Melanocyte stem cells*

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Abstract

Pigmentation of mammalian hairs is mediated by specialized pigment-producing cells referred as melanocytes. Hair pigmentation is tightly linked with hair regeneration cycles, where melanocytes proliferate and differentiate during the hair growth phase, but depleted by apoptosis during the regression phase. This periodical appearance of melanocytes is maintained by a small pool of immature stem cells residing in the hair follicle. Melanocyte stem cells offer an advantageous model by which to elucidate molecular basis of stem cell regulation, since genetic alterations affecting melanocyte regulation result in visible and yet viable phenotypes. Hence, by integrating melanocyte-specific *in vivo* gene manipulation approach and the subsequent phenotype analysis, melanocyte stem cell system provide an irresistible clue to identify key molecules for the stem cell regulation. Consolidation of such data would ultimately reveal an entire molecular network underlying the regulation of stem cells, which would not only contribute complete understanding of stem cell biology, but also provide valuable insights into medical application of stem cells.

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1. Introduction

Elucidation of molecular mechanisms underlying the regulation of stem cells is of great importance for their clinical applications in regenerative medicine and cancer therapy. Stem cells are thought to be regulated by their specialized microenvironment known as the stem cell niche (Spradling et al., 2001). Despite recent intensive studies of the stem cell niche, the molecular mechanisms of stem cell regulation have incompletely understood. As locating and manipulating stem cells is still challenging in many tissues, one drawback that hampers our basic understanding of the stem cell regulation is the difficulty in defining of stem cell behavior at the niche. Hence, development of a novel model system that allows us to identify and manipulate individual stem cells is highly anticipated to clarify molecular basis of stem cell regulations at the niche.

Melanocyte stem cells reside in the hair follicle, and play an essential role in the maintenance of peridical cycles of hair pigmentation (Nishimura et al., 2002). Because of the capabilities of locating and manipulating individual stem cells, melanocyte system offers an ideal model for the study of the stem cell regulation at the niche. This review summarizes recent advances in studies of melanocyte stem cells. Their advantages in the study of molecular basis of stem cell regulation are also discussed.

2. Melanocyte biology

2.1. Functions

Melanocytes are specialized melanin producing cells, and are responsible for skin, hair, and eye pigmentation in vertebrate organisms. Melanocytes synthesize pigment melanin within a special organelle termed a melanosome, where a number of specific enzymes and structure proteins are assembled to synthesize melanin from tyrosine or phenylalanine (Slominski et al., 2004). Once synthesized, melanin is deposited in melanosomes to form melanin granules. These melanin granules are transferred from melanocytes via their dendrite to adjacent keratinocytes, where melanins are accumulated to generate pigmented skin or hairs. While the physiological roles of pigmentation may vary among animal species, in humans the principal function of melanocytes is to protect skin form genotoxic stress of ultraviolet (UV) radiation. This protection is enabled by the ability of melanin granules to absorb UV radiation and quench the UV-induced intracellular free radicals. Indeed, it has been demonstrated that individuals carrying hypomorphic mutation in the melanocortin-1 receptor (MC1R), which is one of critical molecules for the induction of the melanin synthesis pathway, show not only abnormal pigmentation phenotypes, but also higher incidence of melanoma formation and other skin carcinogenesis (Box et al., 2001; Healy et al., 2001), indicating a critical link between melanogenesis and protection against skin carcinogenesis.

In addition to the UV-protection, melanocytes also play an essential role in hearing system (Tachibana, 1999). Patients with Waardenburg Syndrome exhibit varying combinations of abnormal pigmentation as well as auditory defect (Newton, 2002). These phenotypes are caused by a deficiency of melanocytes. It is known that melanocytes also present in the stria vascularis where they consist of an intermediate cell layer, and act to generate the electrophysiological potential in the endolymph fluid (Price and Fisher, 2001; Takeuchi et al., 2000). In this case, melanin pigment formation is not required for their auditory function, but a K^+ channel family, Kir4.1, on melanocyte plasma membrane plays a critical role for the generation of K^+ gradient between plasma membrane and the endolymph fluid, which is essential for the transduction of sound by hair cells (Ando and Takeuchi, 1999).

In contrast to the clear functions of human melanocytes, physiological functions of melanocytes in other organisms are less clear and appear to be variable depending on distinct organisms. In lower vertebrates such as reptiles, amphibians and fish, melanocytes can generate diverse pigment patterns in the skin. Such pigment patters could be important in mediating color adaptation, predator evasion, feeding priority, or sexual selection in individual animals. Hence, in these organisms, melanocytes appear to play more socio-environmental functions in addition to their direct functions in protecting the skin from UV radiation.

2.2. Melanocyte development

Melanocytes in vertebrates are derived from the neural crest, which arises during gastrulation of embryogenesis at the dorsal edge of the neural plate (Thomas and Erickson, 2008). Once emerged, these neural crest cells migrate intensively to the specific sites where they differentiate into a wide range of lineages including peripheral neurons, endocrine cells, bone, cartilage, connective tissue, and melanocytes (Anderson, 2000). While the mechanisms by which multi-potent neural crest cells are specified into the melanocyte lineage remain unclear, it has been suggested

that immature precursors for melanocytes, referred as melanoblasts, are originated from bi-potent glial-melanoblast progenitors, whose existence has been demonstrated in avian and mouse at the neural crest premigratory and migratory stages (Dupin et al., 2000; Mollaaghababa and Pavan, 2003). Recent studies indicate that melanoblast fate decision in these bi-potent progenitors is regulated by, at least in part, Wnt signaling pathway (Dunn et al., 2000). Disruption of Wnt signaling in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ combinatory knockout mice result in a dramatic reduction of several neural crest derivatives, including melanoblasts (Ikeya et al., 1997). Similarly, the mice in which β-catenin, an essential component of the canonical Wnt signaling pathway, is conditionally ablated in neural crest exhibit loss of embryonic melanoblasts (Hari et al., 2002), indicating an indispensable role of the canonical Wnt signaling pathway in melanoblast development. By contrast, gain-of-function of Wnt signaling by forced expression of Wnt1 in mouse neural tube explants culture shows marked increase of melanoblasts at the expense of glial cells (Dunn et al., 2000). Consistently, in zebrafish, overexpression of a constitutive active form of β-catenin in migratory neural crest cells also induces pigment cell formation along with a dramatic reduction of neuronal and glia lineages (Dorsky et al., 1998). Hence, these data clearly indicate a crucial role of Wnt signaling in restricting the glial-melanoblast progenitors toward a melanoblast fate.

Once melanoblasts arise, they migrate along the dorsolateral pathway between the dermatome and the epidermis toward the ventral midline (Steel et al., 1992). In mammalians, most of the melanoblasts further invade the overlying epidermis, where they proliferate and migrate extensively to distribute the entire epidermis (Steel et al., 1992; Wilkie et al., 2002). This is clear contract to those in fish, in which all of these migrated melanoblasts stay in the dermis. In the hairy region of mammalian skin, melanoblasts further enter the newly developing hair follicles where they finally become localized (Figure 1). Once in the hair follicles, melanoblasts are segregated into two populations: one consists of hair matrix melanocytes, which is responsible for pigmentation of the initial hairs; the other population consists of melanocyte stem cells, which are localized at the lower permanent portion of the hair follicle (the bulge region) (Nishimura et al., 2002) and are responsible for the maintenance of the hair follicle pigmentary system in the subsequent hair cycles (Figure 1). In non-hairy regions of the human skin, melanoblasts stay immature and reside on the basement membrane of the epidermis where they undergo terminal differentiation into mature melanocytes upon stimulation from keratinocytes.

