Stem cell factor rescues tyrosinase expression and pigmentation in discreet anatomic locations in albino mice

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Summary
The K14-SCF transgenic murine model of variant pigmentation is based on epidermal expression of stem cell factor (SCF) on the C57BL/6J background. In this system, constitutive expression of SCF by epidermal keratinocytes results in retention of melanocytes in the interfollicular basal layer and pigmentation of the epidermis itself. Here, we extend this animal model by developing a compound mutant transgenic amelanotic animal defective at both the melanocortin 1 receptor (Mc1r) and tyrosinase (Tyr) loci. In the presence of K14-Scf, tyrosinase-mutant animals (previously thought incapable of synthesizing melanin) exhibited progressive robust epidermal pigmentation with age in the ears and tails. Furthermore, K14-SCF Tyr⁻²/c⁻² animals demonstrated tyrosinase expression and enzymatic activity, suggesting that the c2j Tyr defect can be rescued in part by SCF in the ears and tail. Lastly, UV sensitivity of K14-Scf congenic animals depended mainly on the amount of eumelanin present in the skin. These findings suggest that c-kit signaling can overcome the c2j Tyr mutation in the ears and tails of aging animals and that UV resistance depends on accumulation of epidermal eumelanin.

Introduction
Mouse models of human epidermal diseases have been limited by the fact that, unlike human skin, melanocytes are not present at the murine dermal–epidermal junction. Indeed, murine epidermis is devoid of melanocytes except for follicular melanocytes present in the dermis where they impart pigment to the fur. Because epidermal pigmentation depends on melanin production by interfollicular epidermal melanocytes and accumulation of pigment in the keratinocyte layers, the dorsal skin of mice is unpigmented, even in strains such as C57BL/6J with darkly pigmented fur. We previously reported an animal model of "humanized skin" of defined epidermal pigmentation, wherein constitutive secretion of stem cell factor (SCF) by epidermal keratinocytes resulted in

Significance
Many cases of oculocutaneous albinism are caused by mutations in tyrosinase, the rate-limiting enzyme required for synthesis of melanin. From careful observations of pigment rescue by stem cell factor (SCF) in C57BL/6J mice, we hypothesized that SCF signaling through the c-kit tyrosine kinase receptor rescued defective tyrosinase function in the setting of a classic pigment mutation, the c2j tyrosinase mutation. We found that SCF signaling up-regulated tyrosinase mRNA, protein and enzymatic activity. These findings introduce the novel concept of restoring pigmentation in tyrosinase-defective albinism pharmacologically via c-kit signaling.
the retention of large numbers of melanocytes in the interfollicular epidermal basal layer (D’Orazio et al., 2006; Kunisada et al., 1998a,b), mimicking their epidermal localization in human skin. In this transgenic model, constitutive epidermal production of SCF did not seem to affect the melanin type produced by the melanocytes. Rather, the type and quantity of melanin produced by interfollicular epidermal melanocytes paralleled that of the follicular melanocytes, determined by classic pigment-determining loci such as tyrosinase (Tyrs), the rate-limiting synthetic enzyme required for melanin production, or the melanocortin 1 receptor (Mc1r) which encodes the melanocytic G_coupled cell surface receptor on melanocytes that binds melanocyte stimulating hormone (MSH) and mediates growth and differentiation signals via cAMP second messenger production. Roughly a decade ago, Kunisada and others described the phenotype of the K14-Scf transgenic murine strain wherein stem cell factor (SCF), the ligand for the receptor tyrosine kinase c-kit, is constitutively produced by epidermal keratinocytes. As a consequence of epidermal SCF production, melanocytes were maintained in the interfollicular epidermis and imparted pigmentation to the skin itself through melanin production and transfer to keratinocytes (Kunisada et al., 1998a,b).

