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Cell Stem Cell. Author manuscript; available in PMC 2012 September 10.

Published in final edited form as:
Cell Stem Cell. 2010 February 5; 6(2): 130–140. doi:10.1016/j.stem.2009.12.010.

Key Roles for Transforming Growth Factor β in Melanocyte Stem Cell Maintenance

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SUMMARY

Melanocyte stem cells in the bulge area of hair follicles are responsible for hair pigmentation, and defects in them cause hair graying. Here we describe the process of melanocyte stem cell entry into the quiescent state and show that niche-derived transforming growth factor β (TGF-β) signaling plays important roles in this process. In vitro, TGF-β not only induces reversible cell cycle arrest, but also promotes melanocyte immaturity by downregulating MITF, the master transcriptional regulator of melanocyte differentiation, and its downstream melanogenic genes. In vivo, TGF-β signaling is activated in melanocyte stem cells when they reenter the quiescent noncycling state during the hair cycle and this process requires *Bcl2* for cell survival. Furthermore, targeted TGF-β type II receptor (*TGFbRII*) deficiency in the melanocyte lineage causes incomplete maintenance of melanocyte stem cell immaturity and results in mild hair graying. These data demonstrate that the TGF-β signaling pathway is one of the key niche factors that regulate melanocyte stem cell immaturity and quiescence.

INTRODUCTION

Somatic stem cells are generally present in a special cellular organization called the niche (Watt and Hogan, 2000; Li and Xie, 2005). In a niche, stem cells are able to self-renew with high probability and they divide infrequently. The important roles of the niche for stem cell maintenance have been demonstrated in several stem cell systems (Scadden, 2006; Nystul and Spradling, 2006; Nishimura et al., 2002). Genetic studies have revealed a variety of

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.stem.2009.12.010.

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genes essential for stem cell regulation, especially in *Drosophila* germline stem cells, mouse hematopoietic stem cells, and epidermal stem cells. However, the intricate molecular mechanisms that underlie maintenance of stem cells by the surrounding niche environment are not well understood in many vertebrate stem cell systems, at least partly because of the difficulties in identifying and visualizing stem cells and their niches in tissue structures.

Melanocyte stem cells have been identified in a distinct tissue structure called the bulge (Nishimura et al., 2002), an area that is mostly populated by follicular keratinocyte stem cells (Cotsarelis, 2006; Tumbar et al., 2004) and that lies on the basement membrane to which the arrector pili muscle is attached. Our previous study demonstrated that the niche plays a dominant role in melanocyte stem cell fate determination (Nishimura et al., 2002). Indeed, the melanocyte stem cell compartment becomes regionally segregated away from the hair matrix where the amplifying and differentiating cell compartment resides after stem cell division (a schematic is shown in Figure 1L). Thus the different fates of the stem cell compartment and the amplifying and differentiating compartments can be explained by stem cell niche-specific cues in the bulge area and/or by activating signals from the maturing cell niche, the hair matrix in hair follicles.

We previously identified two genes, *Bcl2* and *Mitf*, that are essential for melanocyte stem cell maintenance (Nishimura et al., 2005). Melanocyte stem cells are usually maintained in a quiescent (noncycling) state and are activated only at early anagen (the growing phase) to undergo stem cell division. *Bcl2* is essential for the survival of melanocyte stem cells at the stage where they return to the quiescent state (Nishimura et al., 2005). *Mitf,* the master transcriptional regulator of melanocyte development (Steingrímsson et al., 2004; Levy et al., 2006), is also essential for melanocyte stem cell maintenance by preventing premature differentiation/pigmentation of melanocyte stem cells in the niche (Nishimura et al., 2005). However, extrinsic signals that act dominantly upon the melanocyte stem cell niche have not been thus far identified.

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that plays important roles in the induction of apoptosis, cellular growth arrest, and other physiological and pathological responses (Siegel and Massagué, 2003; Massagué and Gomis, 2006). The role of TGF-β in stem cell quiescence has been suggested in hematopoietic stem cells (Batard et al., 2000; Larsson et al., 2003; Karlsson et al., 2007; Yamazaki et al., 2007), prostate stem cells (Kim et al., 2004), and neural stem cells (Falk et al., 2008) with compelling supportive evidence, although in vivo evidence for the indispensability of TGF-β in stem cell maintenance is still lacking. There are three mammalian isoforms of TGF-β (1, 2, and 3) that signal through type I and type II TGF-β receptors. Signaling can occur only in the presence of both types of receptors and their downstream intracellular mediators (Smad2/3) that subsequently enter the nucleus where transcription of their target genes is regulated. In the skin, TGF-βs are expressed by the regressing hair follicle strand and their role in hair follicle regression through the induction of apoptosis has been well established (Foitzik et al., 2000; Soma et al., 2003). Recent gene expression profiling studies of epidermal label-retaining keratinocytes in the hair follicle bulge region suggest that TGF-β signaling is active in hair follicle keratinocyte stem cells (Tumbar et al., 2004) that reside together in the niche microenvironment with melanocyte stem cells. The significant expression of TGF-β-induced factors in the bulge area and the growth inhibitory functions of TGF-β (Massagué and Gomis, 2006) suggested to us that TGF-β is a candidate niche-derived factor that might transmit quiescence signals to melanocyte stem cells. To examine this, we tested the effects of TGF-β on melanocytes in vitro and in vivo and demonstrate that TGF-β signaling plays dual roles in melanocyte stem cell maintenance through the inhibition of stem cell differentiation in the stem cell niche and the induction of stem cell quiescence.

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RESULTS

Melanocyte Stem Cell Entry into the Dormant State

Melanoblasts that colonize the hair follicle bulge are inactivated at the final stage of hair follicle morphogenesis and thereafter function as the melanocyte stem cell population (Nishimura et al., 2002, 2005). That population is activated at the beginning of each hair regeneration cycle (early anagen) to self-renew but is inactivated again by midanagen. Thus, we hypothesized that melanocyte stem cells enter the quiescent (noncycling) inactivated state in response to external cues from the stem cell niche at a particular stage. To test this hypothesis and to understand the mechanism of stem cell entry into the dormant noncycling state, we first examined changes in morphology and expression of melanogenic genes and the cell cycle progression marker Ki67 in melanocyte stem cells after colonization of the hair follicle bulge. As shown in Figures 1A–1H and Figure S1 (available online), profound downregulation of melanogenic factors including MITF (Figures 1A and 1B; Figure S1A), tyrosinase-related protein 1 (TRP1) (Figures 1C–1F; Figure S1B), tyrosinase (Figures 1G and 1H; Figure S1C), and dopachrome tautomerase (DCT) (Figures 1A and 1B; Figure S1A) were detected by the end of hair follicle morphogenesis (stage 8). Expression of PAX3 and KIT were maintained longer (Figures 1C–1H; Figure S1B) but were subsequently downregulated gradually (data not shown). We then found that these events are preceded by a loss of Ki67 expression at approximately stage 5 (standardized hair follicle stages based on Paus et al. [1999]) of hair follicle morphogenesis (Figure 1K) and the loss of BrdU incorporation by melanocyte stem cells from this stage (Nishimura et al., 2002, and data not shown). Interestingly, downregulation of Ki67 and melanogenic gene expression at the time of stem cell entry into quiescence was similarly found in the following cycles (Figures 1I, 1J, and 1L and data not shown).

TGF-β Signaling in Melanocyte Stem Cells at Their Entry into the Dormant State

To examine the possible involvement of TGF-β in the process of stem cell entry into the noncycling state (stem cell dormancy), we first examined the expression of TGF-βs in hair follicles at different follicle stages. As shown in Figures 2A and 2B, TGF-β1/2 expression was found in the bulge area of follicles starting at around stage 6 and was significantly high at stage 7 (Figures 2A and 2B). Smad2, which is directly phosphorylated by TGF-β receptors, was also found in transgenically tagged ($LacZ^+$) immature melanoblasts and surrounding keratinocytes in the bulge area (Figures 2C and 2E–2G). The phospho-Smad2 signal was strong in those immature melanoblasts in the bulge area of stage 6 and 7 follicles and was then gradually downregulated at stage 8 (Figures 2F and 2G). Interestingly, TGF-β1/2 expression was upregulated again after stem cell division and was maintained in the bulge area during anagen, followed by a gradual downregulation from catagen to telogen (data not shown). On the other hand, the expression of TGF-β1/2 and phospho-Smad2 was low and often undetectable in the pigmented junctional epithelium, such as the mucocutaneous junction of the mouse eyelid where pigmented melanocytes are abundantly located in the basal layer of the epithelium (Figures S2Ab–S2Ae). Taken together, the coincident expression patterns of TGF-β1/2 and phospho-Smad2 in the stem cell niche suggest that TGF-β signaling is active when melanocyte stem cells enter the dormant state and maintain their immaturity.

