP2 (green fluorescence) + *Ki*-67 (pink fluorescence). TRP2 expression in the secondary hair germ (arrows) and single *Ki*-67+ keratinocytes in the epidermis (arrowhead). Inset shows a high magnification of the secondary hair germ (labeled area). *C*) TRP2 (green fluorescence) + c-kit (pink fluorescence). Two out of three TRP2+ melanocytes express c-kit (yellow fluorescence, arrows), whereas one melanocyte is only TRP2+ and does not show c-kit expression (arrowhead). In panels *A–C*, nuclei are counterstained by TO-PRO-3 (blue fluorescence). *D–F*) Early anagen skin. *D*) TRP1 (green fluorescence) + TRP2 (pink fluorescence). TRP2+ and TRP1− cells are located in the distal part of the secondary hair germ (pink fluorescence, arrows). Two out of three TRP2/TRP1 double-positive cells show predominance of TRP2 (pink/yellow fluorescence, small arrowheads). One out of three TRP2/TRP1 double-positive cells located in the proximal part of secondary hair germ show equal expression of both antigens (yellow fluorescence, large arrowhead). Nuclei are counterstained by TO-PRO-3 (blue fluorescence). *E*) TRP1 (green fluorescence) + c-kit (pink fluorescence) + *Ki*-67 (blue fluorescence). Co-expression of TRP1 with c-kit and *Ki*-67 in the secondary hair germ (yellow cytoplasmic fluorescence and blue nucleus, arrow). Absence of *Ki*-67 immunostaining in the TRP1+ and c-kit+ melanocyte (yellow cytoplasmic fluorescence and dark nucleus, large arrowhead). Single c-kit+ and *Ki*-67+ cell does not show TRP1 expression (pink cytoplasmic fluorescence and blue nucleus, small arrowhead). *F*) TRP2 (green fluorescence) + c-kit (pink fluorescence) + *Ki*-67 (blue fluorescence). Single TRP2+ melanocyte seen in the HF bulge does not show c-kit and *Ki*-67 expression (green cytoplasmic fluorescence and dark nucleus, small arrowhead). One of the TRP2+ and c-kit+ cells shows *Ki*-67+ nucleus (pink/yellow cytoplasmic fluorescence and blue nucleus, large arrowhead), whereas two others are *Ki*-67− (yellow cytoplasmic fluorescence and dark nuclei, arrows). *G–K)* Mid-anagen skin. *G*) TRP1 (green fluorescence) + TRP2 (pink fluorescence). TRP2+ and TRP1− cell is located in bulge (pink fluorescence, arrowhead). Four TRP2/TRP1 double-positive cells in the outer root sheath and hair matrix show predominance of TRP2 (pink/yellow fluorescence, small arrowheads). TRP2/TRP1 double-positive cells located in the hair matrix show predominance of TRP1 (green/yellow fluorescence, large arrowhead). HF is indicated by dotted line. *H*) TRP2 (green fluorescence) + *Ki*-67 (pink fluorescence). TRP2+ melanocyte in the HF bulge is *Ki*-67− (arrow), whereas the TRP2+ one in the outer root sheath shows *Ki*-67+ nucleus (large arrowhead). Unproliferating TRP2+ cells in the hair matrix are indicated by small arrowheads, whereas double *Ki*-67/TRP2+ cells are labeled by lines. Dermal papilla is indicated by asterisk. *I*) TRP2 (green fluorescence) + c-kit (pink fluorescence). TRP2/c-kit double-positive cells in the hair matrix (arrows) above the dermal papilla (asterisk) and along the outer root sheath (arrowheads). *J*) TRP1 (green fluorescence) + c-kit (pink fluorescence). TRP1 is co-expressed with c-kit in the hair matrix (arrows) above the dermal papilla (asterisk). C-kit predominates over TRP1 in cells along the outer root sheath (pink fluorescence, small arrowhead). c-kit+ mast cell in the dermis is shown by large arrowhead. *K*) tyrosinase (green fluorescence) + *Ki*-67 (pink fluorescence). Tyrosinase is expressed in proliferating (arrow) and nonproliferating melanocytes (arrowheads) only in the hair matrix above the dermal papilla (asterisk). *L*) TRP1 (green fluorescence) + c-kit (pink fluorescence) + *Ki*-67 (blue fluorescence). C-kit+ cell in the HF outer root sheath is *Ki*-67+ (arrow). Many of the TRP1/c-kit double-positive

bulb again showed distinct patterns in the expression of melanogenic proteins and c-kit. Single melanocytes seen in the HF bulge showed only TRP2 expression and were negative for TRP1, tyrosinase, and c-kit (not shown). Melanocytes located in the outer root sheath expressed TRP2 and c-kit and were TRP1 and tyrosinase− (Fig. 1*M*, Fig. 2*D*). Melanocytes in the hair matrix above dermal papilla that were actively producing pigment showed prominent expression of all three melanogenic proteins (TRP1, TRP2, tyrosinase) and expressed c-kit (Fig. 1*N–P*, Fig. 2*D*). None of the melanocyte subpopulations of the anagen VI HF showed *Ki*-67 immunoreactivity and, therefore, were nonproliferative (not shown). However, c-kit expression was also seen in hair matrix keratinocytes, which is consistent with the expression patterns described before (34, 46).

Thus, during cyclic regeneration of the hair pigmentary unit, follicular melanocytes can be divided into three distinct subpopulations characterized by expression patterns of distinct combinations of melanogenic proteins, c-kit receptor, and proliferative activity. First, HF bulge melanocytes show only TRP2 expression, are c-kit−, and do not proliferate. These most likely represent the stem cell melanocyte population of the HF. Second, HF outer root sheath melanocytes are both TRP2+ and c-kit+, weakly express TRP1, and proliferate during early/mid-anagen. We assume that this subpopulation is stem-cell derived and differentiates into the third subpopulation of melanogenically active melanocytes, located in the hair matrix, that express all three melanogenic proteins, c-kit, and proliferate only during mid-anagen. This population probably represents fully differentiated melanocytes.