Using this model system, pheomelanotic and amelanotic mice were generated by introducing mutations in either Mc1r (Robbins et al., 1993) or Tyr (Le Fur et al., 1996) in otherwise congenic C57BL/6J mice. As in human skin, epidermal pigmentation in this animal model was determined mainly by epidermal deposition of varying amounts of eumelanin, the darkly colored potent UV-blocking pigment found naturally in dark-skinned individuals, and pheomelanin, a red/blonde sulfated pigment found as the dominant melanin species in the skin of UV-sensitive individuals of fair complexion. Thus, K14-Scf C57BL/6J animals with intact Tyr and Mc1r genes were found to produce abundant eumelanin in the skin and were resistant to acute and chronic UV-induced skin damage. In contrast, K14-Scf animals defective at the Mc1r locus instead produced abundant pheomelanin in the skin and albino animals lacking tyrosinase produced neither type of melanin pigment and were amelanotic (D’Orazio et al., 2006). Because pigmentation depended on the function of Mc1r and Tyr rather than the presence of stem cell factor, we concluded production of the specific melanin species in these mice was independent of c-kit (SCF receptor) signaling. Since that original report, we have noticed interesting pigmentation phenotypes that emerged as animals aged. Specifically, K14-Scf transgenic animals harboring either the Mc1r+/E or Tyr^22/E2 skin lightening mutations developed progressive epidermal darkening in the ear and tail skin. In this report, we describe the compound mutant Mc1r^+/E2 Tyr^22/E2 C57BL/6J K14-Scf animal and show that ‘albino’ animals defective at the Tyr locus, previously thought to be incapable of making melanin due to tyrosinase deficiency, demonstrate progressive and robust darkening of the ears and tail through tyrosinase-mediated melanin production in the presence of the K14-Scf transgene. We hypothesize the c2 Tyr loss-of-function mutation, classically thought to result in functional loss of tyrosinase gene expression, can be overcome by c-kit-mediated signaling. These data also suggest that c-kit signals are biochemically distinct from Mc1r signaling and cannot rescue eumelanin synthesis in the presence of a defective Mc1r.

Results

K14-Scf transgenic C57BL/6J animals (Kunisada et al., 1998a,b) were crossed with known C57BL/6J coat color variants to generate amelanotic or pheomelanotic fair-skinned K14-Scf animals as previously described (D’Orazio et al., 2006). The presence of the K14-Scf transgene did not rescue pigmentation differences caused by either the defective Mc1r or Tyr genes, as fair-skinned and albino animals harboring the K14-Scf transgene displayed a similar pigmentation phenotype to their non-transgenic counterparts (Figure 1). Thus, tyrosinase-null animals appeared amelanotic despite the presence of the K14-Scf transgene, and Mc1r defective animals appeared pheomelanotic regardless of whether the K14-Scf transgene was present. Our original model included wild type (Mc1r^+/E2, Tyr^+/+), extension (Mc1r^+/E, Tyr^+/+) and albino wild (Mc1r^+/E2, Tyr^22/E2) strains, and therefore was unable to distinguish Mc1r-mediated responses independent of pigment effects. Specifically, if pheomelanin contributes to UV-mediated damage as others have suggested (Hill and Hill, 2000; Samokhvalov et al., 2005; Takeuchi et al., 2004; Ye and Simon, 2003), then the presence of pheomelanin might confound assessment of other Mc1r-mediated cutaneous responses. Thus, to distinguish pigment-independent aspects of Mc1r function, we generated a K14-Scf transgenic animal system defective at both the Mc1r and Tyr loci. We now report interesting pigmentation phenotypes that emerged in this murine model system.

In all four genotype crosses (wild type Mc1r^+/E2, Tyr^+/+, extension Mc1r^+/E, Tyr^+/+, albino wild Mc1r^+/E2, Tyr^22/E2, and albino extension Mc1r^+/E, Tyr^22/E2) we noted little difference in fur color between K14-Scf transgenic and their non-transgenic counterparts. However, differences in skin color were observed at one or more anatomic sites between K14-Scf animals and their Mc1r and Tyr-matched non-transgenic equivalents. The effect was most pronounced in the wild type (Mc1r^+/E2, Tyr^+/+) background wherein non-transgenic animals exhibited little pigmentation of the skin whereas K14-Scf transgenic animals had jet-black skin throughout the epidermis (Figures 1 and 2). In otherwise congenic animals, loss of Mc1r function caused loss of brown/black pigmentation of both the fur and
Typic differentiation of the K14-Scf animals from their non-transgenic counterparts. Notably, the same discreet areas of hyperpigmentation were noted in very young K14-Scf wild type animals (Figure 2, red triangles), but gradually the distinction between these sites and the rest of the epidermis was lost due to the generalized deposition of eumelanin in the epidermis that occurred in the first to second week of life in the setting of fully functional Mc1r and Tyr genes.

When K14-Scf transgenic Tyr-defective animals were compared, we noted distinct differences in skin pigmentation depending on Mc1r status. Despite the fact that neither strain (Mc1r^{E/E}, Tyr^{2/2}/c2) or Mc1r^{E/e}, Tyr^{2/2}/c2) should have been capable of producing melanin due to the c2j Tyr mutation, we noticed that the skin of the ears and tails of K14-Scf animals darkened in both strains over time (Figure 1). This epidermal darkening was never observed in K14-Scf negative Tyr^{2/2}/c2) animals (Figure 2), suggesting a SCF-mediated requirement for epidermal melanocytes. Furthermore, there seemed to be a difference in the nature of the pigmentation between MC1r-intact and MC1r-defective K14-Scf albino animals as they aged. Specifically, the ears and tails of K14-Scf albino wild type animals actually blackened over time, whereas the ears and tails of K14-Scf albino extension animals became more pigmented in reddish/brown hues, though more faintly, with age (Figure 1). In albino wild type (Mc1r^{E/E}, Tyr^{2/2}/c2) K14-Scf animals, pigmentation of the ears and tail skin first became apparent at roughly 3 weeks of life, displayed progressive darkening over the next several months, and became maximally and intensely dark at roughly 8–12 months of life (Figure 1). These observations suggested to us that c-kit signaling in melanocytes could rescue tyrosinase expression in the setting of the loss-of-function c2j mutation in discreet anatomic locations (ears and tail but not dorsal skin).