TGF-β Induces Cell Cycle Arrest, Downregulation of MITF, and Suppression of Melanogenic Genes in Melanoblasts/Melanocytes

To examine the possible function of TGF-β in stem cell maintenance, including the maintenance of stem cell immaturity and the induction of stem cell dormancy in vivo, we tested the impact of TGF-βs on melanocyte proliferation and differentiation in vitro. First, we performed cell cycle analysis of primary cultured melanocytes treated with TGF-β1/2.

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As shown in Figure 3A, TGF-β induced a significant accumulation of cells in G1 and a reduction of cells in S/G2M. Furthermore, the reduction by TGF-β could be rescued by concomitant stimulation with SCF (Kit ligand [KITL], a survival/mitogenic factor for the melanocyte lineage), although SCF did not significantly promote accumulation of cells in S/G2M. The standard culture medium used for primary melanocytes contains TPA, which is essential for melanocyte survival and proliferation in vitro. TPA can be replaced by various melanocyte survival factors including SCF, basic fibroblast growth factor (bFGF), or endothelin 1/3 (data not shown). SCF expression has been detected in follicular keratinocyte stem cells of the bulge area (Tumbar et al., 2004), the stem cell niche for melanocyte stem cells. We therefore examined potential interactions of SCF with TGF-β on cell growth/survival. As shown in Figure 3B, melanocyte cell number was balanced by SCF and TGF-β in a dose-dependent manner, with similar results seen in the tetrazolium salt-based proliferation assay (WST assay) or by direct cell counting. Melanocytes treated with TGF-β without any melanocyte survival/growth factors (such as SCF, endothelin1/3, or bFGF) underwent apoptosis within 24–48 hr. Concomitant treatment of melanocytes with those factors completely blocked TGF-β1/2-induced apoptosis (Figure S3A). Melanoma cells, most of which exhibit constitutively active MAPK because of *B-raf* (Davies et al., 2002), *N-ras* (van 't Veer et al., 1989), or *Kit* (Chin et al., 2006; Curtin et al., 2006) mutations, were resistant to TGF-β treatment (Figure S3B). Although multiple potential mechanisms could account for TGF-β resistance in melanoma cells, these data are consistent with the hypothesis that melanoma cells have escaped the physiologic role of TGF-β to induce quiescence.

Functional and Morphologic Consequences of TGF-β

During development, immature melanoblasts show characteristic small and bipolar cell bodies rather than large dendritic cell bodies (Nishimura et al., 1999). Melanocyte stem cells that are entering the quiescent/inactivated state also show reductions in the number of dendrites and in the cell size (Figure 1). Treatment of primary cultured melanocytes with TGF-β significantly reduces the number of dendrites and the cell size and produces a discretely bipolar morphology (Figures 3C and 3D), so TGF-β might explain a set of melanocyte stem cell changes including not only morphological changes but also downregulation of melanogenic genes such as tyrosinase. Tyrosinase is the rate-limiting enzyme involved in melanin synthesis, and its activity is a marker for melanocyte differentiation. The dopa reaction assay was utilized to assess tyrosinase activity in primary melanocytes treated with TGF-β and other (control) factors. The activity was examined with the same number of viable melanocytes. As shown in Figures 3E and 3G, a significant reduction of tyrosinase activity was detected after treatment with TGF-β. An induction of tyrosinase activity was observed with forskolin (FSK), as previously described (Suzuki et al., 1997), and the effect of FSK was partially antagonized by concomitant treatment with TGF-β. Furthermore, TGF-β treatment maintained primary cultured melanocytes in an unpigmented state as shown in Figure 3F. These data suggest that TGF-β downregulates tyrosinase, the critical and rate-limiting enzyme for melanin synthesis, a specific marker for lineage differentiation.

To examine how tyrosinase activity is inhibited by TGF-β treatment, we studied the expression of phospho-Smad2 and MITF, the transcriptional master regulator of the melanocyte lineage. As shown in Figure 4, TGF-β induces Smad2 phosphorylation (Figure 4A) as well as the downregulation of MITF protein expression (Figures 4B and 4C). *Mitf* mRNA expression was also decreased significantly by 3 hr (Figure 4D). Moreover, the downregulation of MITF protein expression was associated with a reduction of downstream target gene expression including tyrosinase (Figure 4C). Thus, the TGF-β induction of cell cycle arrest, loss of dendricity, and reduction in cell size and the functional inactivation of

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the melanocyte lineage are associated with the downregulation of MITF and its downstream genes, strongly suggesting important functional roles of TGF-β in maintaining melanocyte stem cell immaturity and quiescence.

Transforming Growth Factor β Receptor II Is a Key Regulator of Melanocyte Stem Cell Maintenance

To examine the role of the TGF-β signaling pathway in the melanocyte lineage, we bred *TGFBRII*$^{fx/fx}$ mice (Cazac and Roes, 2000) to knockin mice expressing Cre recombinase under control of the *Dct* promoter (*Dct*$^{cre/cre}$ knockin mice). These mice were further bred with *Dct-lacZ* transgenic mice to tag the melanocyte lineage cells. As shown in Figures 5A–5C, *TGFBRII*$^{fx/fx}$; *Dct*$^{cre/cre}$ mice (cKO) exhibited mild but accelerated hair graying (with 73.3% penetrance) within 10 months after birth, whereas *TGFBRII*$^{+/+}$;*Dct*$^{cre/cre}$ control mice (con) did not. Histologically, the bulge area of midanagen hair follicles from homozygous cKO mice began to show ectopically pigmented melanocytes with dendritic morphologies starting from the second hair cycle (Figures 5E, 5G, and 5I). These ectopically differentiated melanocytes were preferentially found in follicles that have pigmentation defects in the hair matrix (Figure 5K and data not shown). Some of those follicles had completely lost Dct-lacZ^{+} cells in the bulge area (Figures 5L and 5M), which suggests that the melanocyte stem cells had prematurely differentiated and were eventually depleted from the stem cell niche. In contrast, Dct-lacZ^{+} melanoblasts are maintained in the bulge area of control follicles in an immature and quiescent state with small cell bodies and few dendrites in the bulge area (Figures 5D, 5F, 5H, and 5J). Thus, these histological findings suggest that melanocyte stem cells in cKO mice either failed to return to quiescence (i.e., were slow to return to the quiescent state undergoing premature differentiation in the niche) or alternatively that a defective stem cell progeny failed to migrate from the bulge to the hair matrix. Because the dendritic melanocytes in the bulge did not continue to accumulate in the bulge area with age but rather were lost in follicles that had lost mature melanocytes in the hair matrix, the latter possibility was unlikely. This phenotype of ectopically pigmented melanocytes (EPM) in the bulge resembles previous observations of rare pigmented melanocytes in the niche in mice under genotoxic stress or in aging wild-type mice, in *Mitf*$^{vit/vit}$ mutant mice, and in *Col17a1*-deficient mice (Nishimura et al., 2005; Inomata et al., 2009; and unpublished data). In each case, “inappropriately” pigmented bulge melanocytes preceded hair graying. Ectopic differentiation of melanocyte stem cell progeny in the niche preceded the onset of hair graying or occurred concomitantly with hair graying, presumably because of the defective maintenance of stem cell immaturity of melanocyte stem cells. Although the frequency of the gray hairs was variable depending on the individual animal, melanocyte stem cells had been lost in the bulge area of almost all follicles that produced white hair. These phenomena strongly suggest that stem cell-depleted follicles give rise to white or gray hairs in the subsequent hair cycle. In contrast, epidermal and dermal melanocytes in the pigmented junctional epithelium, such as in mouse eyelids, are maintained both in cKO mice and in control littermates (Figure S4). Collectively these data suggest that the hair graying phenotype in the setting of TGF-βRII deficiency can be caused by incomplete maintenance of melanocyte stem cells.