Dysregulation in melanoblast development typically results in hypo-pigmentation phenotypes, which is readily characterized in animals as piebaldism, congenital unpigmented spotting in the skin and hairs (Bennett and Lamoreux, 2003). Interestingly, spotting patterns of these mutant animals do not appear to be random: these spotting mutants tend to exhibit characteristic spotting patterns including belly spots, head spots, and piebald spotting (Figure 2) (Baxter et al., 2004). Although the reasons how these characteristic patterns are developed during embryogenesis are not completely understood, recent observations of the process of melanoblast development have provided some mechanistic insights into the formation of these spotting phenotypes. It has been demonstrated that melanoblast distribution in the embryonic epidermis is not uniform: melanoblasts exist at higher density in the head, cervical, and tail regions; whereas their density is much lower in the trunk region (Wilkie et al., 2002). Intriguingly, this distribution pattern is well correlated with pigmented and unpigmented pattern of the mutant animals: pigmented areas coincide with regions containing larger numbers of melanoblasts, while white spotting patterns seem to be paralleled with the regions of lower melanoblast density (Yoshida et al., 1996). Hence, based these observations, it is proposed that the piebald-type white spotting phenotypes are caused by dramatic reduction of total melanoblast number, rather than selectively affecting melanoblast precursor cells responsible for populating a given area (Baxter et al., 2004). By contrast, the belly and head spotting phenotypes would be explained by the failure of melanoblast migration because melanoblasts should travel relatively longer distance to reach the belly and head areas from the areas where melanoblast density is high (Baxter et al., 2004).

Through the extensive studies of the white spotting mutant animals, a number of key genes for melanoblast development have already been identified and characterized. Examples of these genes include *Pax3* (*Paired-box 3*), *Sox10* (*Sex-determining region Y-box 10*), *Mitf* (*Microphthalmia-associated transcription factor*), *Edn3* (*endothelin 3*), *Ednrb* (*endothelin receptor B*), *Kit* (*c-Kit tyrosine kinase receptor*), *Kitl* (*Kit ligand*, also called as *SCF* or *steel factor*), and *Snai2* (also called as *Slug*) (Bennett and Lamoreux, 2003) (Figure 3).

Pax3 is a member of paired-box homeodomain transcription factors which are highly conserved across species (Buckingham and Relaix, 2007). Mice harboring heterozygous loss of functional *Pax3* mutation, which is encoded by the *Splotch* locus, exhibit a ventral white spotting phenotype, reflecting a role of Pax3 in melanoblast development (Moase and Trasler, 1992). In contrast, the homozygous mutant mice are embryonic lethal due to multiple defects during cardio-muscular and neural crest development (Moase and Trasler, 1992). Hence, Pax3 plays key roles in neural crest development as well as muscle and cardio-vasculature formation. Requirement of Pax3 in specification of neural crest is conserved throughout vertebrates (Buckingham and Relaix, 2007). Although the exact role of Pax3 in melanoblast development is less clear, it has been recently suggested that Pax3 may play a key role in maintaining the

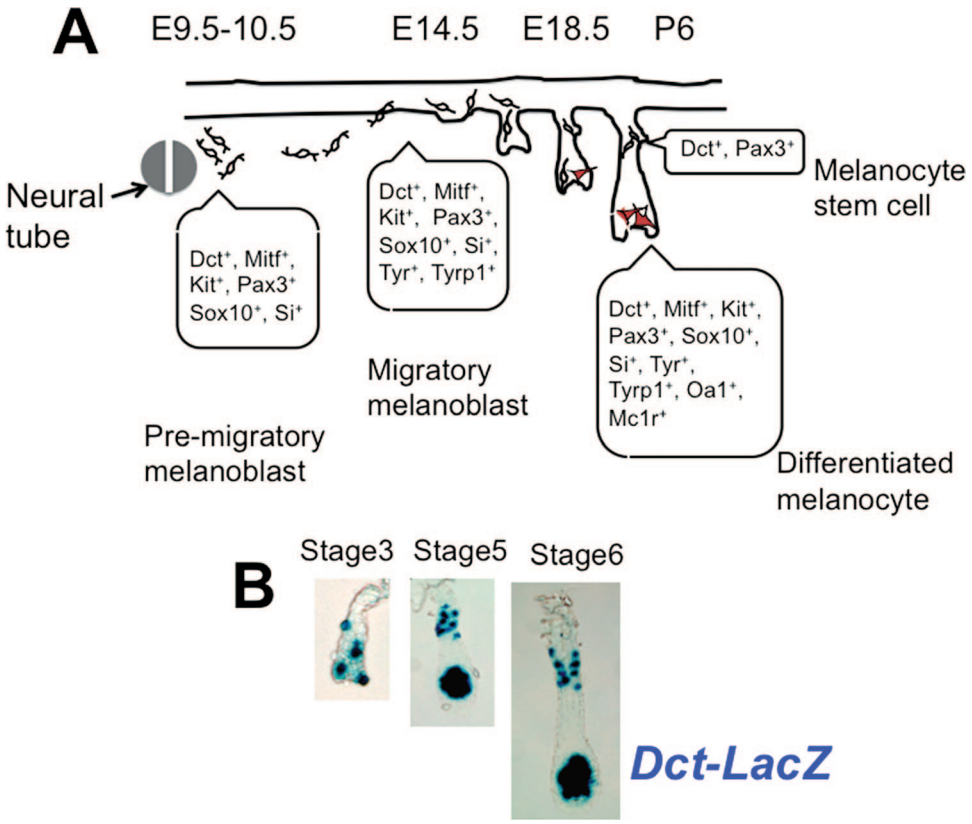

Figure 1. Schematic drawing of melanocyte development in the mouse. (A) During embryogenesis, melanoblasts emerge in the neural crest at embryonic day (E) 9.5–10.5. These earliest stages of melanoblasts are characterized by the expression of Dct, Kit, Mitf, Pax3, Sox10, and Si. Once arise from neural crest, melanoblasts migrate in the developing dermis toward the ventral midline. Subsequently, at E12.5–13.5, they enter the epidermis where they actively migrate and proliferate to distribute the entire body. In addition to the pre-migratory melanoblast markers, these migratory melanoblasts express several melanosomal proteins including Tyr and Tyrp1. Upon initiation of hair follicle morphogenesis, the melanoblasts enter the newly developing and are segregated into two populations: one locates in the hair matrix where melanoblasts are differentiated into mature melanocytes; and the other colonizes at the putative bulge region, where melanoblasts are restricted in a resting status to become melanocyte stem cells. Melanocytes in the hair matrix express Oa1 and Mcr1 in addition to the migratory melanoblast markers, whereas in the bulge melanoblasts, expression of several melanoblast markers including Kit, Mitf, Pax3, Sox10, Tyr, and Tyrp1 are gradually downregulated. **(B)** Melanoblast localization during hair follicle morphogenesis. Skin fragments from *Dct-LacZ* transgenic mice were whole-mount stained with LacZ to visualize melanoblasts in the developing hair follicle. Melanoblasts colonize at the putative bulge region at stage 5–6 of hair follicle morphogenesis, suggesting stem cell specification would take place from stage 5–6 of hair follicle morphogenesis onward.

balance between undifferentiated and differentiated status of melanoblasts (Lang et al., 2005; Watanabe et al., 1998). For instance, several melanocyte-specific genes such as *Mitf* and *Dct* have a consensus-binding motif for Pax3 in their promoter/enhancer regions, and their expression appears to be directly regulated by Pax3 (Lang et al., 2005). *Mitf*, a master regulatory transcription factor for a melanocyte lineage, is transactivated by the synergistic action of Pax3 and Sox10 (Watanabe et al., 1998) (Figure 3), which is critical in mediating cell fate commitment into the melanocyte lineage. By contrast, *Dct*, which is one of the downstream targets of Mitf, is transactivated by the synergism between Mitf and Sox10 (Jiao et al., 2004), while Pax3, acting as a transcriptional repressor, antagonizes Mitf/Sox10-mediated transactivation of *Dct* (Lang et al., 2005) to prevent terminal differentiation of immature melanoblasts. Thus, Pax3 may play a role in mediating melanocyte-fate commitment, while simultaneously maintaining an undifferentiated state of immature melanoblasts (Lang et al., 2005).