To determine whether K14-SCF-induced skin darkening was due to melanin deposition, sections of the skin from different anatomic sites from each of the four murine strains were stained for melanin using the Fontana–Masson method, which stains melanin pigments black in section (Zappi and Lombardo, 1984). Wild type K14-Scf transgenic animals, which are the most heavily pigmented animals among the four groups, revealed abundant epidermal melanin throughout the epidermis (Figure 3). In contrast, fair-skinned extension K14-Scf animals exhibited reduced melanin staining, and Tyr-defective animals had negligible levels of either melanin subtype in their dorsal skin regardless of Mc1r status. When skin sections from the ears and tails of albino K14-Scf animals were stained for melanin by the Fontana–Masson method, we found clear evidence of melanin deposition (as evidenced by black staining) in these anatomic sites as animals aged (Figure 3, white arrows). Melanin staining was much more intense in the ears and tails of aged albino K14-Scf animals with a wild type Mc1r when compared with their Mc1r-defective

![Figure 1. Epidermal pigmentation of C57BL/6J mice variant for the Mc1r and Tyr loci and the K14-Scf transgene: facial characteristics. Animals are otherwise congeneric on the C57BL/6J genetic background. Each photo is a representative image of the particular strain at the specified age. Note the differences in epidermal pigmentation (as can be seen by the skin over the ears and paws) between K14-Scf transgenic and non-transgenic animals are most notable after 1 month of age. The presence of the K14-Scf transgene can be assessed visually in pigmented (i.e., tyrosinase-functional) strains by dark nose spots from day of life 1 (red triangles). K14-Scf animals with wild type pigmentation demonstrate darkly pigmented epidermis (white triangles), and K14-Scf mice of all coat colors develop progressive pigmentation of the ears by 8–12 months of age (blue triangles).](image-url)
counterparts (Figure 3), suggesting that the nature of the melanin deposited may be different between the two strains (i.e., accumulation of pheomelanin in the ears and tail of Mc1r-defective strains, and deposition of eumelanin when Mc1r is functional).

To test this hypothesis, we directly measured melanin type in the skin of the animals, determining the levels of eumelanin and pheomelanin in depleted ear, nose, and tail via HPLC analysis (Ito, 1993). There was abundant eumelanin in the skin of the ears, dorsalum and tail in wild type \(\text{Mc}1^{E/E}, \text{Tyr}^{+/+}\) K14-Scf transgenic animals and eumelanin accumulated with increased age of the animals (Figure 4A). Furthermore, \(\text{Mc}1^{E/E}, \text{Tyr}^{+/+}\) animals demonstrated accumulation of pheomelanin in the skin, with the highest levels in the ears. Unlike the age-dependent eumelanin deposition
observed in wild type strains, however, higher levels of pheomelanin were observed in young adult animals when compared with their older counterparts (Figure 4A). Although the skin of the ears and tails of albino wild (McTyr+/−, Tyr+/−) K14-Scf ears/tail clearly demonstrated age-dependent pigmentation both visually (Figure 1) and by Fontana–Masson melanin staining (Figure 3), we found no appreciable chemical evidence of mature eumelanin or pheomelanin in skin biopsies from these anatomic locations by the HPLC method (Figure 4A) that extrapolates eumelanin and pheomelanin levels by pyrrole-2,3,5-tricarboxylic acid (PTCA) and 4-amino-3-hydroxyphenylalanine (4-AHP) levels, respectively. Nonetheless, spectrophotometric analysis of alkaline lysates of ear skin from older (10–12 month old) K14-Scf wild type and albino animals suggested the presence of melanin pigments especially in Mc1r-intact albino animals (Figure 4B). Together, these data imply that c-kit signaling rescues pigment production in discreet anatomic locations (ears, tail) in animals carrying the tyrosinase c2) mutation but that the chemical nature of the pigment produced may be distinct from conventional melamins made by intact tyrosinase.