Melanocyte Stem Cells Enter Quiescence in a *Bcl2*-Dependent Manner by Responding to Niche-Derived TGF-β

The antiapoptotic molecule *Bcl2* is critical for melanocyte stem cell entry into the dormant state of the hair follicle at around stage 7 (i.e., postnatal days 6–7 for pelage hair follicles) (Nishimura et al., 2005). As shown in Figure S6 and in our previous report, lineage tracing studies revealed sudden and massive loss of bulge melanocyte stem cells at stages 6–8 (Nishimura et al., 2005). At this stage, TGF-β1/2 and phosphorylated Smad2 start to be expressed in the stem cell niche as shown in Figure 2. Thus, we hypothesized that TGF-β

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might be a trigger for melanocyte stem cell apoptosis in the absence of *Bcl2*, whereas TGF-β induces quiescence in wild-type melanocyte stem cells. To test this possibility, an anti-TGF-β blocking antibody (1D11) (Dasch et al., 1989; Ling et al., 2003) or a matched control antibody (13C4) was injected into $Bcl2^{-/-}$ mice during the process of stem cell entry to the dormant state (stage 5–8 of hair follicle morphogenesis). First, we confirmed efficient blocking of Smad2 phosphorylation with the TGF-β blocking antibody but not with the control antibody by immunostaining for phospho-Smad2 (Figures 6A–6D). Next, we examined the viability of melanocyte stem cells in the bulge area of $Bcl2^{-/-}$ mice and of control $Bcl2^{-/+}$ littermates. $LacZ^{+}$ (transgenically tagged) melanoblasts disappeared from the bulge area by P8 in $Bcl2^{-/-}$ mice, either with or without the control antibody (Figure 6F; Figure S5). Our prior studies demonstrated the programmed cell death of bulge melanoblasts at around P7 in *Bcl2* null mice. However, the viability of bulge $LacZ^{+}$ melanoblasts was preserved after administration of the TGF-β blocking antibody in $Bcl2^{-/-}$ mice at P4, P6, and P8 (Figure 6G). Because the loss of bulge melanoblasts at P8 was such a synchronous, high-penetrance feature of $Bcl2^{-/-}$ mice, these data show a dramatic rescue of the bulge melanoblast population upon injection of the 1D11 TGF-β blocking antibody. In contrast, mice treated with the control antibody appeared the same as untreated $Bcl2^{-/-}$ mice, with essentially complete melanoblast loss at P8. It is noteworthy that melanocyte stem cells in *Bcl2* null mice treated with 1D11 at P4, P6, and P8 were still absent from whisker follicles, which develop earlier (during embryonic development) than truncal follicles. Thus, the current findings suggest that niche-derived TGF-β is critical not only for induction of stem cell quiescence and maintenance of stem cell immaturity, but also as a trigger of melanocyte stem cell apoptosis in the setting of *Bcl2* deficiency.

DISCUSSION

Melanocyte Stem Cell Entry into the Quiescent State

In this study, we demonstrate that TGF-β signaling is critical for melanocyte stem cell entry/maintenance in the quiescent state. The stem cell quiescence is preceded by the activation of TGF-β signaling, the loss of Ki67 expression, the downregulation of melanogenic gene expression, and dramatic morphologic changes from a dendritic shape into a slender, oval shape with shrinkage resulting from an increased nuclear/cytoplasmic ratio (summarized in Figure 7). Upregulation of TGF-β1/2 expression in the niche area and phospho-Smad2 expression by melanocyte stem cells was detected prior to morphological changes, downregulation of melanogenic genes, and loss of Ki67 expression by melanocyte stem cells. These changes were found during every hair cycle, which suggests that melanocyte stem cells return to the quiescent state after completion of stem cell division in early anagen. Although we previously reported that Dct-lacZ-positive cells in the bulge area do not express tyrosinase and are maintained in an immature state (Nishimura et al., 2002), it was not clear at which stage melanocyte stem cells lose tyrosinase expression and return to the quiescent state, i.e., after localization in the niche or after stem cell division in early anagen. In this study, we found that the expression of melanogenic genes is significantly downregulated in melanocyte stem cells at stages 6–8 as well as at midanagen stage during hair cycle. Melanocyte stem cells divide only at early anagen (Nishimura et al., 2002), so most melanocyte stem cells are usually kept in a nondividing state—in the Go phase. This long quiescent state gives rise to long-term in vivo labeling with a nucleotide analog that is characteristically seen in a number of somatic stem cells including the well-investigated hematopoietic stem cells (Nystul and Spradling, 2006; Suda et al., 2005) and epidermal stem cells (Cotsarelis et al., 1990; Tumbar et al., 2004). Stem cell quiescence/dormancy is reminiscent of *Caenorhabditis elegans* dauer formation, which promotes longevity with minimum metabolism. Melanocyte stem cell quiescence also might be a strategy for melanocyte stem cell longevity for continuous hair pigmentation.

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TGF-β Induces Melanoblast Immaturity through the Downregulation of MITF and Its Downstream Targets

One of the core defining features of stemness is the maintenance of an immature (undifferentiated) state. In melanocytes, the state of differentiation is most clearly defined in relation to the pigmentation pathway—a pathway that is potently regulated by the lineage selective transcription factor MITF (Steingrímsson et al., 2004; Levy et al., 2006). The studies reported here via primary melanocytes demonstrate that TGF-β1/2 suppresses melanin production as well as the activity of tyrosinase, the rate-limiting melanogenic enzyme, and this is consistent with previously reported observations in an immortalized cell line (Kim et al., 2004). These effects can be explained by the downregulation of *Mitf*, because tyrosinase and many other melanogenic genes have been shown to be its direct downstream transcriptional targets (Steingrímsson et al., 2004; Vance and Goding, 2004; Levy et al., 2006; Buscà and Ballotti, 2000; Lang et al., 2005). Melanoblasts that have just colonized the melanocyte stem cell niche do express tyrosinase. These cells lose the expression of TGF-β within the TGF-β-expressing niche microenvironment, suggesting that TGF-β inhibits the expression of melanogenic genes to prevent melanocyte maturation.

TGF-β Signaling for Melanocyte Stem Cell Renewal

We previously observed a phenomenon that we presumptively referred to as “in situ differentiation” or “ectopic differentiation” of melanocyte stem cells in midanagen follicles during the hair graying process (Nishimura et al., 2005; Inomata et al., 2009). A similar cell population was detected in the current study within *TGFbRII* cKO mice. These ectopically pigmented melanocytes (EPMs) in the niche are characterized by their dendritic, large cell bodies and the production and accumulation of melanin pigment in midanagen follicles. This phenomenon was observed not only during aging but also in an accelerated fashion within the graying mutant $MITF^{vit/vit}$ mice (Nishimura et al., 2005). In nonaged physiological conditions, only immature melanoblasts (melanocyte stem cells) can be found in the stem cell niche. The accelerated appearance of EPMs in the setting of defective TGF-β signaling (i.e., in *TGFBRII* cKO mice) indicates that TGF-β signaling plays an important role in the maintenance of melanocyte stem cell immaturity. These changes were found beginning at the second hair cycle, being distributed frequently in midanagen follicles with defective hair pigmentation.

A role for TGF-β signaling in stem cell quiescence has been previously suggested for hematopoietic stem cells, neural stem cells, and prostate stem cells (Batard et al., 2000; Salm et al., 2005; Larsson et al., 2003; Karlsson et al., 2007; Falk et al., 2008; reviewed in Watabe and Miyazono, 2009). Whereas *TGFbRII* is dispensable for hematopoietic stem cell renewal (Larsson et al., 2003), Smad4 is critical for stem cell renewal in the hematopoietic system and in hair follicle stem cells (Karlsson et al., 2007). *TGFbRII* and *Smad4* are also important for homeostasis of the epidermis and hair follicles (Guasch et al., 2007; Qiao et al., 2006; Yang et al., 2005, 2009). However, the role of TGF-β in stem cell immaturity has not been previously implicated. Our data demonstrate that TGF-β signaling is important for melanocyte stem cell renewal in $TGFbRII^{fx/fx}$; $Dct^{cre/cre}$ mice through the maintenance of melanocyte stem cell immaturity and quiescence.