SCF is prominently expressed in the HF connective tissue sheath and dermal papilla during cyclic regeneration of the hair pigmentation unit

Our above data (Figs. 1, 2) suggest that during cyclic regeneration of the hair pigmentation unit, melanocytes undergoing differentiation and fully differentiated melanocytes are both dependent on the SCF/c-kit signaling, whereas stem cell melanocytes are probably SCF independent.

To define SCF expression during anagen-associated regeneration of the hair pigmentation unit, the highly sensitive tyramide-amplified immunofluorescence method was applied. We found that in telogen HF, SCF was prominently expressed in the follicular connective tissue sheath and in dermal fibroblasts (**Fig. 3*A***) and that this expression pattern was maintained throughout anagen (Fig. 3*B*, *C*). However, during mid- and late anagen, SCF expression also appeared in the HF dermal papilla (Fig. 3*B*, *C*). This correlates with maximal melanin-producing activity of the c-kit+ melanocytes located in the HF matrix above the dermal papilla. Therefore, our data suggest that proliferation, differentiation, and melanogenic activity of the c-kit+ follicular melanocytes depends on the c-kit ligand SCF, locally produced by mesenchymal cells of the HF connective tissue sheath and dermal papilla during HF anagen development.

SCF overexpression increases HF melanocyte proliferation, differentiation, and ectopic expression of TRP1 in follicular bulge melanocytes

To determine the role of SCF in the control of the melanocyte proliferation and differentiation during cyclic regeneration of the hair pigmentation unit, we have compared the number, melanogenic protein expression, and proliferative activity of the HF outer root sheath melanocytes in SCF overexpressing transgenic versus wild-type mice. This subpopulation of the HF melanocytes was selected as having the maximal number of the c-kit+ proliferating and differentiating cells during the anagen IV stage of the induced hair cycle (Figs. 1, 2). SCF expression in the transgenic mice used for this study was also targeted to the HF outer root sheath by the K14 promoter (32), which would maximize effect of SCF on c-kit+ outer root sheath melanocytes.

The hair cycle was induced by depilation, and the back skin of both mouse strains was analyzed at anagen IV stage (i.e., 5 days after depilation). We performed immunostaining for TRP1, TRP2, and tyrosinase. TRP1 was prominently expressed by the numerous outer root sheath melanocytes in the SCF transgenic mice, whereas in the wild-type mice, TRP1 immunoreactivity was seen predominantly in HF matrix melanocytes and only rarely in the outer root sheath (**Fig. 4*A*, *B***). The

melanocytes in the hair matrix show *Ki*-67 expression (pink/yellow cytoplasmic fluorescence and blue nuclei, arrowheads), and some of them are *Ki*-67− (yellow cytoplasmic fluorescence and dark nucleus, asterisk). Single c-kit+ keratinocytes in the hair matrix (pink cytoplasmic fluorescence and blue nuclei) are indicated by large arrowhead. In panels *G–K*, nuclei are counterstained by TO-PRO-3 (blue fluorescence). *M–P)* Late anagen skin. *M*) TRP2 (green fluorescence) + c-kit (pink fluorescence). TRP2/c-kit double-positive cells are expressed in the outer root sheath (pink/yellow fluorescence, arrows). *N*) TRP2 (green fluorescence) + c-kit (pink fluorescence). TRP2 is co-expressed with c-kit in the hair matrix above the dermal papilla (yellow fluorescence, arrows), and c-kit alone is expressed by the hair matrix keratinocytes (pink fluorescence, asterisk). *O*) TRP1 (green fluorescence) + c-kit (pink fluorescence). Co-expression of TRP1 and c-kit in the hair matrix melanocytes (yellow fluorescence, arrows). Hair matrix keratinocytes express c-kit alone (pink fluorescence, asterisk) *P*) Tyrosinase (green fluorescence) + c-kit (pink fluorescence). Co-expression of tyrosinase and c-kit expression in the hair matrix melanocytes above the dermal papilla (yellow fluorescence, arrows). c-kit expression in the hair matrix keratinocytes (asterisk) and dermal mast cells (arrowheads). In panels *M–P*, nuclei are counterstained by TO-PRO-3 (blue fluorescence). DP, dermal papilla; EP, epidermis; HB, hair bulge; HM, hair matrix; ORS, outer root sheath. Scale bars = 100 μm (*A*, *B*), 50 μm (*C*, *G–L*, *N–P*), and 25 μm (*D–F*, *M*).