We next investigated the possibility that the loss of tyrosinase affected melanocyte viability in the skin, utilizing the DCT-LacZ transgene incorporated into our system (Mackenzie et al., 1997) to readily identify melanocytes in skin sections. We found that the inactivating c2) Tyr mutation had little effect on melanocyte accumulation in the epidermis, as evidenced by abundant blue-stained melanocytes in the interfollicular stratum basale from both extension and albino extension animals (Figure 5). Non-transgenic animals demonstrated no localization of melanocytes to the interfollicular epidermis.

As albino animals showed progressive darkening of the skin (ears, tail) with age, we hypothesized that tyrosinase expression might increase in the skin as animals aged. To determine whether tyrosinase expression increased with age, we performed Western analysis on ear skin samples from young versus older albino animals to determine tyrosinase protein expression in this system over time (Figure 6A). We found clear evidence that tyrosinase protein levels were higher in aged albino animals than in their genetically matched younger counterparts, and that non-transgenic albino animals that lacked
the skin of aged mice could be due to SCF-mediated increased tyrosinase expression over time or be simply due to an increase in melanocyte numbers. To differentiate between these two possibilities, we compared expression of tyrosinase with β-galactosidase (hypothesizing that DCT-LacZ expression would reflect total melanocyte number) in young versus aged doubly transgenic K14-Scf and DCT-LacZ Myc1r<sup>F/E</sup> Tyr<sup>c2j/c2j</sup> animals. As shown in Figure 6B, we noted a clear age-dependent induction of tyrosinase independent of DCT-LacZ expression (i.e., melanocyte number). Together, these data argue that the c-kit receptor tyrosine kinase upregulates tyrosinase expression in melanocytes independent of effects on melanocyte accumulation. To clarify whether tyrosinase activity was responsible for pigment production seen in the ears and tail of K14-Scf albino wild and albino extension animals as they aged, we assessed the skin for tyrosinase activity using the tyramide-based tyrosinase assay (TTA) (Han et al., 2002). The TTA is an in situ test for tyrosinase activity based on tyrosinase-mediated in situ deposition of biotin subsequently detected with a streptavidin-conjugated fluorescent dye. Thus, to yield a fluorescent signal, cells must express functionally active tyrosinase. Although the most robust TTA responses were clearly found among biopsies from fully pigmented wild type (Myc1r<sup>F/E</sup>, Tyr<sup>c2j/c2j</sup>) K14-Scf strains, we found unambiguous evidence of functional tyrosinase activity in the epidermis of ears and tails (but not dorsal skin) of K14-Scf albino animals regardless of Myc1R function (Figure 6C). There was no evidence for tyrosinase activity in biopsies from non-transgenic animals, regardless of Myc1R or Tyr status, again demonstrating the requirement for the K14-Scf transgene to support functional melanocytes in the interfollicular epidermis. From these observations, we conclude that the c2j loss-of-function mutation of the Tyr gene can be rescued at least in part by c-kit signaling in vivo. Furthermore, as there was tyrosinase present in both the albino wild type and the albino extension K14-Scf animals, we conclude that loss of Myc1R function does not inhibit tyrosinase expression or enzymatic activity. Because our in vivo data suggested that SCF-induced tyrosinase activity independent of its effect on melanocyte survival, we turned to an in vitro system to directly test whether SCF induces tyrosinase expression and activity. Using B16 murine melanoma cells (Myc1r<sup>F/E</sup>, Tyr<sup>c2j/c2j</sup>), we found that recombinant murine SCF promoted increased levels of tyrosinase protein (Figure 6D). Furthermore, in these cells, SCF potently induced tyrosinase enzyme activity as determined by robust DOPA staining (Figure 6E) and increased melanin accumulation (Figure 6F). Thus, tyrosinase protein levels and enzyme function are induced by SCF regardless of Myc1R status or the c2j tyrosinase mutation.

Lastly, we tested the UV sensitivity of our K14-Scf strains to determine the extent to which Myc1R and Tyr independently affect UV sensitivity in our animal model.
of humanized skin. We exposed cohorts of K14-Scf and non-transgenic Mct1-intact and Mct1-mutant animals to UV radiation, and determined the minimal dose that caused inflammation of the dorsal skin 24 h later. We found that eumelanin accumulation in the skin rather than Mct1r or Tyr status afforded significant photoprotection (Figure 7). Thus, wild type (Mct1r/E, Tyr1c2) K14-Scf animals with their eumelanin-laden epidermics were much more resistant to UV-induced inflammation than any other strain. The contribution of epidermal melanocytes (and their pigment) to UV protection is clearly demonstrated by the fact that wild type (Mct1r/E, Tyr1c2) non-transgenic animals (who lack epidermal melanin; Figure 3) exhibited similar UV sensitivity as Mct1r- or tyrosinase-defective strains. As there was no appreciable difference in the UV sensitivity as measured by minimal erythematous dose (MED) between extension animals (with abundant pheomelanin in the dorsal skin) and albino strains (that lack melanin pigments altogether in the dorsal skin), we conclude that pheomelanin is a very poor blocker of UV radiation (Figure 7). Furthermore, pigment-independent Mct1r effects seemed to have little contribution to UV-induced cutaneous inflammation in our model as albino wild (Mct1r/E, Tyr1c2) and albino extension (Mct1r/E, Tyr1c2) K14-Scf strains displayed similar UV sensitivities.