There are some possible explanations for the gradual (rather than abrupt) and partial loss of melanocyte stem cells observed in the melanocyte-targeted *TGFbRII*-deficient mice ($TGFbRII^{fx/fx}$; $Dct^{cre/cre}$) studied. One possibility is that only a small fraction of melanocyte stem cells (rather than the entire population) becomes activated at the beginning of anagen. Indeed, a small fraction of melanocyte stem cells can be labeled by the nucleotide analog BrdU at the beginning of the anagen stage when melanocyte stem cells undergo cell division. This suggests that a small fraction of melanocyte stem cells are activated during the

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hair cycle and only those activated melanocyte stem cells need to go back into the quiescent state for self-renewal. Another possibility is that the Cre recombination may not have completely and homozygously deleted the floxed *TGFbRII* gene in all melanocyte stem cells with a resultant incomplete penetrance of the phenotype. In either case, the mild hair graying phenotype in spite of the striking in vitro effects of TGF-β as well as the histological abnormalities observed in affected follicles of cKO mice suggest compensatory mechanisms for the homeostatic regulation of the melanocyte stem cell pool. The TGF-β signaling pathway may be cooperating with other signals from the niche cells as well as stem cells for stem cell maintenance/survival and size control of the stem cell pool. Stem cells can renew themselves only when they survive and produce progeny that maintain stem cell features. An antiapoptotic role of Dct/TRP2 also has been reported (Nishioka et al., 1999; Chu et al., 2000; Pak et al., 2004; Michard et al., 2008) but we have not observed any significant hair graying phenotype in $Dct^{cre/cre}$ mice (Guyonneau et al., 2004). Based upon the in vitro and in vivo consequences of TGF-β signaling on melanocytes, its effects in melanocyte stem cells appear to include the induction of quiescence and the maintenance of immaturity, which might be synergistically promoted by other niche-specific environmental factors.

A separate function of TGF-β demonstrated in this study is the induction of melanocyte stem cell apoptosis upon entry into quiescence when Bcl2 is not functioning or under conditions of growth/survival factor starvation. Phospho-Smad2 is detectable in melanocyte stem cells when they return to the quiescent state and the anti-TGF-β-blocking antibody blocks the expression and rescues *Bcl2*-deficient melanocyte stem cell death in mice. These findings demonstrate that TGF-β is responsible for the apoptosis of melanocyte stem cells in *Bcl2* deficiency as well as melanocyte stem cell maintenance. Thus TGF-β signaling may alternatively determine quiescence versus death signaling, depending upon the state of the Bcl2-related antiapoptotic machinery downstream in the cells. Indeed, Bcl-2 protection of cells from TGF-β-induced apoptosis has been reported (Huang and Chou, 1998; Bruckheimer and Kyprianou, 2002). Thus, such a mechanism of TGF-β-induced melanocyte stem cell death could in principal contribute to certain instances of human hair graying (e.g., in settings of insufficient local expression of Bcl-2 or growth/survival factors).

Relationship between Stem Cell Maintenance and Melanomagenesis

Regional segregation of the melanocyte stem cell compartment from the amplifying and differentiating compartment is quite distinct within the hair follicle. Our data suggest that SCF and TGF-β can balance the size of the melanocyte stem cell pool. *Bcl2,* whose expression can be maintained by SCF via MITF (McGill et al., 2002), is likely to be important in protecting against TGF-β-induced apoptosis of melanocyte stem cells at stages 6–8, when they go into the quiescent state at the transition from hair follicle morphogenesis to hair cycle progression. These data are consistent with our previous findings that once melanocyte stem cells enter dormancy (after colonization of the stem cell niche in hair follicles), they survive in a manner independent of KIT-blocking-antibody treatment (Nishimura et al., 2002). Stem cell quiescence induced by TGF-β in the presence of Bcl2 might be necessary to obtain this KIT-independent quiescence.

TGF-β signaling can induce either quiescence or apoptosis, the latter occurring in $Bcl2^{-/-}$ melanocytes or in wild-type melanocytes deprived of serum growth factors in vitro. Cytokines capable of activating a variety of receptor tyrosine kinases potently protect against this TGF-β-induced death, suggesting a potential mechanism for balancing the size of the stem cell pool. Nearly all human melanocytic neoplasias (including benign nevi) contain oncogenic mutations predicted to constitutively activate the same pathway(s), in particular mutations of *KIT, B-RAF*, and *N-Ras* (Davies et al., 2002; van 't Veer et al., 1989; Chin et al., 2006; Curtin et al., 2006). It will be of interest to determine whether TGF-β signaling would be antagonized by the actions of these mutations, comparable to TGF-β

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antagonism by KIT signaling. If so, this could represent a fundamental mechanism whereby such oncoproteins induce growth/survival advantages to the melanocytic lineage within these neoplasms.

EXPERIMENTAL PROCEDURES

Animals and Antibody Teatment

Dct-lacZ transgenic mice (gift of Dr. I. Jackson) have been previously described (Mackenzie et al., 1997; McGill et al., 2002; Nishimura et al., 2002). The *Dct-lacZ* transgenic colony (CBA/C57BL6) was backcrossed to C57BL/6J. *Bcl-2*$^{-/+}$ (C57BL/6J) and wild-type (C57BL/6J) mice were purchased from the Jackson Laboratory or Charles River. *TGFBRII*$^{fx/fx}$ (Cazac and Roes, 2000), *Dct*$^{cre/cre}$ (Guyonneau et al., 2004), and Tyr-cre (Delmas et al., 2003) have been described previously. A murine IgG1 monoclonal antibody, 1D11, which neutralizes all three mammalian TGF-β isoforms (1, 2, and 3), was produced and purified at Genzyme Corporation (Framingham, MA). This antibody has a circulatory half-life of 34 hr in mice when administered by intra-peritoneal injection (Genzyme Corp.). An isotype-matched irrelevant murine IgG1 monoclonal antibody, 13C4, also produced by Genzyme Corporation, directed against Shigela toxin, was used as a control antibody. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Kanazawa University and Dana-Farber Cancer Institute).

Immunohistochemical Analysis

Immunohistochemical staining and whole-mount β-galactosidase staining were performed as previously described (Nishimura et al., 2002, 2005; Inomata et al., 2009). Additional details on the methods and antibodies used are provided in the Supplemental Information.

Primary Melanocyte Culture

Primary human melanocytes from neonatal foreskins (provided by Dr. Ruth Halaban, Yale University) were maintained between passages 1 and 4 in F10 medium containing (GIBCO-BRL), 7% fetal bovine serum, penicillin/streptomycin/glutamine (GIBCO-BRL), 1×10^{-4} M 3-isobutyl-1-methyl xanthine (IBMX)(Sigma), 50 ng ml^{-1} 12-O-tetradecanoyl phorbol-13-acetate (TPA, Sigma), 1 μM Na_3VO_4, and 1×10^{-3} M N^6,2′-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP, Sigma). A day before stimulation with TGF-β, primary melanocytes were plated in medium containing a lower concentration of TPA (10 ng ml^{-1}). For the Annexin V assay, the medium was replaced with one supplemented with 10 ng ml^{-1} TPA but without dbcAMP a day before stimulation with TGF-β. Another source of primary human melanocytes (KUR-ABO, Osaka, Japan) were maintained in medium254 (KURABO) supplemented with 3 ng/ml recombinant bFGF, 5 μg/ml insulin, 0.18 μg/ml hydrocortisone, 10 ng/ml phorbol-12-myristate-13-acetate (PMA), 0.2% (v/v) bovine pituitary extract (BPE), and 0.5% (v/v) fetal bovine serum (FBS) (KURABO). For the Annexin V assay, the medium was replaced with one without dbcAMP and bFGF a day before stimulation with TGF-β. Primary melanocytes were stimulated with human TGF-β1 or TGF-β2 (R&D systems), 20 ng/ml recombinant human SCF (R&D Systems), recombinant bFGF (Promega), 50 ng/ml TPA, or 20 μM Forskolin (Sigma).

Tyrosinase Assay

Enzymatic assays for tyrosinase (dopa oxidase) were performed according to a published procedure (Modern Experimental Biochemistry) (Boyer, 1993) with 0.1 M phosphate buffer (pH 6.8) and 25 mM L-dopa (Sigma). Cells were trypsinized and counted. The same number (3×10^5 for Figure 3G) of cells were lysed with 1% TritonX/PBS at room temperature for 1

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hr with shaking. Absorbance (475 nm) was analyzed with a multiplate reader (Bio Rad). The data were analyzed with commercially available software MPM III versus 1.57 al. (Biorad Multiplate Manager III).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction/TaqMan

The details on the methods are described in the Supplemental Information.