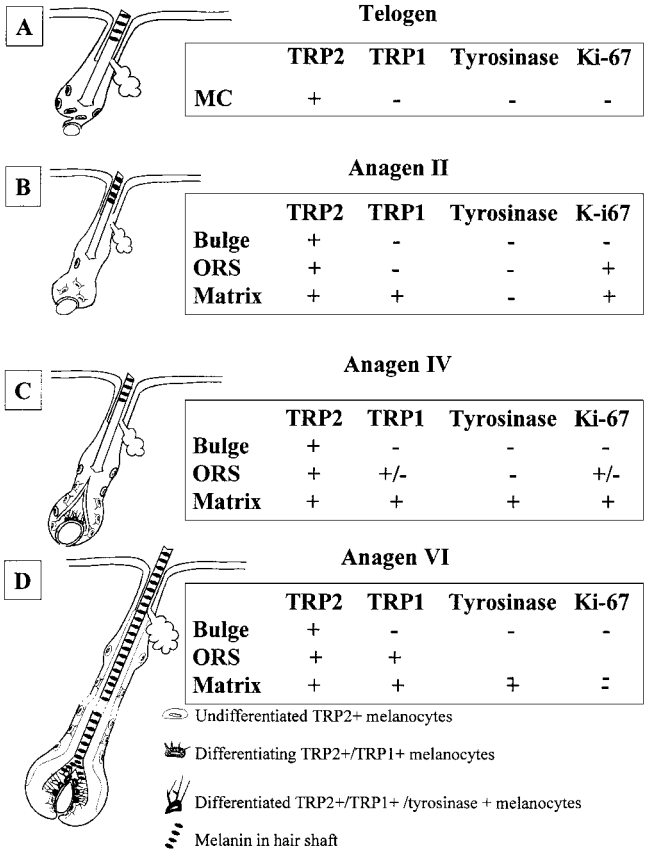

Figure 2. Summary of the expression patterns for TRP1, TRP2, tyrosinase, and *Ki*-67 in different subpopulations of the HF melanocytes during hair cycle-associated regeneration of hair pigmentation unit. Schematic drawings represent localization of different subpopulations of the HF melanocytes during distinct hair cycle stages (telogen, early, mid-, and late anagen). Undifferentiated TRP2+ melanocytes are located in the secondary hair germ and bulge, the differentiating TRP+ and TRP2+ melanocyte are mostly located in the outer root sheath, while differentiated melanogenically active melanocyte (TRP1+, TRP2+, and tyrosinase+) are located in the hair bulb.

number of TRP1+ cells in the HF outer root sheath of SCF transgenic mice was significantly higher ($P<0.001$) than in wild-type mice (Fig. 4*C*). In addition, TRP1+ cells were ectopically located in the HF bulge of SCF transgenic mice, whereas such cells were not seen in the bulge of wild-type HF.

Similarly, the number of TRP2+ cells in the HF outer root sheath of SCF overexpressors was also significantly higher ($P<0.001$), compared with that in wild-type mice (Fig. 4*D–F*). However, no increase in the number of TRP2+ cells was seen in the HF bulge of SCF transgenic mice, compared with the wild-type HF (not shown). Also, no tyrosinase-positive (tyrosinase+) cells were seen in the HF outer root sheath of SCF overexpressors (not shown). Similarly, as in wild-type HF, tyrosinase was exclusively expressed in the HF matrix melanocytes of SCF transgenic mice.

Proliferative activity in outer root sheath melanocytes, assessed by double immunovisualization of the TRP2 and K_i-67, revealed a significant increase ($P<0.01$) in the percentage of double-positive (proliferating) cells in the HF outer root sheath of the SCF transgenic mice, compared with that in wild-type mice (53.7±5.3 vs. 34.1±4.9%, respectively). However, no proliferative TRP2+ melanocytes were seen in the HF bulge of SCF transgenic mice (not shown). This significant increase in the number of melanocytes and in their proliferative activity in SCF-overexpressing mice suggests that SCF is required for melanocyte proliferation during early and mid-anagen. Furthermore, ectopic expression of TRP1 in the HF bulge of SCF transgenic mice suggests that SCF/c-kit signaling also promotes melanocyte differentiation.

Blockade of the SCF/c-kit signaling during early and mid-anagen results in the formation of gray, partially depigmented hairs

To determine the functional significance of the SCF/c-kit signaling in the control of cyclic regeneration of the hair pigmentary unit, anti-c-kit monoclonal antibody (ACK45) was intradermally administered into the C57BL/6 mouse skin at various times points of the depilation-induced hair cycle. This commercially available analog of the ACK2 antibody blocks c-kit signaling *in vitro* and *in vivo* (27, 30). In the first experiment, the requirement of SCF/c-kit signaling for melanocyte pro-

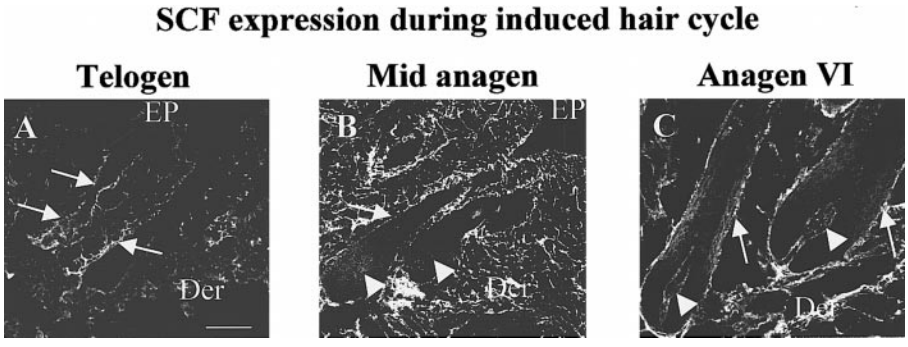

Figure 3. SCF expression during hair cycle in C57BL/6 mice. Back skin cryostat sections (8-μm thickness) of postnatal C57BL/6 mice in defined hair cycle stages were processed for SCF immunovisualization. *A*) Telogen (unmanipulated skin). SCF expression in the perifollicular connective tissue sheath (arrows) and in the dermis. *B*) Mid-anagen (day 5 after depilation). SCF expression in the perifollicular connective tissue sheath (arrows), in the dermal papilla (arrowheads), and in the dermis. *C*) Late anagen (day 8 after depilation). SCF expression in the HF dermal papilla (arrowheads), in the perifollicular connective tissue sheath (arrows), and in the dermis. Der, dermis; EP, epidermis. Scale bar = 100 μm.