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genetic variants of this transgenic murine model: (1) wild type Mct1^{P/E}, Tyr^{1+/4}, (2) extension Mct1^{P/E}, Tyr^{+/-}, (3) albino wild Mct1^{P/E}, Tyr^{P2/12/c2} and (4) albino extension Mct1^{P/E}, Tyr^{P2/12/c2} animals. We found that the presence of the K14-Scf transgene promoted interesting pigmentation phenotypes among these strains. First, we noted distinct areas of hyperpigmentation, including the entrance to the nares, the footpads, the ears and the genitalia, among tyrosinase-intact strains. We also observed consistent K14-Scf-mediated age-dependent pigmentation of the skin of the ears and tail in albino (Ty^{P2/12/c2}). Interestingly, these are the same anatomic locations in which melanocytes can be found in the epidermis in scant numbers in normal, non-transgenic animals (Rosenthal, 1979). Together, these data suggest that there may be a pro-melanocyte survival factor or a pigmentation signal distinct from MSH-Mct1r cAMP signaling in these sites. Although the factor(s) that promotes darkening in the ears and tail in our transgenic model remain to be elucidated, our data indicate that melanin synthesis is not dependent on the Mct1r/cAMP axis, as the ears and tails of the K14-Scf animals darkened both in the presence and absence of Mct1r function.

Constitutive SCF production in the epidermis as contributed by the K14-Scf transgene also rescued melanin production in animals harboring the c2j Tyr mutation that was previously thought incapable of melanin production because of tyrosinase deficiency. Specifically, the Mintz group reported that the c2j mutation consisted of a G → T point mutation at nucleotide 291 that resulted in an arginine → leucine substitution in codon 77 and a change of the gene’s splicing site, causing no change in mRNA levels but much reduced tyrosinase protein instability (Le Fur et al., 1996). Our transgenic K14-Scf Ty^{P2/12/c2} animals displayed overt pigmentation of the epidermal skin of the ears and tail (but not dorsal skin or fur) as they aged. We found clear evidence of age-dependent and K14-Scf-dependent tyrosinase protein expression and functional activity in the stratum basale of ears and tail of K14-Scf Ty^{P2/12/c2} animals, strongly suggesting that c-kit signaling somehow rescued the tyrosinase defect caused by the c2j mutation. Using B16 murine melanoma cells, we found SCF-mediated upregulation of tyrosinase protein expression (by Western blotting) as well as induction of tyrosinase function as determined by DOPA staining and increased cellular pigmentation, further supporting a role for SCF-mediated tyrosinase and pigment induction in melanocytes. The SCF/c-kit-mediated induction of tyrosinase seemed to be independent of melanocyte accumulation and could be demonstrated in vitro in B16 melanocytes.

c-kit is a receptor tyrosine kinase found on a wide variety of cells, including melanocytes and their precursors. Functional interaction of SCF with c-kit is critical to the survival of melanocytes, and the c-kit pathway has been implicated in UV-induced pigmentation (Hachiya et al., 2001) and in melanoma oncogenesis (Smalley et al.,

Discussion

The K14-Scf transgenic murine model is a useful model in which to study pigment and melanocyte responses in the epidermis. Because of constitutive production of SCF by keratinocytes in the basal layer of the epidermis in this model, melanocytes are recruited to the interfollicular stratum basale, similar to their epidermal location in normal human skin. In this manner, melanocytes produce melanin and transfer the pigment to adjacent keratinocytes in the epidermis, imparting color to the skin of K14-Scf mice and facilitating studies of UV responses in skin. Furthermore, as pigmentation in this system is dictated by the melanocortin 1 receptor (Mct1) or tyrosinase (Tyr) and all strains are on a pure C57BL/6J genetic background, this model is a practical means to study cutaneous UV responses in epidermis of variant melanin composition in an otherwise congenic system. In this report, we describe the pigmentation phenotype of four
SCF binding to c-kit causes homodimerization and auto-tyrosine phosphorylation that creates docking sites for a number of Src-homology 2 (SH2)-containing signal transduction molecules. Mutations in c-kit that reduce the receptor tyrosine kinase activity cause deficiencies in melanoblast migration and proliferation as demonstrated by piebaldism (melanocyte deficiency), the clinical consequences of c-kit deficiency (Giebel and Spritz, 1991; Yoshida et al., 2001). c-kit activation stimulates a host of downstream pathways including PI3-kinase/AKT (Blume-Jensen et al., 1998; Feng et al., 2000), PLC-γ (Maddens et al., 2002), Src kinase (Timokhina et al., 1998), JAK/STAT (Deberry et al., 1997) and MAP kinase pathways (Funasaka et al., 1992). In addition to these downstream signaling and transcriptional pathways, c-kit signaling upregulates the master melanocytic differentiation and survival transcription factor Mitf through the MAP kinase pathway and phosphorylation of Mitf itself (Hemesath et al., 1998; Wu et al., 2000). Although, we have shown that SCF induces tyrosinase in the presence or absence of the c2) loss-of-function mutation, further work must be performed to identify the specific molecular pathways involved in SCF-mediated tyrosinase activation.