Sox10 is a member of the high mobility group (HMG) family of transcription factors (Kelsh, 2006). *Sox10* expression in pre-migratory neural crest cells is conserved throughout vertebrates (Kelsh, 2006), and it expression is gradually restricted in glial and melanocyte lineages (Hou and Pavan, 2008). Sox10 has broad functions during neural crest development including neural crest formation, fate specification, and stem cell maintenance (Hou and Pavan, 2008; Kelsh, 2006). These functions of Sox10 are also well conserved in vertebrates. For instance, in mice, heterogeneous *Sox10* dominant negative mutant, which is encoded by *dominant megacolon* (*Dom*) locus, exhibits white spotting and megacolon (Potterf et al., 2001) (Figure 2C). In homozygous, the mutant mice are embryonic lethal due to multiple failures of neural crest derivatives including loss of melanoblasts, enteric ganglia and peripheral nerve cells (Potterf et al., 2001). Intriguingly, these similar defects are also found in homozygous *Sox10* mutants

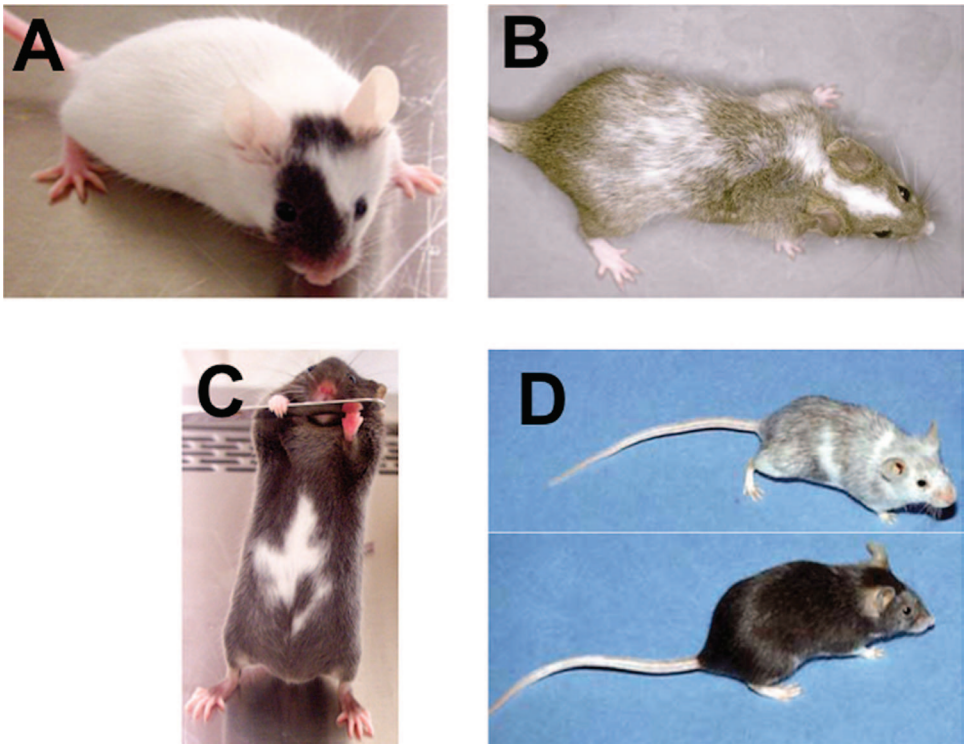

Figure 2. Defects in melanocyte development cause white spotting, while the stem cell defect results hair graying. (A) A *Ednrb*$^{s-l}$/*Ednrb*$^{s-l}$ mouse demonstrating extensive piebald spotting. **(B)** A *Kit*$^{W-2J/+}$ mouse demonstrating a white head blaze, and small dorsal spot on the back. **(C)** A *Sox10*$^{Lacz/+}$ mouse exhibiting the characteristic white belly spot. **(D)** A *Mitf*$^{vit/vit}$ mouse (upper) exhibits gradual hair graying. A lower mouse is age-matched control. Adapted from the WEB site of the European Society for Pigment Cell Research (ESPCR), "Color genes": http://www.espcr.org/micemut/.

in zebrafish (Dutton et al., 2001). Hence, in the melanocyte lineage, Sox10 plays indispensable roles in promoting survival, migration and differentiation (Wegner, 2005). Although its downstream targets are not completely clarified, it has been demonstrated that Sox10 preferentially binds enhancer regions of several melanogenic genes including *Mitf, Dct, Tyr*, and *Tyrp1,* and enhances their expression (Murisier and Beermann, 2006). Thus, Sox10 represents one of key players in orchestrating melanocyte differentiation and maturation by directly regulating expression of genes required for melanogenesis (Wegner, 2005).

The *microphthalmia* (*mi*) mutant mice exhibit hypopigmentation, small eyes, and osteopetrosis phenotypes in homozygous (Levy et al., 2006). This mutant locus encodes Mitf, a member of the Myc supergene family of basic–helix–loop–helix–leucine–zipper (bHLH-Zip) transcription factors. Mitf is thought to be a master regulatory transcription factor for the melanocyte lineage (Levy et al., 2006). Mitf directly regulates various melanocyte-specific genes including *Dct, Tyr, Tyrp1, Si, Aim1, Mc1r, Mlana*, and *Trpm1* (Levy et al., 2006) (Figure 3). In the melanocyte lineage, Mitf has broad implications for survival, cell cycle regulation, migration, and differentiation, and these functions of Mitf appear to be well conserved in vertebrates (Hou and Pavan, 2008). Fate mapping studies of the *Mitf*-deficient mice showed dramatic reduction of migratory melanoblasts soon after the emergence of melanoblasts (Nakayama et al., 1998), suggesting that Mitf is required for the survival of melanoblasts at the early stage of their development. Consistently, in the zebrafish *mitfa/nacre* mutants, melanocytes are specifically lost in both embryo and adult, indicating essential role of mitfa in the maintenance of the melanocyte lineage (Lister et al., 1999). Mitf expression is also seen in retinal pigment epithelial (RPE) cells, osteoclasts, and mast cells, and plays important roles in these cell lineages (Hershey and Fisher, 2004; Planque et al., 2004). Interestingly, *Mitf*-homologous genes are also found in *Drosophila* and *C. elegans*, and mutation analysis *Drosophila Mitf* (*Dmel*) showed its potential role in eye imaginal disc formation, suggesting that the role of Mitf in eye development may be partially conserved between flies and vertebrates (Hallsson et al., 2004).