FACS Analysis for Annexin V Staining and Cell Cycle Analysis

Apoptosis was detected by Annexin V staining (BD PharMingen). Attached and floating cells were harvested after 48 hr treatment with TGF-β and other factors. Annexin V-FITC-positive cells were analyzed by FACS Caliber (Becton Dickinson). For cell cycle analysis, cells were pelleted and fixed in 70% ethanol for 30 min. Cells were washed once with PBS and then were stained with 1 mg/ml propidium iodide and 25 μg/ml RNase in PBS for 30 min. The cell cycle profiles were collected with FACS Caliber with Cellquest (Becton Dickinson) and were analyzed with ModFit (Verity Software House) software.

Cell Proliferation Assays

Viable cell counting with trypan blue staining and WST-1 cell proliferation assays (Roche) were carried out to measure cell proliferation/viability according to manufacturer's instructions.

Statistical Analysis

All experiments were performed with at least three different primary cultures or animals in independent experiments. Significance was evaluated by Student's t test. Data are presented as mean ± SD or SEM, as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Tetsuo Watabe, Dr. Kohei Miyazono, Dr. Elaine Fuchs, Dr. Vincent Hearing, and Dr. Elizabeth Hearing for antibodies, Dr. Ian Jackson for the Dct-LacZ transgenic mice, and Dr. Hans Widlund for discussion. The research was funded by grants from the Uehara Memorial Foundation, the Kato Memorial Bioscience Foundation (to E.K.N.), and grants from the NIH and the Melanoma Research Alliance (to D.E.F., who is also a Distinguished Clinical Scholar of the Doris Duke Medical Foundation).

References

Batard P, Monier MN, Fortunel N, Ducos K, Sansilvestri-Morel P, Phan T, Hatzfeld A, Hatzfeld JA. TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. J Cell Sci. 2000; 113:383–390. [PubMed: 10639326]

Boyer, RF. Modern Experimental Biochemistry. Redwood City, CA: Benjamin/Cummings Pub. Co; 1993.

Bruckheimer EM, Kyprianou N. Bcl-2 antagonizes the combined apoptotic effect of transforming growth factor-beta and dihydrotestosterone in prostate cancer cells. Prostate. 2002; 53:133–142. [PubMed: 12242728]

Buscà R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res. 2000; 13:60–69. [PubMed: 10841026]

Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. Immunity. 2000; 13:443–451. [PubMed: 11070163]

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Chin L, Garraway LA, Fisher DE. Malignant melanoma: Genetics and therapeutics in the genomic era. Genes Dev. 2006; 20:2149–2182. [PubMed: 16912270]

Chu W, Pak BJ, Bani MR, Kapoor M, Lu SJ, Tamir A, Kerbel RS, Ben-David Y. Tyrosinase-related protein 2 as a mediator of melanoma specific resistance to cis-diamminedichloroplatinum(II): therapeutic implications. Oncogene. 2000; 19:395–402. [PubMed: 10656687]

Cotsarelis G. Epithelial stem cells: A folliculocentric view. J Invest Dermatol. 2006; 126:1459–1468. [PubMed: 16778814]

Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell. 1990; 61:1329–1337. [PubMed: 2364430]

Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol. 2006; 24:4340–4346. [PubMed: 16908931]

Dasch JR, Pace DR, Waegell W, Inenaga D, Ellingsworth L. Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. J Immunol. 1989; 142:1536–1541. [PubMed: 2537357]

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949–954. [PubMed: 12068308]

Delmas V, Martinozzi S, Bourgeois Y, Holzenberger M, Larue L. Cre-mediated recombination in the skin melanocyte lineage. Genesis. 2003; 36:73–80. [PubMed: 12820167]

Falk S, Wurdak H, Ittner LM, Ille F, Sumara G, Schmid MT, Draganova K, Lang KS, Paratore C, Leveen P, et al. Brain area-specific effect of TGF-beta signaling on Wnt-dependent neural stem cell expansion. Cell Stem Cell. 2008; 2:472–483. [PubMed: 18462697]

Foitzik K, Lindner G, Mueller-Roever S, Maurer M, Botchkareva N, Botchkarev V, Handjiski B, Metz M, Hibino T, Soma T, et al. Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo. FASEB J. 2000; 14:752–760. [PubMed: 10744631]

Guasch G, Schober M, Pasolli HA, Conn EB, Polak L, Fuchs E. Loss of TGFbeta signaling destabilizes homeostasis and promotes squamous cell carcinomas in stratified epithelia. Cancer Cell. 2007; 12:313–327. [PubMed: 17936557]

Guyonneau L, Murisier F, Rossier A, Moulin A, Beermann F. Melanocytes and pigmentation are affected in dopachrome tautomerase knockout mice. Mol Cell Biol. 2004; 24:3396–3403. [PubMed: 15060160]

Huang YL, Chou CK. Bcl-2 blocks apoptotic signal of transforming growth factor-beta in human hepatoma cells. J Biomed Sci. 1998; 5:185–191. [PubMed: 9678489]

Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, Iseki S, Hara E, Masunaga T, Shimizu H, Nishimura EK. Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. Cell. 2009; 137:1088–1099. [PubMed: 19524511]

Karlsson G, Blank U, Moody JL, Ehinger M, Singbrant S, Deng CX, Karlsson S. Smad4 is critical for self-renewal of hematopoietic stem cells. J Exp Med. 2007; 204:467–474. [PubMed: 17353364]

Kim DS, Park SH, Park KC. Transforming growth factor-beta1 decreases melanin synthesis via delayed extracellular signal-regulated kinase activation. Int J Biochem Cell Biol. 2004; 36:1482–1491. [PubMed: 15147727]

Lang D, Lu MM, Huang L, Engleka KA, Zhang M, Chu EY, Lipner S, Skoultchi A, Millar SE, Epstein JA. Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature. 2005; 433:884–887. [PubMed: 15729346]

Larsson J, Blank U, Helgadottir H, Björnsson JM, Ehinger M, Goumans MJ, Fan X, Levéen P, Karlsson S. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood. 2003; 102:3129–3135. [PubMed: 12842983]

Levy C, Khaled M, Fisher DE. MITF: Master regulator of melanocyte development and melanoma oncogene. Trends Mol Med. 2006; 12:406–414. [PubMed: 16899407]

Li L, Xie T. Stem cell niche: Structure and function. Annu Rev Cell Dev Biol. 2005; 21:605–631. [PubMed: 16212509]

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Ling H, Li X, Jha S, Wang W, Karetskaya L, Pratt B, Ledbetter S. Therapeutic role of TGF-beta-neutralizing antibody in mouse cyclosporin A nephropathy: morphologic improvement associated with functional preservation. J Am Soc Nephrol. 2003; 14:377–388. [PubMed: 12538738]

Mackenzie MA, Jordan SA, Budd PS, Jackson IJ. Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. Dev Biol. 1997; 192:99–107. [PubMed: 9405100]

Massagué J, Gomis RR. The logic of TGFbeta signaling. FEBS Lett. 2006; 580:2811–2820. [PubMed: 16678165]

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell. 2002; 109:707–718. [PubMed: 12086670]

Michard Q, Commo S, Belaidi JP, Alleaume AM, Michelet JF, Daronnat E, Eilstein J, Duche D, Marrot L, Bernard BA. TRP-2 specifically decreases WM35 cell sensitivity to oxidative stress. Free Radic Biol Med. 2008; 44:1023–1031. [PubMed: 18206123]

Nishimura EK, Yoshida H, Kunisada T, Nishikawa SI. Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. Dev Biol. 1999; 215:155–166. [PubMed: 10545227]

Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S. Dominant role of the niche in melanocyte stem-cell fate determination. Nature. 2002; 416:854–860. [PubMed: 11976685]

Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: Incomplete melanocyte stem cell maintenance in the niche. Science. 2005; 307:720–724. [PubMed: 15618488]

Nishioka E, Funasaka Y, Kondoh H, Chakraborty AK, Mishima Y, Ichihashi M. Expression of tyrosinase, TRP-1 and TRP-2 in ultraviolet-irradiated human melanomas and melanocytes: TRP-2 protects melanoma cells from ultraviolet B induced apoptosis. Melanoma Res. 1999; 9:433–443. [PubMed: 10596909]

Nystul TG, Spradling AC. Breaking out of the mold: Diversity within adult stem cells and their niches. Curr Opin Genet Dev. 2006; 16:463–468. [PubMed: 16919446]