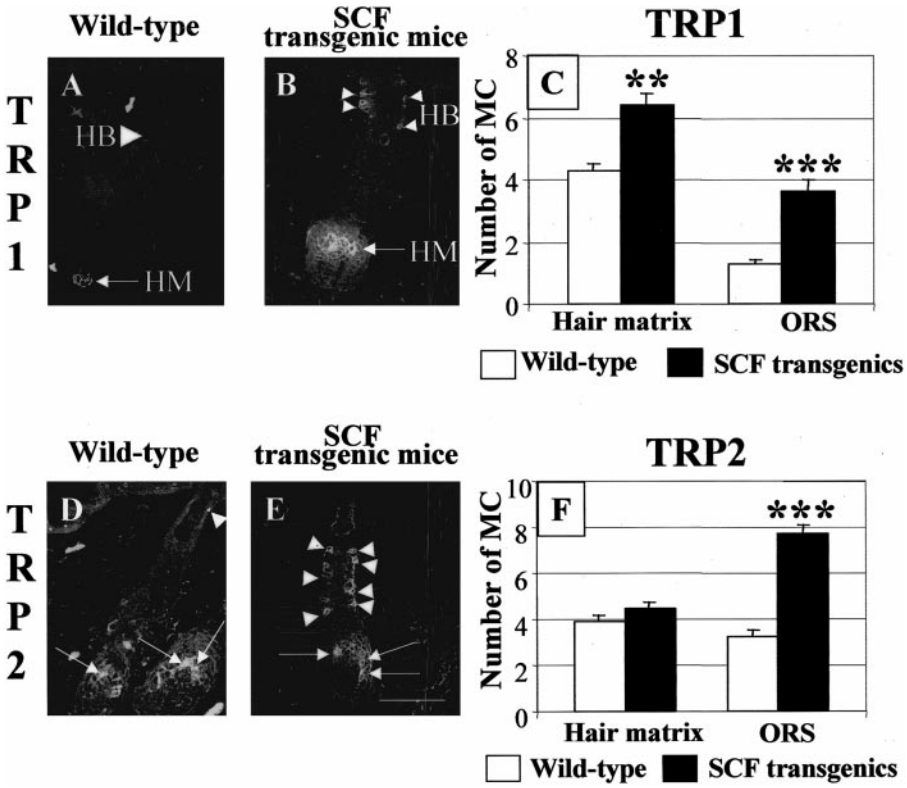

Figure 4. Increase of TRP1+ and TRP2+ melanocytes in the HF outer root sheath in the SCF overexpressing mice. *A*—*C*) TRP1 expression in SCF transgenic and wild-type mice. *A*) Wild-type mice. Expression of TRP1 in the HF matrix (arrows). *B*) SCF transgenic mice. Numerous TRP1+ cells in the HF outer root sheath and bulge (arrowheads). Up-regulation of TRP1 expression in the HF matrix (arrow). *C*) Comparison of the number of melanocytes expressing TRP1 in the hair matrix and in the ORS between SCF transgenic and corresponding control mice (mean±SE, $n=3$ animals per group, Student t test, asterisk indicates significant differences to the vehicle control, $^{**}P<0.05$, $^{***}P<0.001$). *D*–*F*) TRP2 expression in SCF transgenic and wild-type mice. *D*) Wild-type mice. TRP2 expression in the HF matrix (arrows) and in the ORS (arrowhead). *E*) SCF transgenic mice. TRP2 expression in the HF matrix (arrows) and increase of TRP2 expression in the outer root sheath (arrowheads). *F*) Comparison of the number of TRP2+ melanocytes in the hair matrix and in the ORS of SCF transgenic mice vs. corresponding control (mean±SE; $n=3$ animals per group; Student t test; asterisk indicates significant differences to the vehicle control; $^{***}P<0.001$). HB, hair follicle bulge; HM, hair matrix.

liferation and differentiation during early and mid-anagen was assessed by administration of ACK45 antibody on days 1, 3, and 5 after depilation (**Fig. 5*A***). This resulted in the formation of gray, partially depigmented hairs in mice treated with ACK45, whereas control mice had black, fully pigmented hair shafts (Fig. 5*B*). On day 5 after depilation, the number of TRP1+ and TRP2+ melanocytes in the HF outer root sheath and hair matrix was significantly reduced in the ACK45-treated skin ($P<0.05$), compared with the vehicle control skin (Fig. 5*C–F, I, J*). Importantly, no TUNEL+ (apoptotic) cells were seen in the HF after ACK45 treatment, whereas as a positive control TUNEL cells were seen in the HF during its transformation from anagen to catagen (Fig. 5*G, H*). Instead, proliferation of HF melanocytes was significantly decreased ($P<0.005$) in the ACK45-treated HF versus controls (Fig. 5*K*).

This suggests that SCF/c-kit signaling is required for melanocyte proliferation and differentiation during early and mid-anagen. This is also consistent with the patterns of c-kit expression in proliferating and differentiating melanocytes during the various hair cycle stages and with our data demonstrating increased numbers of proliferating HF melanocytes in SCF-overexpressing mice, described above (Figs. 1, 2, 4).

Continuous blockade of the SCF/c-kit signaling throughout anagen leads to the complete hair depigmentation

To determine the dependence on SCF of the melanogenically active follicular melanocytes located in the HF matrix above dermal papilla, ACK45 was administered intradermally on days 1, 3, 5, 7, 9, and 11 after depilation (**Fig. 6*A***). On days 12–14 after depilation, control mice had black, fully pigmented hairs, whereas ACK45-treated mice had white, completely depigmented hairs over the entire back (Fig. 6*B–D*), associated with absence of melanin microscopically in skin cross-sections (Fig. 6*D*).