Endothelin signaling plays an indispensable role at the early stage of melanocyte development. In fact, it is known that classical hair spotting loci, *piebald* and *lethal spotting* (*ls*) encode *Ednrb* and its ligand *Edn3*, respectively (Giller et al., 1997; Matsushima et al., 2002) (Figure 2A). Consistently, hypomorphic mutations in *EDN3* or *EDNRB* genes result in Waardenburg syndrome in humans (Attie et al., 1995; Edery et al., 1996). Hence, it is clear from these

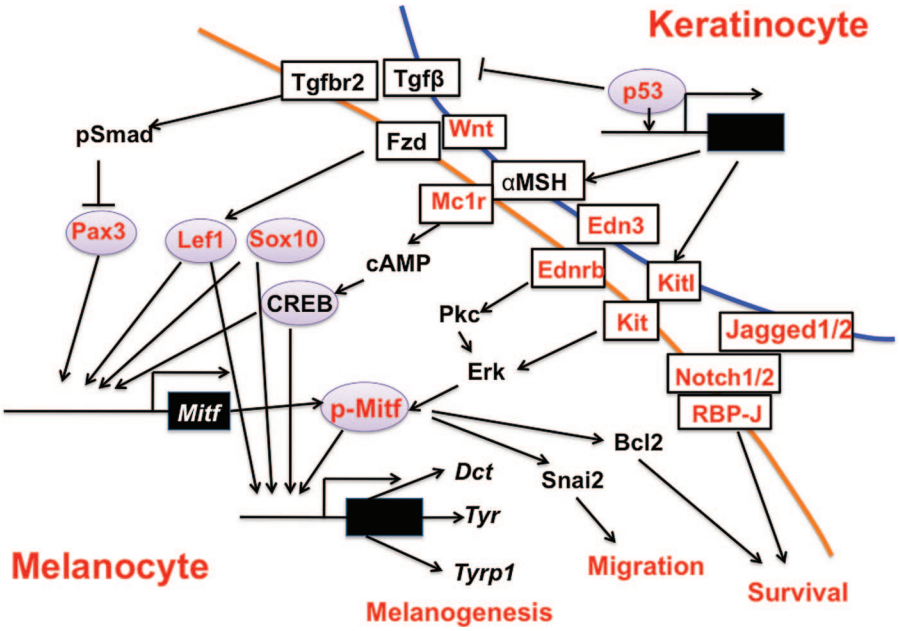

Figure 3. Simplified schematic showing of key molecules and signaling pathways implicated in melanocyte-keratinocyte interactions. In the melanocyte lineage, a series of transcription factors, including Pax3, Sox10, Creb, Lef1, and Mitf play crucial roles in the regulation of melanocyte proliferation, differentiation, and survival. Expression the melanocyte master regulatory gene *Mitf* is regulated by synergistic action of Pax3, Sox10, Lef1, and Creb on its promoter/enhancer. Mitf activates its own promoter by positive feedback loop. Once translated, Mitf protein is phosphorylated by the Erk kinase downstream activation of c-Kit signaling pathway. Phosphorylation of Mitf results in stabilization of Mitf-p300 transactivation complex, and thereby upregulating its transcriptional activity to stimulate expression of the target genes including *Dct, Typ*, and *Tyrp1*. c-Kit signaling also stimulates expression of Bcl2 to mediate melanocyte survival. Activation of melanocortin signaling pathway increases cytoplasmic cAMP concentration, which results in activation of CREB. This activated CREB directly binds the cAMP-responsive elements present on the promoter regions of *Mitf* and various melanosomal genes, and stimulates their gene expressions. Wnt signaling is required for melanocyte development. Activation of Wnt signaling results in the stabilization of β-catenin/Lef complex, which leads in transactivation of downstream target genes such as *Mitf* to promote melanocyte-fate specification and melanocyte differentiation. In contrast, keratinocyte expression of TGF-β plays a role in suppressing melanogenesis. Activation of TGF-β signaling results in the repression of *Pax3* through phosphorylated Smads, which leads to *Mitf* repression to block melanocyte activation. Activation of Notch signaling is essential for the survival of melanoblasts, while underlying molecular mechanism is unclear. pSmad, phosphorylated Smads; Fzd, Frizzeled; cAMP, adenosine 3′:5′-cyclic monophosphate; CREB, cAMP responsive element binding protein, Pkc protein kinase C; Erk, extracellular signal-regulated kinase/mitogen-activated protein kinase, and pMitf, phosphorylated Mitf. Signaling molecules and transcription factors whose physiological roles in melanocytes are evidenced by genetic mutant animals are shown in red.

phenotypes that endothelin signaling is implicated in the melanocyte development. More precise studies using the mice in which *Ednrb* gene was conditionally ablated in the melanocyte lineage have shown that endothelin singling is transiently required for melanoblast development between E10.5 and E12.5, when melanoblasts are dispersing from the neural tube (Shin et al., 1999). Thus, these data suggests an essential role of endothelin signaling in promoting proper migration of melanoblasts at the early stage of melanocyte development rather than mediating fate-specification of melanoblasts. Consistently, in avian embryo, *Ednrb2* expression is upregulated in melanoblasts prior to migrating into the dorsolateral pathway, and loss of *Ednrb2* results in dramatic reduction of migrating melanoblasts (Harris et al., 2008). This indicates conservatory function of endothelin signaling between mammalian and avian species in promoting melanoblast migration. Interestingly however, in zebrafish, loss of function mutant of *ednrb1/rose* exhibits abnormal pigmentation pattern only in an adult stage, while leaving early larval pigment patterns intact (Parichy et al., 2000). This suggests that there may be certain species-specific variations in the requirement of endothelin signaling during melanocyte development.

Both melanocytes and melanoblasts highly express c-Kit tyrosinase kinase receptor, whose activation is mediated by Kitl (also known as steel factor or SCF) expressed in the epidermis (Yoshida et al., 1996; Yoshida et al., 1996). c-Kit signaling is implicated in diverse aspects of melanocyte regulation including proliferation, survival, migration, and differentiation (Jordan and Jackson, 2000; Mackenzie et al., 1997; Yoshida et al., 1996). Administration of an antagonistic antibody against c-Kit into mice depletes actively proliferating melanoblasts/ melanocytes, indicating an essential role of c-Kit signaling in proliferation and survival of these cells (Nishikawa et al., 1991; Yoshida et al., 1996). c-Kit signaling activates the MAP kinase pathway, which eventually phosphorylates Mitf by Erk kinase (Hemesath et al., 1998; Wu et al., 2000). Phosphorylation of Mitf results in stabilization of Mitf-p300 transactivation complex, and thereby enhancing its transcriptional activity to stimulate expression of the target genes (Hemesath et al.,

1998; Price et al., 1998; Wu et al., 2000). These target genes include *Bcl2* and *Snai2*, whose expressions are critical for melanoblast survival and migration, respectively (McGill et al., 2002; Sanchez-Martin et al., 2002) (Figure 3). This activation of Mitf is also implicated in induction of melanocyte terminal differentiation. The pleiotropic role of c-Kit signaling in the melanocyte lineage seems to be highly conserved among vertebrate species. In the mouse, *Kit* and *Kitl* mutations correspond to dominant spotting *W* and *Steel (Sl)* loci, respectively (Chabot et al., 1988; Huang et al., 1990; Miller et al., 2007) (Figure 2B). In humans, *KIT* and *KITLG* mutations are identified in piebaldism, a dominant ventral depigmentary disorder (Giebel and Spritz, 1991; Miller et al., 2007). Analysis of zebrafish *c-Kit* mutant *sparse* has demonstrated crucial functions of zebrafish c-Kit in survival and migration of melanocyte precursors (Parichy et al., 1999). Thus, from these phenotypes of Kit signaling mutants it is evident that Kit signaling plays a crucial role in melanocyte development.