Pak BJ, Lee J, Thai BL, Fuchs SY, Shaked Y, Ronai Z, Kerbel RS, Ben-David Y. Radiation resistance of human melanoma analysed by retroviral insertional mutagenesis reveals a possible role for dopachrome tautomerase. Oncogene. 2004; 23:30–38. [PubMed: 14712208]

Paus R, Müller-Röver S, Van Der Veen C, Maurer M, Eichmüller S, Ling G, Hofmann U, Foitzik K, Mecklenburg L, Handjiski B. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J Invest Dermatol. 1999; 113:523–532. [PubMed: 10504436]

Qiao W, Li AG, Owens P, Xu X, Wang XJ, Deng CX. Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. Oncogene. 2006; 25:207–217. [PubMed: 16170355]

Salm SN, Burger PE, Coetzee S, Goto K, Moscatelli D, Wilson EL. TGF-beta maintains dormancy of prostatic stem cells in the proximal region of ducts. J Cell Biol. 2005; 170:81–90. [PubMed: 15983059]

Scadden DT. The stem-cell niche as an entity of action. Nature. 2006; 441:1075–1079. [PubMed: 16810242]

Siegel PM, Massagué J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer. 2003; 3:807–821. [PubMed: 14557817]

Soma T, Dohrmann CE, Hibino T, Raftery LA. Profile of transforming growth factor-beta responses during the murine hair cycle. J Invest Dermatol. 2003; 121:969–975. [PubMed: 14708594]

Steingrímsson E, Copeland NG, Jenkins NA. Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet. 2004; 38:365–411. [PubMed: 15568981]

Suda T, Arai F, Hirao A. Hematopoietic stem cells and their niche. Trends Immunol. 2005; 26:426–433. [PubMed: 15979407]

Suzuki I, Tada A, Ollmann MM, Barsh GS, Im S, Lamoreux ML, Hearing VJ, Nordlund JJ, Abdel-Malek ZA. Agouti signaling protein inhibits melanogenesis and the response of human melanocytes to alpha-melanotropin. J Invest Dermatol. 1997; 108:838–842. [PubMed: 9182807]

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Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. Defining the epithelial stem cell niche in skin. Science. 2004; 303:359–363. [PubMed: 14671312]

van ’t Veer LJ, Burgering BM, Versteeg R, Boot AJ, Ruiter DJ, Osanto S, Schrier PI, Bos JL. N-ras mutations in human cutaneous melanoma from sun-exposed body sites. Mol Cell Biol. 1989; 9:3114–3116. [PubMed: 2674680]

Vance KW, Goding CR. The transcription network regulating melanocyte development and melanoma. Pigment Cell Res. 2004; 17:318–325. [PubMed: 15250933]

Watabe T, Miyazono K. Roles of TGF-β family signaling in stem cell renewal and differentiation. Cell Res. 2009; 19:103–115. [PubMed: 19114993]

Watt FM, Hogan BL. Out of Eden: Stem cells and their niches. Science. 2000; 287:1427–1430. [PubMed: 10688781]

Yamazaki S, Iwama A, Morita Y, Eto K, Ema H, Nakauchi H. Cytokine signaling, lipid raft clustering, and HSC hibernation. Ann N Y Acad Sci. 2007; 1106:54–63. [PubMed: 17442772]

Yang L, Mao C, Teng Y, Li W, Zhang J, Cheng X, Li X, Han X, Xia Z, Deng H, Yang X. Targeted disruption of Smad4 in mouse epidermis results in failure of hair follicle cycling and formation of skin tumors. Cancer Res. 2005; 1:8671–8678. [PubMed: 16204035]

Yang L, Wang L, Yang X. Disruption of Smad4 in mouse epidermis leads to depletion of follicle stem cells. Mol Biol Cell. 2009; 20:882–890. [PubMed: 19073883]

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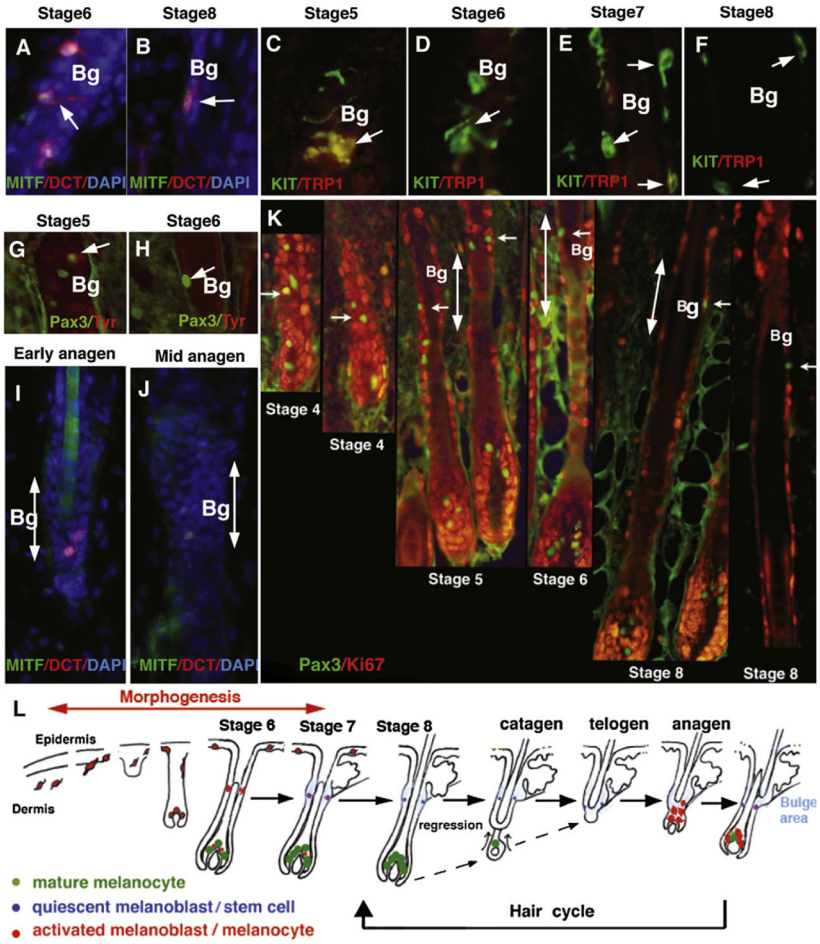

Figure 1. The Process of Melanocyte Stem Cell Entry into a Dormant State

(A–H) Morphological changes of bulge melanoblasts and the downregulation of melanogenic genes at the final stage of hair follicle morphogenesis.

(A and B) Expression of MITF (green) and DCT (red) by bulge melanoblasts at stages 6 and 8, respectively.

(C–F) TRP1 (red) and KIT (green) expression in follicles from stage 5 to stage 8 (X400).

(G and H) Immunostaining of tyrosinase (red) and PAX3 (green) (X400).

(I and J) Immunostaining of early anagen follicle (I) and midanagen follicle (J) for Dct-lacZ (red) and MITF (green) (X400). Note the significant downregulation of MITF and DCT by bulge melanoblasts by midanagen.

(K) Immunostaining of hair follicles of different stages for Ki67 (red) and Pax3 (green) (X200).

(L) Schematic of melanocyte stem cell development and their entry into a dormant state after development and during the hair cycle.

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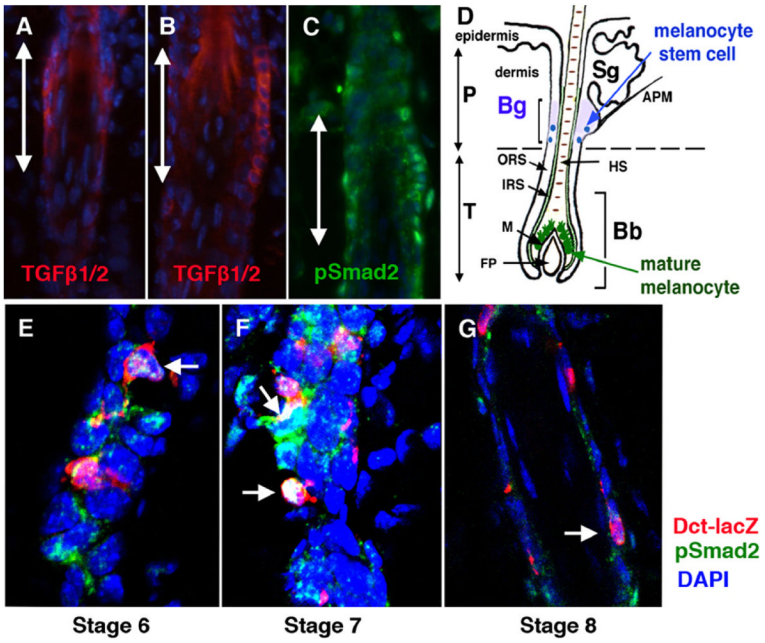

Figure 2. Activated TGF-β Signaling in the Bulge Area at the Time of Melanocyte Stem Cell Entry into the Dormant State

(A and B) TGF-β1 (red) expression in the pelage hair follicle (A) and in the sensory hair follicle at stage 7 (B). Double arrows indicate bulge (Bg).