Western blotting of proteins extracted from full-thickness skin treated with ACK45 showed no tyrosinase

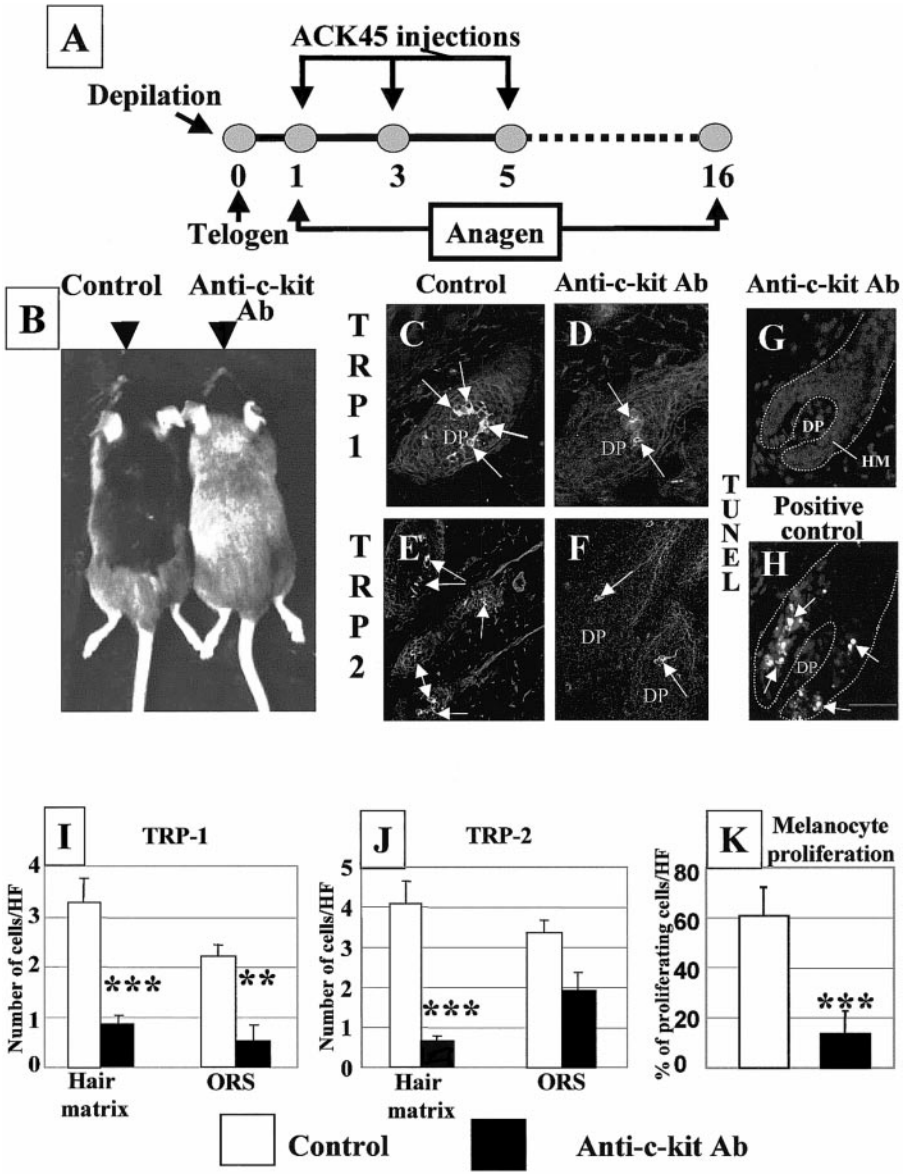

Figure 5. Blocking of SCF/c-kit signaling is required for melanocyte proliferation and differentiation during early and mid-anagen. Hair cycle was induced by depilation in the back skin of 8-wk-old C57BL/6 mice, and anti-c-kit antibody (ACK45) or vehicle control were administered intracutaneously at days 1, 3, and 5 after depilation *A*) Skin was harvested at day 5 after depilation, and cryostat sections were immunostained with antisera against TRP1 and TRP2 (*C–F*). *B*) Control mouse shows appearance of new black fur over the entire back, whereas ACK45-treated mice display growth of new partially depigmented gray hairs in the entire back skin (arrows). *C*) Control. Numerous TRP1+ melanocytes in the hair matrix of the control HF (arrows). *D*) ACK45. Single TRP1+ cells in the HF matrix of the ACK45-treated skin (arrows). *E*) Control. Numerous TRP2+ melanocytes in the HF matrix (arrows) and outer root sheath (arrowhead). *F*) ACK45. Single HF matrix cells expressing TRP2 (arrows). Absence of TRP2 expression in the outer root sheath. *G*) Absence of TUNEL+ cells in the ACK45-treated HF. *H*) Positive control. Numerous TUNEL+ cells in the hair matrix of the control catagen II HF (day 17 after depilation). *I*) The comparison of the number of TRP1+ melanocytes in the hair matrix and outer root sheath between the control and anti-c-kit AB-treated anagen IV HF (mean±SE, n=4–5 animals per group, Student t test, asterisk indicates significant differences to the vehicle control, $^{**}P<0.01$, $^{***}P<0.005$). *J*) The comparison of the number of TRP2+ melanocytes in the hair matrix and outer root sheath between the control and anti-c-kit AB-treated anagen IV HF (mean±SE, n=4–5 animals per group, Student t test, asterisk indicates significant differences to the vehicle control, $^{***}P<0.005$). *K*) Percentage of the proliferating *Ki*-67+ melanocytes in the control and ACK45-treated anagen IV HF (mean±SE, n=4–5 animals per group, Student t test, asterisk indicates significant differences to the vehicle control, $^{***}P<0.005$). In panels *G* and *H*, HF are indicated by dotted line. DP, dermal papilla; HM, hair matrix; ORS, outer root sheath. Scale bar = 100 μm.