2.3. Genetics of melanocytes

Melanocytes afford an advantageous model in understanding molecular basis of diverse cellular processes, since alterations in genes involved in the melanocyte regulation are easily identifiable as coat color mutant in animals. Indeed, currently more than 120 different loci have been identified as coat color mutants in mice (Bennett and Lamoreux, 2003). While some exceptions, these classical pigmentation mutants fall into roughly two distinct functional subgroups (Bennett and Lamoreux, 2003). The first group is comprised of mutations of genes involved in melanocyte development. As described in detail in the previous section, these mutations typically exhibit diverse degree of congenital white spotting of hair and skin due to reduction of melanoblast number and/or a defect in melanoblast migration during development. The other group consists of mutations of genes implicated in melanocyte functions including melanin biosynthesis, melanosome generation/maturation, and melanosome transfer. Mutations in such genes result in alterations in coat color, or hypo- or hyper-pigmentation phenotype without developing any white spotting. The examples of genes in this group are: *Atrn, Dct, Mcr1, Myo5a, Oa1, P, Rab27a, Si, Tyr*, and *Tyrp1* (Bennett and Lamoreux, 2003).

In addition to these two subgroups, a new class pigmentation mutants, which exhibit a premature hair graying phenotype from the second hair cycles, has recently been proposed particularly from the gene knockout approach in the mouse (Mak et al., 2006; Moriyama et al., 2006; Nishimura et al., 2005; Ruzankina et al., 2007; Schouwey et al., 2007). In these mutants, hair pigmentation appears to be normal in the initial hairs, while it is prematurely lost from the subsequent hair cycles. As hair pigmentation from the second hair cycle is exclusively derived from melanocyte stem cells, this hair graying phenotype is attributed to the improper maintenance or emergence of melanocyte stem cells in the initial hair follicles. A classical mutant, $Mitf^{vit/vit}$ is categorized in this group (Lerner et al., 1986; Nishimura et al., 2005) (Figure 2D). Although the responsible genes are not clarified, *Faded (fe)* (Oh et al., 1986) and *London Gray (lgr)* loci (Nishina et al., 1994) have been reported to exhibit hair graying phenotype. In addition to these spontaneous mutants, recent gene ablation studies indicate that loss of *Bcl2*, *Notch1* and *Notch2*, or *RBP-J* results in premature hair graying starting from the second hair cycle (Aubin-Houzelstein et al., 2008; Moriyama et al., 2006; Schouwey et al., 2007) (Figure 6). Hence, by combing genetic manipulations and phenotypical identification of hair graying mutants, hair pigmentary system would offer an advantageous opportunity for elucidating genetic basis of stem cell regulation.

Taking advantage of mouse pigmentary system where genetic alterations affecting melanocyte regulation are readily identifiable by abnormal coat and/or skin color phenotypes, a large-scale chemical mutagenesis screen has been conducted to dissect new molecular pathways implicated in homeostatic regulation of mouse pigmentation (Fitch et al., 2003). This approach has allowed researchers to identify several new dark skin and dark coat color loci that have been previously unrecognized as pigmentation mutants (Fitch et al., 2003; McGowan et al., 2008; Van Raamsdonk et al., 2004). Importantly, this random mutagenesis approach offers a significant opportunity not only for identifying melanocyte endogenous molecules, but also for elucidating external molecular mechanisms of the surrounding microenvironment by which melanocyte homeostasis is regulated.

Because of visibility of the clear pigmentation pattern and feasibility of gene manipulations, zebrafish is another excellent model organism that allows identifying pigment genes by phenotype-driven genetics approaches. Because many of genes and biological processes specific for vertebrate development such as those in neural crest development appear to be conserved in zebrafish, genetic programs identified in zebrafish would be directly applied for human genetics. Indeed, lots of pigmentation mutants including *rose/ednrb1, sparse/kit, nacre/mitfa, colourless/sox10*, and *golden/slc24a5* are found to be conserved human pigmentary disorders (Pickart et al., 2004). Hence, with large-scale forward genetics screens and identification and characterization of pigmentation mutants, the zebrafish offers invaluable opportunities in understanding of not only pigment cell biology, but also human disease.

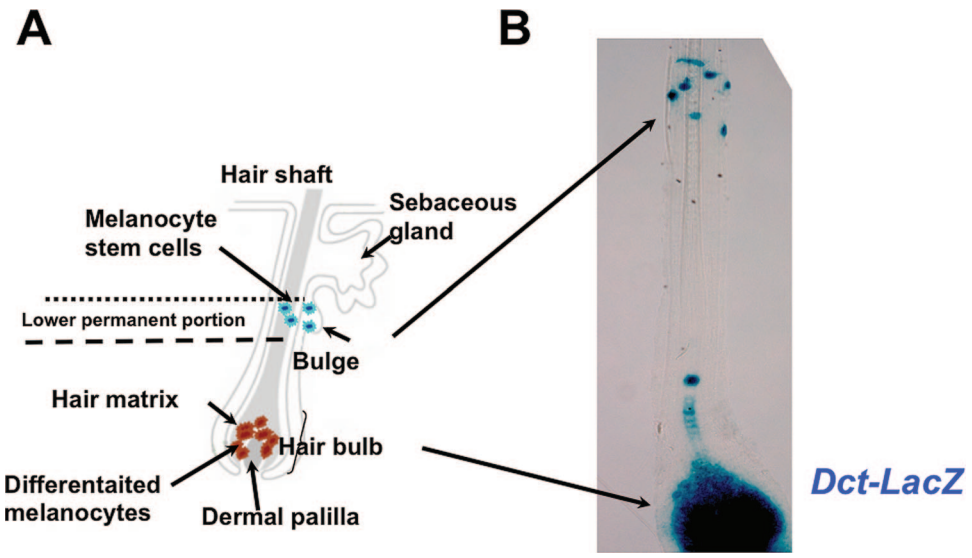

Figure 4. Localization of melanocyte stem cells in the hair follicle. (A) Schematic illustration of the hair follicle. The hair follicle is divided into two portions: two-thirds of lower hair follicle (transit portion) that completely reforms itself over the hair cycle; and the upper permanent portion of the follicle that is maintained throughout hair cycling. Melanocyte stem cells reside in the lower permanent protion including the bulge region, while differentiated melanocytes are localized in the hair matrix. **(B)** Whole mount staining of the *Dct-LaZ* hair follilce. Using *Dct-LaZ* transgenic mice, melanocyte stem cells can be located at the lower permanet protion of the hair follicle, which is anatomically seggregated from differentiated melanocytes in the hair bulb.

3. Melanocyte stem cells and regeneration of pigmentary system

The hair follicle is a continuously replenishing mini-organ, where two-thirds of the lower follicle (transit portion) completely is regenerated over the hair cycle, whereas the remaining upper permanent portion is maintained (Stenn and Paus, 2001) (Figure 4). In this regard, the hair follicle offers an ideal model system by which the process of tissue regeneration is analyzed under the physiological condition. The periodic hair cycle consists three distinct phases of hair follicle growth phase (anagen), followed by a regression phase (catagen), and a resting phase (telogen). Melanocytes appear at the onset of anagen phase in the hair matrix where they actively proliferate and differentiate into mature melanocytes. During catagen, these melanocytes are depleted from the follicles by apoptosis, and finally melanocytes become absent in telogen hair follicle until the next melanogenesis initiated in the subsequent anagen phase. Given this regenerative cycle of melanogenesis, the presence of the stem cells for follicular melanocytes had been suggested over a decade (Yoshida et al., 1996).