As pigmentation and tyrosinase activity were observed in Mc1r-intact or -deficient mice, SCF-mediated tyrosinase rescue must be independent of Mc1r signaling. Furthermore, Mc1r function seemed to influence the type of pigment produced as the nature of the coloration of the ears and tails differed among Mc1r−/− and Mc1r+/− K14-Scf Tyr²⁰/²⁰ animals (i.e., dark pigment in the Mc1r-intact state). Intriguingly, while Fontana–Masson staining indicated melanin deposition in the ears and tails of Tyr²⁰/²⁰ K14-Scf animals (Figure 3) and alkaline lysis of ears from aged K14-Scf animals showed increased pigment that absorbed spectrophotometrically at 470 nm, HPLC analysis revealed no appreciable accumulation of eumelanin or pheomelanin pigments therein as defined by conventional degradation studies based on formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) by permanganate oxidation of eumelanin and 4-amino-3-hydroxyphenylalanine (4-AHP) by hydriodic acid reductive hydrolysis of pheomelanin. This is a perplexing experimental result, and further investigations must be carried out to clarify these discrepancies, however, these findings may suggest that the chemical nature of SCF-induced melanin pigments in the ears and tails of albino (Tyr²⁰/²⁰) animals may be of a distinct chemical composition when compared with mature eumelanin and pheomelanin produced in the tyrosinase-intact state. Tyrosinase is reported to catalyze the first two steps of the biosynthesis of either eumelanin or pheomelanin, namely conversion of tyrosine to DOPA and then oxidation of DOPA to dopaquinone. If the chemical nature of melanin pigment(s) deposited in the presence of a c2)-mutant tyrosinase differs from conventional eumelanin or pheomelanin, then this finding might imply a hitherto unappreciated role for tyrosinase in later biosynthetic steps of melanogenesis. As the HPLC methods used in this study do not discriminate between melanin intermediates and mature melanin, the difference in melanin composition may be one explanation, however other possibilities (e.g., distribution of melanosomes) must also be considered. Nonetheless, because pigmentation of the skin of the ears and tail of K14-Scf Mc1r−/− Tyr²⁰/²⁰ mice was visually much darker than in K14-Scf Mc1r−/− Tyr²⁰/²⁰ animals (as well as by A่าv), it seems likely that the nature of the SCF-induced pigment produced by c2)-mutant tyrosinase may depend on the function of the melanoctlin 1 receptor (as it does in the tyrosinase-intact state).

Despite the presence of abundant melanocytes in the basal layer of the dorsal skin of the K14-Scf albino animals, we did not observe age-related darkening in the dorsal skin, regardless of Mc1r status. Only melanocytes found in the ears and tail produced epidermal melanin with age (Figures 1 and 2), suggesting that there may be additional factor(s) in the skin of the ears and the tail that promote tyrosinase expression/activity in addition to c-kit signaling. Whether these are the same factor(s) that promote accumulation of interfollicular melanocytes in the absence of K14-Scf remains to be determined. However, as epidermal interfollicular melanocytes are present in the basal layer in each of the four animal strains studied herein, we conclude neither Mc1r nor tyrosinase function is required for de facto melanocyte survival.