protein (molecular weight 75 kDa) as early as day 8 after depilation, compared with the control skin (Fig. 6*E*). SCF/c-kit blockade also dramatically decreased TRP1 and TRP2 expression in the hair matrix melanocytes, compared with the control HF (Fig. 6*F–I*). Therefore, these data suggest that SCF/c-kit signaling is also

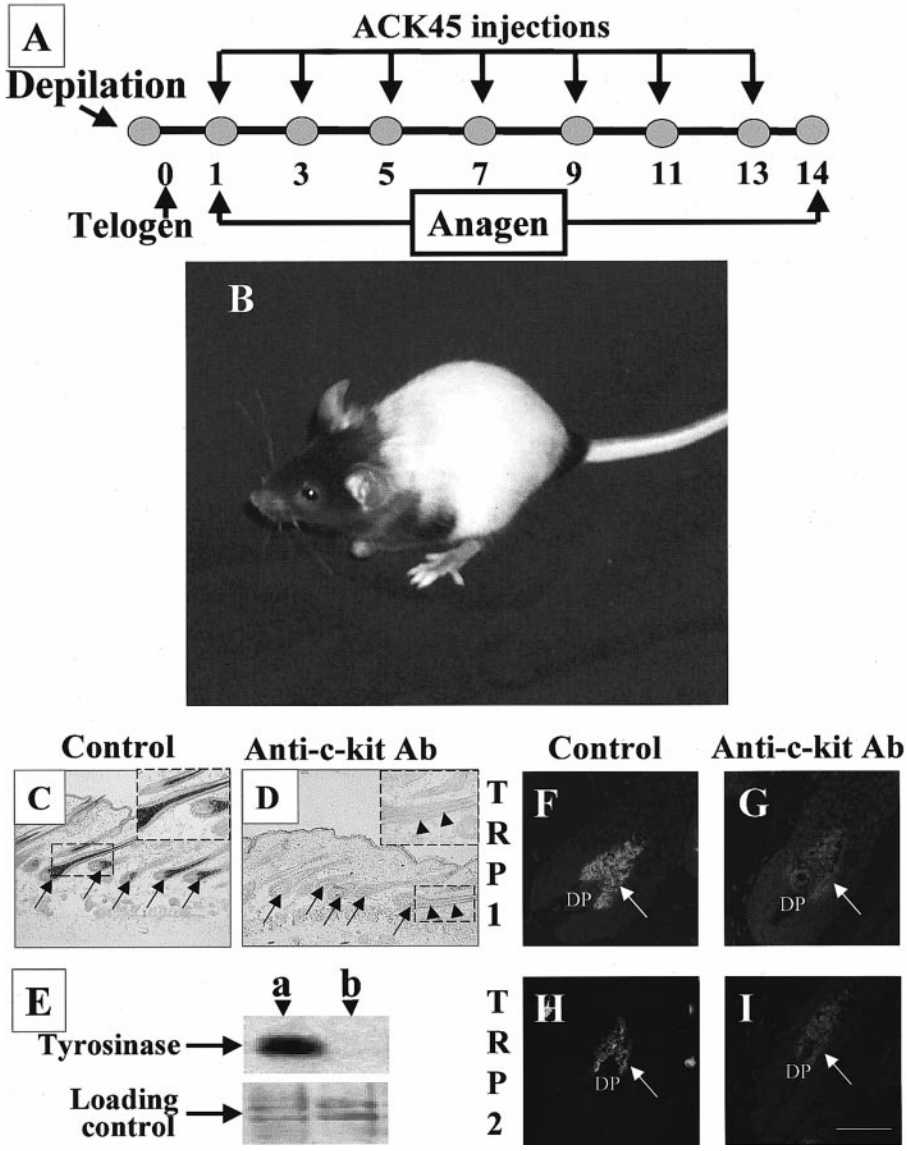

Figure 6. SCF/c-kt signaling is required for the pigment-producing activity of the melanocytes during late anagen. Hair cycle was induced by depilation in the back skin of 8-wk-old C57BL/6 mice. ACK45 or vehicle control were administered intracutaneously at days 1, 3, 5, 7, 9 and 11 after depilation A) Skin was harvested at days 8–12 after depilation. Cryostat sections were processed for the determination of endogenous alkaline phosphatase (*C, D*) or immunostained with antisera against TRP1 and TRP2 (*F–I*). Western blotting assay of the extracts of the full-thickness skin either treated with anti-c-kit AB or with vehicle control was performed to detect tyrosinase protein (*E*). *B*) Mouse treated with ACK45 shows completely depigmented white hairs over the entire back. *C*) Microscopically, HF of control mice show apparently normal hair pigmentation (arrows). *D*) HF of ACK45-treated mice do not show melanin granules in the hair bulb and depigmented hair shafts (arrows). *E*) Western blot analysis: a, control skin; b, ACK45-treated skin. *F*) Strong TRP1 expression in the hair bulb of the control HF (arrow). *G*) Down-regulation of the TRP1 expression in the hair bulb of the ACK45-treated HF (arrow). *H*) Strong TRP2 expression in the control HF (arrow). *I*) Down-regulation of the TRP2 expression in the ACK45-treated HF (arrow). DP, dermal papilla. Scale bar = 100 μm.

critical for pigment production by HF melanocytes during HF anagen development.

SCF/c-kit blockade does not affect the capacity of the hair pigmentation unit to regenerate during the subsequent hair cycle

To assess whether blockade of the SCF/c-kit signaling once affects hair pigmentation during subsequent hair cycles, a new hair cycle was induced by depilation in mice that previously received ACK45 treatment. A second depilation was performed in these mice after completion of the HF anagen-catagen-telogen transition of the previous hair cycle (i,e., on day 30 after the first depilation) (**Fig. 7*A***). To compare the color of the new and old hairs, depilation was performed only in selected areas of the back skin, and after the second depilation mice were not treated with ACK45 (Fig. 7*A*).