Proliferation and differentiation of melanocytes in the hair follicle is highly dependent on c-Kit signaling pathway. In fact, administration of an antagonistic anti-c-Kit antibody into neonatal mice depletes virtually all the proliferating melanocytes from the hair matrix (Nishikawa et al., 1991), resulting in loss of hair pigmentation in the initial hairs. Importantly, however, despite the obvious depletion of melanocytes in the initial hair follicles, the hair pigmentation is restored in the subsequent hair cycles. These data strongly suggest that the stem cells, which are resistant against c-Kit antibody treatment and is capable of regenerating the hair pigmentary unit, are present somewhere in the hair follicle. Using *Dct-LacZ* transgenic mice, which allow visualizing melanocyte lineage by the specific expression of the *LacZ* transgene (Mackenzie et al., 1997), a small subset of melanoblasts were found to remain localized at the bulge region of the hair follicle in c-Kit antibody treated animals (Nishimura et al., 2002). These melanoblasts are immature, infrequently dividing, and capable of regenerating follicular pigmentary unit when they are grafted onto neonatal skin. These capacities of the melanoblasts satisfy the definitions for stem cells. Hence, it is evident that the melanoblasts localizing at the bulge region of the hair follicle represent a stem cell population in the melanocyte lineage (Nishimura et al., 2002) (Figure 4). Because these bulge stem cells are anatomically segregated from their differentiated progenies in the hair matrix (Figure 4), this distribution pattern of follicular melanocytes allows locating the individual stem cells at this specific location. Hence, melanocyte stem cells offer an advantageous model in which to understand how stem cell behavior is regulated by their surrounding microenvironment. Interestingly, the bulge melanoblasts can also be found in human hair follicles, while whether they are corresponding to stem cells or not awaits further investigation.

In the human skin, immature melanocytes reside on the basement membrane of the interfollicular epidermis, where they play a role in formation of skin pigmentation. Whereas the origin of the human epidermal melanocytes

has not been determined yet, several lines of evidence suggests that these melanocytes may also arise from the bulge melanoblasts in the hair follicle. Vitiligo is a condition where the epidermal melanocytes are lost from the skin. Intriguingly, it is known that repigmentation process of vitiligo is initiated in a perifollicular manner (Falabella and Barona, 2009), supporting a follicular origin of the epidermal melanocytes. Indeed, one of the therapeutic treatments of vitiligo is to perform autologous transplantation of pigmented follicles, which can efficiently achieve repigmentation of the vitiligo skin (Rusfianti and Wirohadidjodjo, 2006). These facts raise the possibility that the hair follicle could serve as a reservoir for the melanocytes in the interfollicular epidermis. This notion was further supported by the histological analysis of the repigmentation process of the vitiligo skin where upward migration of the bulge melanoblast toward the interfollicular epidermis was observed (Cui et al., 1991). Similar upward migration of the bulge melanoblast was also observed in *Keratin14-KitL* transgenic mice (Nishimura et al., 2002) by which the humanized pigmented skin can be mimicked in mice via the forced expression of KitL in the basal layer of the epidermis (Kunisada et al., 1998). Hence, these observations would provide compelling evidence that the bulge melanoblasts in human follicles may represent a reservoir population not only for the bulb melanocytes in the hair follicle but also for the epidermal melanocytes in the skin, although more precise fate analysis is needed to prove the capacity of the bulge melanoblasts in regenerating the epidermal melanocytes.

As discussed in the previous section, the feasibility of phenotype-driven genetic approaches would be one of the advantageous features of melanocyte regeneration system. While regeneration of mouse hair pigmentation offers a model for the study of stem cell regulation, genetic manipulations in mice are still laborious and time consuming. Therefore, establishment of a melanocyte regeneration model in other organisms in which a functional genetics approach is easily achieved is highly anticipated. Accumulating evidence demonstrates that zebrafish melanocyte regeneration system would be one promising candidate for such a model.

It is known that zebrafish can regenerate the caudal fin following amputation. Intriguingly, along with the fin regeneration, the melanocyte pattern is also recovered as it becomes undistinguishable from normal fins (Rawls and Johnson, 2000). This melanocyte regeneration does not appear to be mediated by multiplication of the pre-existing differentiated melanocytes, but such regeneration seems like being facilitated by melanocyte progenitors or stem cells. In support of this idea, preventing new melanin synthesis by a specific melanin synthesis inhibitor, phenylthiourea (PTU) resulted in completely blocked visible melanocyte regeneration without affecting the distribution of pre-existing pigment melanocytes, but soon after the removal of PTU, pigmented melanocytes appeared within the regenerating fin (Rawls and Johnson, 2000). This result strongly suggests that the cells responsible for melanocyte regeneration are originated from unpigmented precursors, but not existing pigmented melanocytes. This initial wave of melanocyte regeneration is largely dependent on c-Kit signaling as *kit* null mutants do not exhibit melanocyte recovery during initial stages of melanocyte regeneration (Rawls and Johnson, 2000). Interestingly though, these *kit* mutants can eventually regenerate their pigmentation pattern in the fin regenerate, suggesting the existence of two distinct classes of melanocyte precursor/stem cell populations in terms of requirement of c-Kit signaling (Rawls and Johnson, 2000). Melanocyte regeneration is possible even after multiple rounds of fin amputation. While melanocyte stem cells have not been identified in zebrafish, this remarkable capacity of melanocyte regeneration strongly suggests the presence of self-renewing melanocyte stem cells.

In addition to the reconstitution of melanocytes during fin regeneration in adult fish, melanocyte regeneration also occurs in zebrafish larvae. It has been demonstrated that melanocytes are efficiently depleted by the treatment of the larvae with a melanocyte-specific cytotoxic agent, (2-morpholinobutyl)-4-thiophenol (MoTP) (Yang and Johnson, 2006). Once in cells, MoTP is converted to a cytotoxic form only when it is hydroxylated by tyrosinase. This feature allows MoTP to selectively deplete pigmented melanocytes in which tyrosinase-mediated melanin biosynthesis is highly activated, while leaving immature melanocytes intact (Yang and Johnson, 2006). Interestingly, following depletion of melanocytes by MoTP treatment, it has been found that melanocytes reappear in the larvae. BrdU incorporation assay indicates that these regenerated melanocytes are derived from proliferating melanocyte precursors that are otherwise maintained in a quiescent status (Yang and Johnson, 2006). Hence, these data suggest that melanocyte precursors or stem cells existing in the larvae may participate melanocyte regeneration following depletion of mature melanocytes by MoTP treatment, although it is not clear why and how melanocyte regeneration occurs following melanocyte depletion in larvae. Further characterization of these putative stem cells in terms of their localization will help to understand how the stem cells are regulated at the niche.

Although stem cells responsible for melanocyte regeneration in zebrafish are less characterized, the clear pigmentation phenotypes are deeply proved to be advantageous in investigating underlying molecular mechanisms of melanocyte regeneration. Zebrafish is a valuable model organism for functional genetics, which is partly due to its feasibility of performing large scale genetic screens such as random forward mutagenesis and chemical genetic

screening (Pickart et al., 2004). Hence, by combining such genetic approaches and phenotype-based screens to identify abnormal melanocyte regeneration phenotypes, zebrafish would provide an unrivaled opportunity to elucidate molecular basis of melanocyte regeneration. Because key molecular pathways involved in neural crest development are highly conserved across vertebrate species (Anderson, 2000), such molecular information obtained from zebrafish studies is supposed to be directly applicable in understanding human melanocyte regulation.