Lastly, we compared UV sensitivity of the four K14-Scf strains of animals to determine whether Mc1r-mediated UV protection was pigment-dependent in our animal model. By measuring the minimal erythematous dose (MED) of the dorsal skin in response to UV radiation (Mackenzie, 1983; Sayre et al., 1981), we directly tested the ability of eumelanin or pheomelanin to protect the skin against acute inflammatory effects of UV in otherwise congenic animals. Our data revealed that epidermal eumelanin was profoundly UV-protective whereas pheomelanin did little to prevent UV-induced inflammation. In fact, the UV sensitivity of pheomelanic skin (in extension K14-Scf animals) was essentially identical to that of Mc1r-matched amelanotic skin (albino extension K14-Scf animals; Figure 7), suggesting that the UV sensitivity of Mc1r-defective skin occurs mainly because of lack of eumelanization rather than non-pigment Mc1r effects. Some have argued that the presence of pheomelanin in the skin, produced in part as a result of defective Mc1r signaling, may exacerbate UV-mediated cutaneous injury by promoting reactive oxygen species (Hill and Hill, 2000; Smit et al., 2008; Takeuchi et al., 2004). Although we have not directly measured UV-induced reactive oxygen damage in our animals, our MED data argue that pheomelanin-containing skin is equally sensitive to UV radiation as...
amelanotic skin, at least as measured by global inflammation 24 h after UV exposure in this animal model. Nonetheless, due to the poor UV-protective properties of pheomelanin, we posit that approaches that upregulate eumelanin in the skin, for example by topical application of either MSH analogs (Abdel-Malek et al., 1995, 2006) or by pharmacologic manipulation of cAMP signaling (D’Orazio et al., 2006), would likely result in enhanced cutaneous UV protection. Nonetheless, these data provide a rationale for possible translational induction of pigmentation of tyrosinase-defective individuals (e.g., patients with certain forms of oculocutaneous albinism) by c-kit activation. Future experiments are planned to investigate whether SCF-induced pigmentation is UV-protective in our animal model. Overall, we conclude that the K14-SCf congenic model of variant pigmentation is useful to study UV effects in the skin that c-kit-mediated signals partially overcome the pigmentation defect caused by the c2j tyrosinase mutation and that Mc1r protects against UV-mediated inflammation mainly by promoting eumelanin deposition in the setting of functional pigment synthesis.

Materials and Methods

Animals
C57BL/J mice of varying pigment phenotype were crossed with K14-SCf transgenic animals (also on the C57BL/6 background) as described previously (D’Orazio et al., 2006; Kunisada et al., 1998a,b). The pigmentation phenotypes used were: C57BL/J Mc1r+/+ Tyrc−/− (wild type, black pigmentation), C57BL/J Mc1r+/− Tyrc−/− (extension mutant, blonde pigmentation), and C57BL/J Mc1r+/− Tyrc+/+ (albino wild, non-pigmented). Presence of the K14-SCf transgene was assessed either by phenotype (obvious skin color characteristics in the case of wild type or extension animals) or, in the albino animals, by PCR amplification of a fragment specific to the K14-SCf transgene (DNA obtained by tail snip), as described (Kunisada et al., 1998a). Transgenic dopachrome tautomerase-β-galactosidase mice (Mackenzie et al., 1997) were obtained from Dr. Ian Jackson’s Laboratory (Edinburgh, UK). All experiments were carried out in accordance with institutionally approved animal protocols.

Reagents
Recombinant murine stem cell factor (SCF) was purchased from Chemicon Millipore (Billerica, MA, USA). l-DOPA (3,4-Dihydroxyphenyl-l-alanine) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Melanin staining, β-galactosidase melanocyte quantification, and the tyramide tyrosinase assay (TTA)

Animals were either killed by CO2 narcosis or anesthetized with isoflurane anesthesia prior to skin sampling. Approximately 1 cm2 skin biopsies were obtained from sheared skin using institutionally approved protocols. For Fontana-Masson melanin staining, samples were fixed in 10% buffered formalin (Sigma) and were paraffin embedded and sectioned (6 microns) by the University of Kentucky histopathology core laboratory before being stained for melanin using the Fontana-Masson Staining Kit (American Master*Tech Scientific, Inc., Lodi, CA, USA) (Zappi and Lombardo, 1994), which stains mel-
nin black. For LacZ-mediated identification of melanocytes in DCT-LacZ transgenic animals and the TTA assay, frozen sections (6 microns) were prepared. β-galactosidase staining and counterstaining with nuclear fast red were performed as described (Franco et al., 2001; Mackenzie et al., 1997), and numbers of blue-stained cells were quantified. For in situ TAA-mediated determination of tyrosinase activity, frozen sections were treated with avidin/biotin blocking reagents (Vector Laboratory, Burlingame, CA, USA) and stained for tyrosinase using Perkin Elmer’s Tyramide Reagent Pack as described (Han et al., 2002). Microscopic evaluation of skin biopsies was performed using an Olympus BX51 microscope and images were captured using the QCapture Pro program (QImaging Software, Surrey, BC, Canada). For whole mount β-gal stained, images were taken with a dissecting scope (Olympus SZ61) before sectioning.