(C) Immunostaining for phospho-Smad2 (green).

(D) Schematic representation of a mouse pelage hair follicle.

(E–G) Localization of phospho-Smad2 (green)- in Dct-lacZ (red)-expressing cells (arrows) in the bulge area of stage 6–8 follicles (X600).

Abbreviations: P, permanent portion; T, transient portion; Sg, sebaceous gland; Bg, bulge area (double arrows in A–C); Bb, hair bulb; APM, arrector pili muscle; HS, hair shaft; ORS, outer root sheath; M, hair matrix; FP, follicular papilla.

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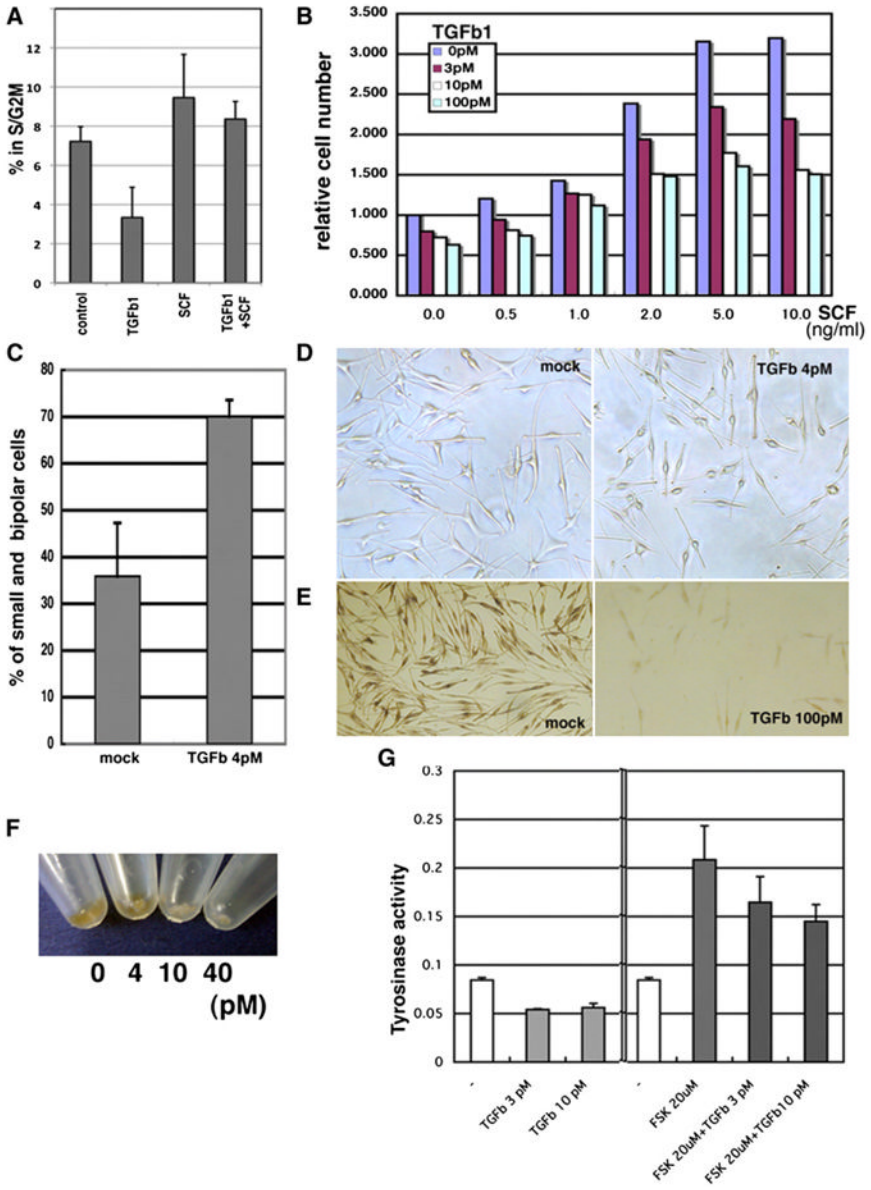

Figure 3. Induction of Cell Cycle Arrest and Maintenance of Undifferentiated Status by TGF-β

(A) Percentage of cultured melanocytes at $S/G_2/M$ phases. Primary cultured melanocytes were stimulated with 4 pM TGF-β1 or 10 ng/ml SCF.

(B) TGF-β and SCF mutually balance melanocyte cell numbers in a dose-dependent manner. Primary cultured melanocytes were treated with different concentrations of TGF-β1 and/or SCF. The relative cell numbers for each condition are shown.

(C) Percentage of large or dendritic melanocytes (nonbipolar cells) with or without TGF-β1 treatment of primary cultured melanocytes.

(D) Representative morphology of primary cultured melanocytes in the presence or absence of 4 pM TGF-β1.

(E) Representative appearance of DOPA-stained melanocytes treated with TGF-β1 for 1 week. (F) Appearance of melanocyte pellets after incubation of primary human melanocytes with TGF-β1 for one week.

(G) Tyrosinase activity of primary melanocytes stimulated with TGF-β and 20 μM forskolin (FSK). Data are presented as mean ± SD.

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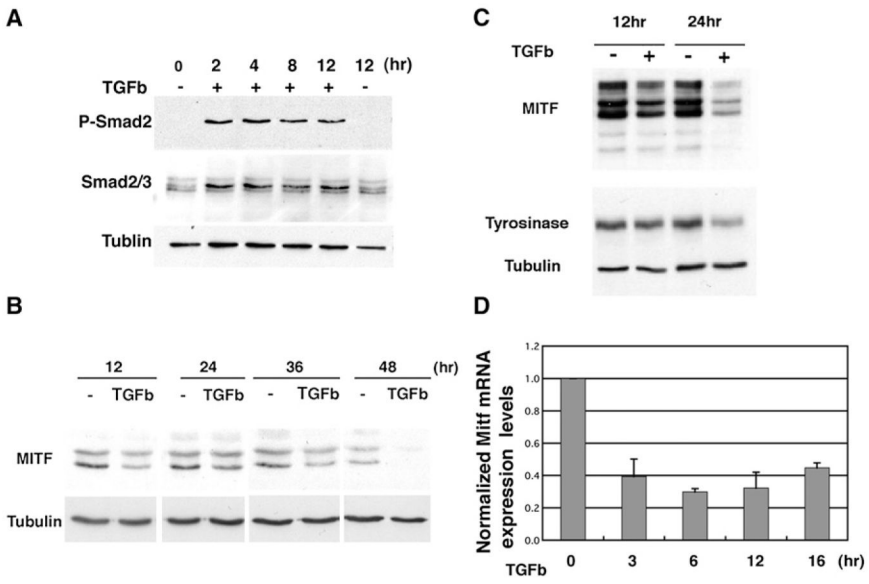

Figure 4. Downregulation of MITF and Its Downstream Target Tyrosinase by TGF-β

(A) Western blotting for phospho-Smad2 after stimulation of primary cultured human melanocytes with TGF-β1.

(B) Western blotting for MITF after TGF-β1 stimulation.

(C) Tyrosinase expression is downregulated by TGF-β1.

(D) Real-time PCR analysis of primary melanocytes after stimulation with TGF-β1. Data are presented as mean ± SD.