4. Molecular basis of melanocyte stem cell regulation

4.1. Molecular characterization of melanocyte stem cells

Understanding molecular mechanisms underlying stem cell regulation within the niche is critical for stem cell biology. However, this has been largely hampered by the lack of systematic approaches to dissect the complete molecular makeup of stem cells at the niche. In an effort to characterize the molecular basis of the regulation of melanocyte stem cells at the niche, one attempt has been made to characterize molecular expression profile in melanocyte stem cells (Osawa et al., 2005). The gene expression and immunohistological analyses indicate that several key melanogenic transcription factors including S*ox10* and *Mitf* are dramatically downregulated in melanocyte stem cell population (Osawa et al., 2005) (Figure 1). Intriguingly, in addition to these functional genes for melanocytes, the expression of several house keeping genes is also shown to be downregulated, suggesting an idea that basal transcription is globally down-modulated in the melanocyte stem cells.

Given critical roles of Mitf and Sox10 in the promotion of melanogenesis, their downregulation is supposed to lead to suppression of the melanocyte proliferation and differentiation. Indeed, it has been demonstrated that down-modulation of *Mitf* in a melanoma cell line results in induction of a G1 cell-cycle arrest which is associated with a dramatic morphological change into formation of a small and rounded-cell morphology (Carreira et al., 2006). Hence, these data indicate a crucial role of the Mitf downregulation in induction of the cell cycle exit in melanocytes (Carreira et al., 2006). Intriguingly, the fact that the *Mitf* downregulation induces a rounded-cell morphological change suggests the idea that *Mitf* downregulation may also contribute to the induction of a stem-cell like phenotype. Likewise, Sox10 has been shown to orchestrate melanoblast development and melanogenesis by directly upregulating melanogenic genes required for melanocyte maturation (Wegner, 2005). Thus, its downregulation is supposed to result in attenuation of melanocyte differentiation as it is seen in the $Sox10^{dom}$ mutant mouse (Potterf et al., 2001).

Why are these transcription factors downregulated in melanocyte stem cell population? One possibility is the implication of Wnt signaling. Recent molecular expression studies have revealed the preferential expression of Wnt inhibitors in the bulge epidermal cells, suggesting that Wnt signaling might be constitutively downregulated within the bulge region (Morris et al., 2004; Tumbar et al., 2004) (Figure 5B). Given Wnt/Lef1 pathway-dependent expression of *Sox10* and *Mitf* genes in melanocytes (Aoki et al., 2003; Saito et al., 2003), the inhibition of Wnt signaling may represent one cause of the downregulation of *Sox10* and *Mitf* in the bulge melanocayte stem cells. In addition to the Wnt signaling, the c-Kit signaling pathway also appears to be downregulated at the same region (Mak et al., 2006) (Figure 5C, D), which may also lead in down-modulation of *Mitf* transcriptional activity (Hou et al., 2000; Wu et al., 2000). Although the physiological role of the downregulation of *Sox10* and *Mitf* is unclear, their downregulation might be important for the maintenance of immature and resting states of the stem cells to ensure their long-term survival (Osawa et al., 2005).

Melanocyte stem cells appear to be maintained in a quiescent status at the niche (Nishimura et al., 2002). One of the central questions of stem cell biology is why and how stem cell quiescence is maintained at the niche. Recent loss of function studies have clarified key roles of negative cell-cycle regulators, such as p21, p27, and Pten, in the maintenance quiescent stem cells (Cheng et al., 2000; Groszer et al., 2001; Walkley et al., 2005; Yilmaz et al., 2006). In the melanocyte lineage, loss of *Pten* expression is commonly (∼20%) observed in human melanoma (Wu et al., 2003), suggesting a role of Pten in the regulation of melanocyte growth. Melanocyte-specific ablation of *Pten* gene ameliorates the hair depilation-induced premature hair graying (Inoue-Narita et al., 2008). This is due to abnormal expansion of the stem cell number in the *Pten*-deficient mice. *In vitro* studies, *Pten*-deficient melanocytes showed a dramatic reduction of the cell cycle inhibitor $p27^{kip1}$, suggesting a role of Pten through $p27^{kip1}$ in the induction of cell cycle arrest (Inoue-Narita et al., 2008). Similarly, loss of *Pten* results in expansion of stem-cell pool in hematopoietic stem cells and neural stem cells, but stem cells are eventually depleted due to exhaustion (Groszer et al., 2001; Yilmaz et al., 2006). Therefore, these data indicate that Pten negatively regulates stem cell proliferation by restricting cell cycle entry. Importantly, disruption of such mechanism would ultimately lead to stem cell exhaustion, suggesting a critical role of relative quiescence in the maintenance stem cell population.

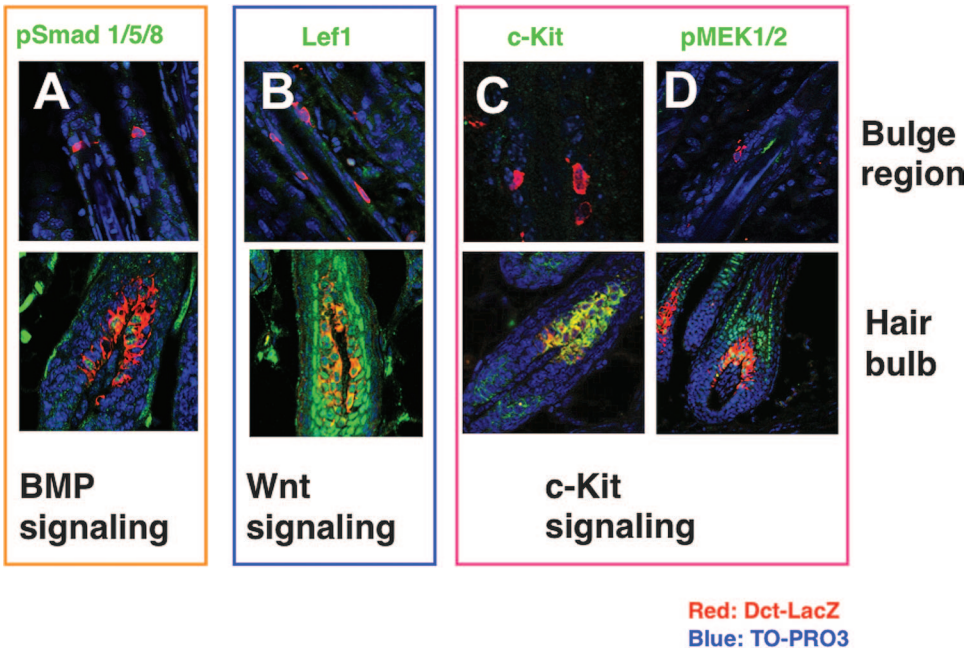

Figure 5. BMP, Wnt, and c-Kit signaling are activated in melanocytes in the hair bulb, but not in melanocyte stem cell population at the bulge region. Skin sections from *Dct-LacZ* transgenic mice were stained with antibody against either phosphorylated Smad1/5/8, Lef1, c-Kit, or phosphorylated MEK1/2 (shown in green). Melanoblasts/melanocytes in the hair bulb or the bulge region are visualized by anti-LacZ antibody (shown in red). Melanocytes in the hair bulb are positively stained with these antibodies, indicating that BMP, c-Kit, and Wnt signaling pathways are activated in these cells. In contrast, none of these markers are detectable in the bulge melanoblasts, suggesting that these signaling pathways may not be implicated in melanocyte stem cell regulation.