Western blotting

In the case of whole skin biopsies, skin sections were taken and flash frozen in liquid nitrogen. Biopsies were then homogenized and lysed in lysis buffer (50 mM HEPES pH 7.5, 1% nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 0.1 mM sodium orthovanadate) with protease inhibitors (CompleteMini™ Roche Diagnostics, Indianapolis, IN, USA). For B16 cells, cells were washed and lysed in the aforementioned lysis buffer, SDS-PAGE and immunoblot analysis was then performed by conventional methods (30 μg protein per lane). Bands for tyrosinase and GAPDH (loading control) were identified by polyclonal rabbit anti-mouse tyrosinase (clone H-109, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal anti-GAPDH (Ambion, Foster City, CA, USA), respectively with appropriate peroxidase-labeled secondary reagents and developing with ECL Pls (Amersham, Buckinghamshire, UK).

DOPA staining

B16 mouse melanoma cells were grown to 50–70% confluence and were incubated (37°C, 5% CO2) either with media alone (DMEM + 10% FCS + glutamine) or with media containing recombinant stem cell factor (5 ng/ml) for 48 h. Cells were then washed in PBS, fixed in 4% formaldehyde (4%), incubated in l-DOPA (10 mM in PBS pH 7.3) for 2 h and photographed (magnification 100×) using an Olympus BX51 microscope (Center Valley, PA, USA) with image capture using the QCapture Pro program (QImaging Software).

Melanin quantification

(A) NaOH-based spectrophotometric method (Yoshida et al., 2007): 1 × 106 B16 cells were solubilized in 1N NaOH/10% DMSO for 2 h at 80°C. Spectral absorbances of supernatants were measured (470 nm) as an estimation of the amount of melanin present in each sample. In other experiments, whole depilated skin samples were lysed and data are described as A470 per mg tissue weight. (B) HPLC-based direct quantification: Eumelanin and pheomelanin were quantitatively analyzed by HPLC based on the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) by alkaline hydrogen peroxide oxidation of eumelanin and 4-amino-3-hydroxyphenylalanine (4-AHP) by hydriodic acid reductive hydrolysis of pheomelanin, respectively. PTCA determination was performed by a method modified from a previous report (Wakamatsu et al., 2003). The eumelanin and pheomelanin content were calculated by multiplying those of PTCA and 4-AHP by factors of 25 and 9, respectively (Wakamatsu and Ito, 2002).

gRT-PCR

Ear biopsies were taken from K14-SCF young (1 month), young adult (5 months), and aged adult (12 months) doubly transgenic K14-SCf and DCT-LacZ Mc1r+/− Tyrc−/− animals and flash frozen in liquid nitrogen. RNA was extracted using Trizol (Invitrogen, Carls-
bad, CA, USA) after homogenization and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using SuperScript III First Strand Synthesis Super Mix for qRT-PCR (Invitrogen). Primers and corresponding probes were designed using the Universal Probe Library (Roche, Basel, Switzerland). Primers for tyrosinase were ttgctcctcaatcttc (position 823–843) and tgtctctgtgctcagcttc (877–899), primers for DCT-LacZ were ttcgtctctctctcgttcctc (702–719) and ctctctctctctctc (744–764). Multiplex reactions were performed using a FAM-labeled probe to the gene of interest and a Y555-labeled probe to the β-actin reference gene (UPL Mouse ActB Gene Assay; Roche). Results (normalized to actin B expression) are shown as fold change compared with levels in 1 month old animals.

**Minimal erythematous dose (MED) determination**

Animals of the indicated genetic status were depilated with surgical shears and topical depilatory cream (Nair®; Church & Dwight Co., Inc., Lakewood, NJ, USA) used as directed 1 day prior to irradiation. Mice were then sedated with ketamine/xylazine according to standard veterinary dosing (Xu et al., 2007) and UV-occlusive tape with holes punched in it was applied to the dorsal skin to facilitate multiple UVB dosing on the same animal. Mice were exposed to UV irradiation in a custom-made lucite chamber (Plastic Design Corporation, MA, USA) outfitted with a double bank of UVB lamps (UV Products, Upland, CA). The tube lamps used were UVP product no. 34-0098-01, and were 15 W T8 mercury bulbs that emit at 302 nm. The UV spectral chart provided by the company shows a bell-shaped emittance curve with energy emitted from between roughly 280–375 nm with a peak at 302 nm. UV emittance was measured with the use of a UV photometer (UV Products). Accordingly, we find the spectral output of the lamps to be roughly 75% UV-B (290–320 nm) and 25% UV-A (315–400 nm). Delivered doses are calibrated for UVB emittance. Erythema and/or erythema of the UV-exposed areas was scored visually 24 h after irradiation, and the minimal erythematous dose (MED) was calculated as the minimal dose of radiation needed to cause erythema and/or edema of the entire circle of exposed skin.

**Statistical analysis**

Statistical comparisons of MED between cohorts of animals were evaluated by a Tukey’s post-test. Differences were considered statistically significant if the P-value was <0.05.

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**References**