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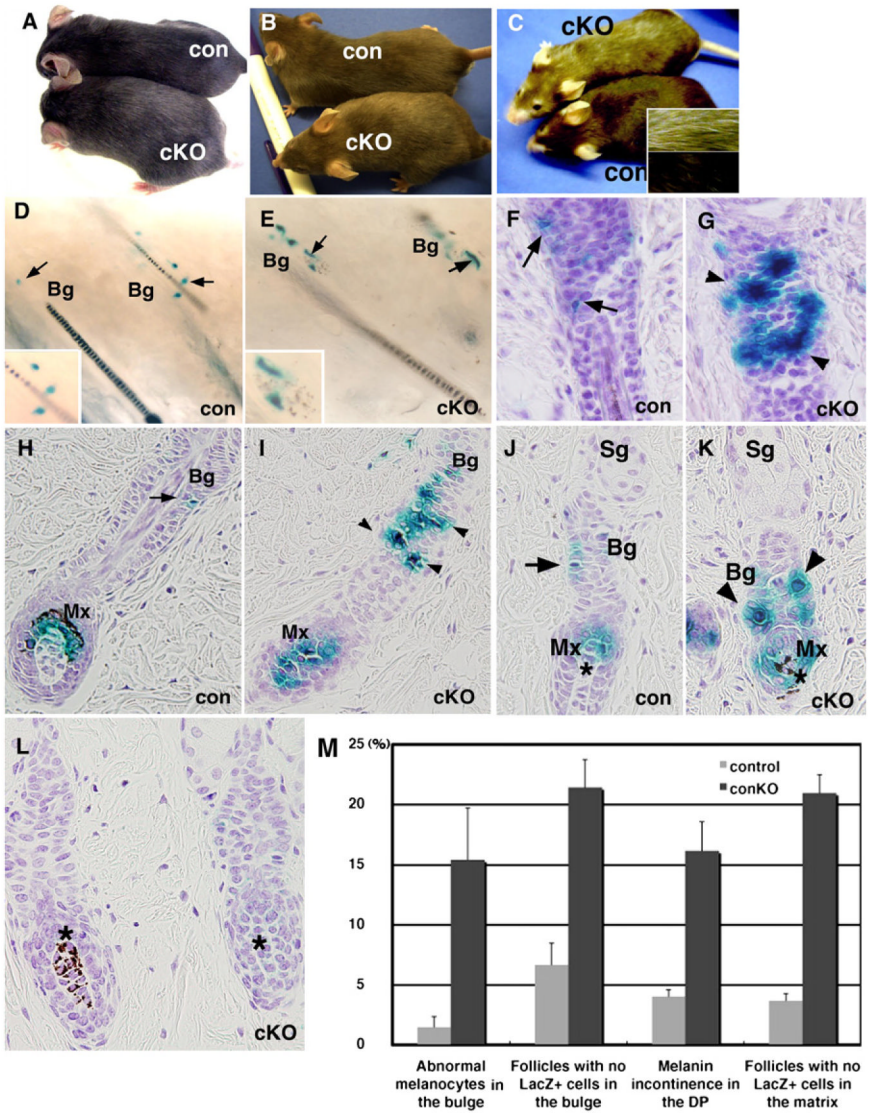

Figure 5. Melanocyte Stem Cell Maintenance through *TGFbRII*

Dct$^{cre/cre}$; *TGFBRII*$^{fx/fx}$ conditional knockout mice (cKO) are compared to *Dct*$^{cre/cre}$; *TGFBRII*$^{+/+}$ (con) littermate controls.

(A–C) Images of hair coats of 6-month-old (A, B) and 10-month-old (C) mice.

(D and E) Whole-mount lacZ staining of the trunk skin from 3-month-old control (E) and *TGFBRII* cKO mice with the *Dct-lacZ* transgene.

(E) Note that LacZ^{+} dendritic and slightly pigmented melanocytes with large cell bodies are found in the bulge-subbulge area of late anagen follicles in cKO mice (E, arrowheads), whereas LacZ^{+} melanoblasts in the bulge area of control follicles are immature and small in size (satisfying the characteristic features of melanocyte stem cells) (D, arrow). Deposition of melanin pigment in the bulge area of cKO follicles (inset).

(F–K) Bright field images of LacZ-stained hair follicles.

(F–I) In midanagen (anagen V) follicles, most LacZ^{+} melanoblasts in the bulge area of control follicles are small in size with minimal dendrites (arrows in F and H), whereas LacZ^{+} dendritic melanocytes with large cell bodies (arrowheads in G and I) instead of small

melanoblasts were sometimes found in the bulge area of cKO follicles at the same stage of the hair cycle.
(J–L) Melanin incontinence was more frequently found in the dermal papillae (asterisk) of cKO follicles (K, L) than in control follicles (J) (×400). Sg, sebaceous gland; Bg, bulge area.
(M) Statistical analysis of histological abnormalities of hair follicle melanocytes in 6-month-old cKO mice with the graying phenotype and in control littermates. The frequency of hair follicles with the typical histological abnormalities was analyzed. Large and dendritic melanocytes or pigmented melanocytes in the bulge area of anagen IV–VI follicles were classified as “abnormal” melanocytes. Significance was evaluated by Student’s t test. Data are presented as mean ± SEM.

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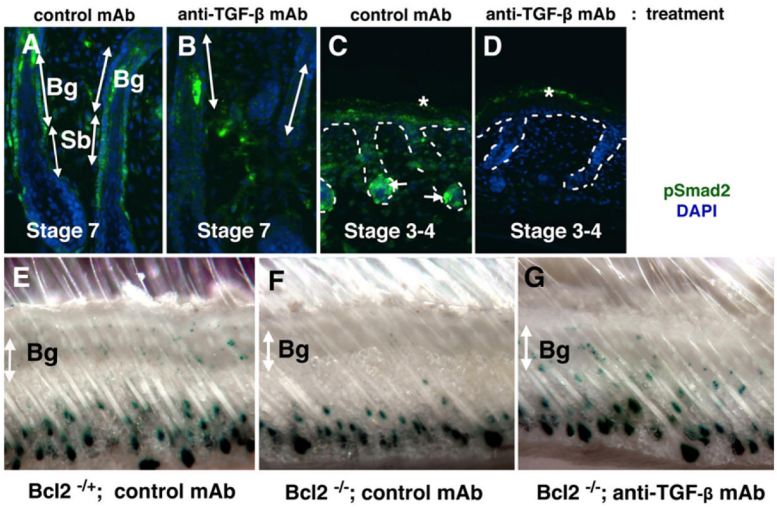

Figure 6. Treatment with an Anti-TGF-β Neutralizing Antibody Promotes the Survival of *Bcl2* Null Melanocyte Stem Cells at Their Entry into the Dormant State

(A–D) Stage 7 hair follicles on the dorsal skin of wild-type mice treated with the control mono-clonal antibody (mAb) (13C4) (A, C) or with the anti-TGF-β neutralizing antibody (1D11) (B, D), respectively, at days 4, 5, 6, and 7 after birth. Asterisks show the nonspecific staining of the cornified layer (X400). (C, D) Stage 4 hair follicles on the ventral skin of animals (A) and (B), respectively (×400).

(E–G) Images of whole-mount X-gal staining of *Bc2*$^{-/-}$ (F, G) and *Bcl2*$^{-/+}$ (E) mice with Dct-lacZ transgene after treatment with the control monoclonal antibody (13C4) (E, F) or with the anti-TGF-β neutralizing antibody (1D11) (G), respectively, at day 4, 5, 6, and 7 after birth. Bg, bulge area; Sb, subbulge area.

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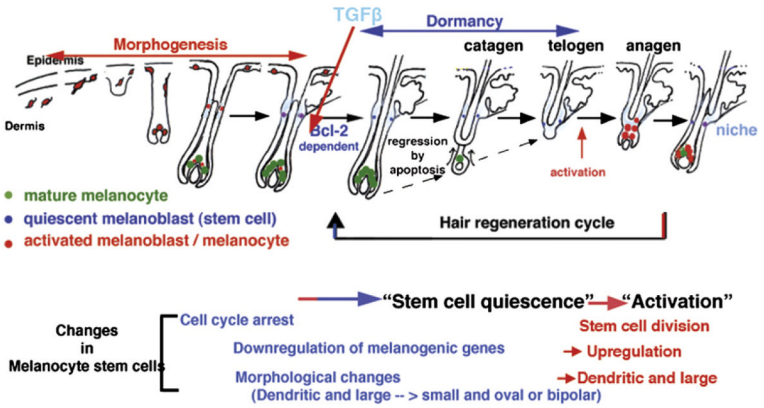

Figure 7. Summary of Melanocyte Stem Cell Status during the Hair Cycle

Dormant status (shown in blue) and activated status (shown in red) of melanocyte stem cells alternate in synchronization with the hair cycles.