After the second (local) depilation, HF produced black, fully pigmented hairs, in striking contrast to the fully depigmented hairs of the previous hair cycle (Fig. 7*B*). After the second depilation, HF had completely normal patterns of expression of melanogenic proteins in follicular melanocytes, as well as normal localization of melanin granules in the HF (Fig. 7*C–D*). This suggests that blocking SCF signaling did not permanently affect the stem cell melanocytes, which repopulate the HF and regenerate the hair pigmentation unit during subsequent hair cycles.

DISCUSSION

Here, for the first time, we systematically study the expression of melanogenic proteins, SCF, and c-kit receptor in the hair cycle-associated regeneration of the hair pigmentation unit. Using transgenic and pharmacological models, we demonstrate that SCF/c-kit signaling is critically important for the HF melanocyte proliferation, differentiation, and pigment production during anagen. Furthermore, we provide here a simple and fully reversible model for programming hair depig-

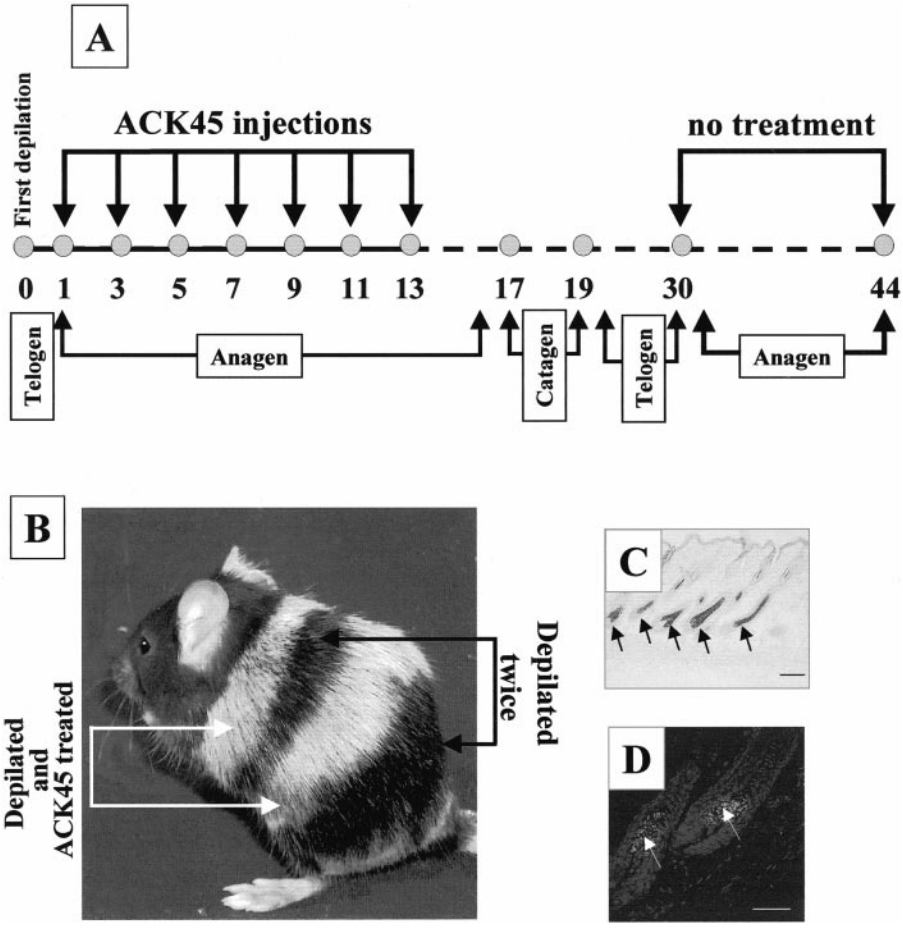

Figure 7. Blockade of the SCF/c-kit signaling does not affect the capacity of the HF to restore hair pigmentation unit during subsequent hair cycle. *A*) Hair cycle was induced by depilation in the back skin of 8-wk-old C57BL/6 mice. ACK45 was administered intracutaneously at days 1, 3, 5, 7, 9, and 11 after depilation. After the completion of HF anagen–catagen–telogen transition, hair depilation was performed again in selected areas of back skin. Mice were not treated with ACK45 after second depilation. *B*) After second depilation, HF produce new pigmented hairs in contrast to white one. *C*) Melanin granules and pigmented hair shafts in the HF after second depilation (arrows). *D*) Tyrosinase is present in the HF matrix after second depilation (arrows). Scale bar = 100 μm.

mentation that might be broadly used for the experimental pharmacological correction of hair pigmentation disturbances.

We demonstrate that during HF telogen–anagen transformation, resting melanocytes proliferate, differentiate, and migrate within the HF synchronously with regeneration and construction of HF bulb. Whereas resting melanocytes in the telogen HF may only be detected by the expression of TRP2, their transition to melanogenic competence is accompanied by the appearance of TRP1 and tyrosinase proteins (Figs. 1, 2). These data are in line with the dynamics of the expression patterns for these markers in melanoblasts and mature melanocytes during embryonic development (44). In addition, hair cycle–dependent expression of melanogenic proteins demonstrated in our study is consistent with molecular data published previously (7, 8). It was shown that only dopachrome tautomerase (TRP2) activity was detected in telogen, whereas tyrosinase, dopa oxidase activities, and gp75 protein (TRP1) become detectable in early anagen, reaching the highest activity during late anagen. (7, 8).

Based on melanogenic protein expression patterns and proliferative activity during cyclic regeneration of the hair pigmentation unit, HF melanocytes may be divided into three distinct subpopulations. The first is located in the HF bulge, expresses only TRP2, does not proliferate, and presumptively represents HF melanocyte stem cells. The second is located in the HF outer root sheath, expresses TRP2 and relatively weak TRP1, displays proliferative activity during early and mid-anagen, and represents differentiating melanocytes. The third is located in the hair matrix above the dermal papilla; expresses TRP2, TRP1, and tyrosinase; proliferates only during mid-anagen; actively produces melanin during mid- to late anagen; and progressively disappears during catagen, presumably via apoptosis and/or dedifferentiation (6, 47, 48).

We show here that the two latter melanocyte subpopulations express c-kit and show dependence on SCF/c-kit signaling during anagen. In the anagen HF, SCF is expressed by mesenchymal cells of the HF connective tissue sheath and the dermal papilla (Fig. 3) (i.e., in close vicinity to the proliferating, differentiating, and pigment-producing melanocytes; Figs. 1, 2).

We show that the HF melanocytes are maximally proliferative during early and mid-anagen, which is associated with c-kit expression on their surface (Figs. 1, 2). Overexpression of the locally produced SCF in transgenic mice (promoter: K14) (32) significantly increases the number of HF melanocytes and their proliferative activity (Fig. 4). In contrast, the short-term administration of the ACK45 antibody blocking c-kit signaling dramatically reduces melanocyte proliferation and cell number in mid-anagen HF (Fig. 5), which leads to the partial hair depigmentation. These data are consistent with previous reports demonstrating increase of the melanocyte proliferation in the early

anagen HF (6, 9, 49). This is also in line with the observation that SCF/c-kit signaling is critically important for the stimulation of melanocyte proliferation during embryonic and postnatal development (31, 50–52).

Here, we assume that SCF also promotes HF melanocyte differentiation directly or indirectly via regulation of the melanogenic protein expression. We demonstrate that overexpression of the locally produced SCF amplifies expression of TRP1, a marker of more differentiated melanocytes, in the outer root sheath, and leads to the aberrant appearance of TRP1 expression in HF bulge melanocytes (Fig. 4). This is consistent with the earlier induction of melanocyte differentiation in the HF of transgenic mice, compared to wild-type mice. Also, we show that mature melanocytes expressing c-kit in the HF matrix are strikingly dependent on SCF to maintain their pigment-producing activity. Continuous ACK45 treatment throughout anagen dramatically decreases TRP1, TRP2, and tyrosinase expression in this subpopulation of HF melanocytes and leads to the formation of completely depigmented hair (Fig. 6). These data are consistent with other reports linking regulation of the TRP1 and tyrosinase genes with SCF/c-kit signaling (53). This regulation may occur via recruitment of the Microphthalmia transcription factor (54, 55), which is known to strongly stimulate the transcriptional activities of the melanogenic protein genes (56, 57).

One group has reported that administration of an antibody blocking c-kit signaling during embryonic or neonatal development induces apoptosis in murine melanoblasts and melanocytes (58, 59). However, as we show here, no apoptotic cell death occurs in the HF melanocytes of young adult mice after ACK45 treatment (Fig. 5). This indicates that during cyclic regeneration of hair pigmentation unit, SCF/c-kit signaling is involved in the control of melanocyte proliferation, differentiation, and melanin production, rather than in the inhibiting apoptotic cell death, at least in the postnatal period.

In our study, we also demonstrate that TRP2+ melanocytes located in the HF bulge do not express c-kit receptor (Fig. 1) and, most likely, are not dependent on SCF/c-kit signaling during the anagen-coupled regeneration of the hair pigmentation unit. In telogen HF, these cells are located in the immediate vicinity of those melanocytes that co-express TRP2 and c-kit. During anagen, TRP2+ cells in the bulge do not proliferate and remain c-kit− (Figs. 1, 2). We assume that these HF melanocytes represent a stem cell-like subpopulation, most likely responsible for the restoration of hair pigmentation unit during subsequent hair cycles. It was shown previously that rapid termination of anagen-associated melanogenesis by cyclophosphamide does not prevent a new pigmentary activity during the following hair cycle (60). Recently it was shown that melanocyte stem cells in the skin of SCF transgenic mice are SCF independent (31). Our data, showing that continuous blockade of the SCF/c-kit signaling does not prevent the hair pigmentation unit from generating melanogenically active melanocytes during the next hair cycle (Fig. 7), support this concept.

However, we cannot exclude the importance of other factors, which may be required for cyclic regeneration of the hair pigmentation unit. It was shown that changes in anagen-associated melanogenesis were accompanied by changes in gene expression of MC1-R (melanocortin receptor-1) activated by POMC-derived ACTH and MSH peptides (61). Furthermore, it has been recently demonstrated that HF-associated ACTH, α-MSH, and MC1-R expressions are hair cycle dependent (62, 63). In addition, other proteins known to be involved in melanocyte biology (namely, Agouti signal protein, endothelin family, FGF2, hepatocyte growth factor), may be important for modulating the activity of HF melanocytes during hair cycle (16, 24, 52, 64, 65).

Nevertheless, pharmacological blockade of SCF/c-kit signaling by ACK45 treatment during depilation-induced hair cycles appears to represent a simple and fully reversible model for programming hair pigmentation in mice. This fully supports previous reports of depigmented hairs 8 wk after administration of anti-c-kit antibody to postnatal mice (30). However, our model allows for partial or complete hair depigmentation and, thus, for programing either gray or white hairs in mice, depending on the time course of ACK45 administration during the depilation-induced hair cycle. Finally, this depigmentation is fully reversible and allows exploration of the effects of the experimental modulators of pigmentation during several hair cycles. Therefore, this model appears suited to studies of hair pigmentation. FJ

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