Despite intensive studies, molecular mechanisms underlying regulation of stem cells remain largely unclear. Melanocytes afford an advantageous model by which to define molecules required for the stem cell maintenance, because genetic alterations controlling the stem cell viability are easily identifiable by the hair graying phenotype of the animal. Hence, by integrating melanocyte-specific gene manipulations and phenotype-based screening systems, melanocyte stem cell system can provide an invaluable tool to dissect crucial molecules involved in stem cell regulation.

4.2. Cell-to-cell interaction

The fact that melanocyte stem cells are randomly scattered within the bulge region without forming into cell clusters reminds us a possibility that the bulge region itself serves a niche for the melanocyte stem cells rather than depending on specific niche cells that might be present at the bulge. Histological analysis of melanocyte stem cells indicates their direct cell-cell contact with the surrounding keratinocytes (Nishimura et al., 2002), suggesting the involvement of the cell-to-cell interaction in the regulation of the stem cells.

It has been widely accepted that the homeostatic regulation of melanocytes may occur through cell-cell interactions between keratinocytes and melanocytes within the epidermis (Hirobe, 2005). Keratinocytes provide a complex of paracrine factors and adhesion molecules for melanocytes and plays a dominant role in regulating melanocyte survival, proliferation, and differentiation (Figure 3). Although molecular details of the keratinocyte-melanocyte interactions remain largely elusive, a series of recent studies have provided some mechanistic insight into molecular basis of melanocyte regulation by the epidermal keratinocytes (Cui et al., 2007; Yang et al., 2008).

In the human skin, UV irradiation causes skin pigmentation, which is attributed to acceleration of melanogenesis in the epidermis. By contrast, under a normal skin condition, majority melanocytes are maintained in an undifferentiated and resting status. Accumulating evidence indicates these melanocyte behavior is tightly regulated by the surrounding keratinocytes. For instance, under the normal condition, the growth and differentiation of the epidermal melanocytes is constitutively suppressed by the keratinocyte expression of TGFβ (Yang et al., 2008). Activation of TGFβ signaling in melanocytes leads to downregulation of *Pax3*, which is mediated direct repression of *Pax3* promoter downstream TGFβ signaling pathway (Figure 3). As activation of the melanogenesis program requires the Pax3-Mitf transcription network, the repression of *Pax3* by TGFβ signaling seizes the ability of melanocytes to respond to the external melanogenesis stimuli (Yang et al., 2008). Thus, the melanocytes are restricted in the undifferentiated and resting

status on the normal keratinocytes. On contrary to the suppressive role of the normal keratinocytes, the keratinocytes following UV irradiation exhibit an intensive melanogenesis stimulatory activity. Several lines of studies have clarified a central role of the tumor suppressor protein p53 in the UV-induced melanogenic response (Cui et al., 2007). The activation of p53 leads to *TGF* β repression in the epidermal keratinocytes (Yang et al., 2008), allowing melanocyte to respond to the external melanogenic stimuli. In addition, the p53 also promotes marked induction of keratinocyte expression of *αMSH* and *KITL,* which is responsible for the acceleration of UV-induced melanogenesis in the epidermis (Cui et al., 2007; McGowan et al., 2008). Hence, by integrating *TGF* β repression and *αMSH* and *KITL* upregulation, the irradiated keratinocytes play a dominant role in stimulating proliferation and differentiation of the melanocytes (Figure 3).

In addition to these keratinocyte-derived paracrine factors, direct cell-cell adhesion between melanocytes and keratinocyte is also required for the proper regulation of melanocytes (Haass et al., 2005). Numerous *in vitro* culture studies demonstrate a dominant role of keratinocytes in promotion of the growth and survival of the primary melanocytes (Halaban et al., 1988; Sviderskaya et al., 1995; Yonetani et al., 2008). It is known that co-culture of melanocytes and keratinocytes can efficiently maintain the primary melanocytes *in vitro*, whereas in the absence of keratinocytes, melanocytes are hardly to grow in culture even in the presence of various keratinocyte-derived growth factors. This suggests the strict requirement of direct cell-cell contact to keratinocytes for the maintenance of the melanocytes (Yonetani et al., 2008). The spatial association between melanocytes and keratinocytes is partly mediated by homophilic interaction of E-cadherin molecules. Loss of E-cadherin expression, which is frequently seen in metastatic melanomas, results in aberrant growth and migration of melanoma cells, while conversely, forced expression of E-cadherin in melanoma cells renders them susceptible for keratinocyte-dependent growth controls (Hsu et al., 2000). Hence, these studies provide compelling evidence for a pivotal role of cell-cell interactions between melanocytes and keratinocyte in the proper regulation of melanocytes, while underlying molecular mechanisms remain largely elusive.

These data clearly indicate a predominant role of the keratinocytes in homeostatic regulation of melanocytes in the epidermis (Figure 3). Therefore, it would be speculated that keratinocytes might also be implicated in the regulation of melanocyte stem cells at the niche. In this regard, it is intriguing to note that TGFβ signaling appears to be preferentially activated in the bulge keratinocytes in the hair follicle (Tumbar et al., 2004). Given the important role of TGFβ signaling in restricting differentiation of the epidermal melanocytes (Yang et al., 2008), it would be possible that keratinocytes via TGFβ signaling may play a role in the maintenance of melanocyte stem cells at the niche. In addition to keratinocytes, dermal fibroblasts are also thought to be involved in melanocyte regulation. It is known that dermal fibroblasts secrete various stimulatory factors at the dermal papilla and stimulate proliferation and differentiation of melanocytes. The question of how melanocyte stem cells are regulated by the cells in other lineage(s) has been incompletely understood. This could be an important issue that should be addressed in future.

4.3. Notch signaling

Notch compromises a family of evolutionally conserved receptors, whose activation is mediated by specific cell-cell interaction with cells expressing the ligand, Jagged or Delta (Artavanis-Tsakonas et al., 1999). Once activated, the intracellular domain of the Notch receptors is cleaved and translocated into nucleus, where it forms transactivation complex with the RBP-J transcription factor to initiate transactivation of various target genes. Accumulating evidence indicates pleiotropic roles for Notch signaling in diverse cellular processes, including cell cycle arrest regulation, apoptosis/survival, differentiation, and stem cell maintenance, while the exact function of Notch signaling is highly cell context dependent (Hurlbut et al., 2007).

A series of recent studies have elucidated that Notch signaling represents a key component among keratinocyte-melanocyte interactions (Aubin-Houzelstein et al., 2008; Kumano et al., 2008; Moriyama et al., 2006; Schouwey et al., 2007) (Figure 6). Gene expression and immunohistochemical analyses showed that Notch signaling and its obligate target Hes1 are activated in the embryonic melanoblasts as well as the bulge melanoblast population (Moriyama et al., 2006), suggesting implication of Notch signaling in the regulation of the melanocyte lineage. As one of the Notch lignad, Jagged2, is abundantly expressed in the surrounding epidermal keratinocytes (Moriyama et al., 2006), Notch signaling in the melanoblasts is supposed to be activated as a consequence of melanocyte-keratinocyte interactions. To investigate the physiological role of Notch signaling in the melanocyte lineage, the *RBP-J* gene was conditionally ablated in a melanocyte-specific manner using a *Tyr-Cre* driver transgenic mouse line (Aubin-Houzelstein et al., 2008; Moriyama et al., 2006; Schouwey et al., 2007) (Figure 6). As the *Tyr-Cre* transgene has been demonstrated to be activated in the melanoblasts from E11.5 onward, the Cre-mediated ablation of the *RBP-J* gene could take place in any cells of these melanoblast descendants including melanocyte stem cells and differentiated melanocytes in the

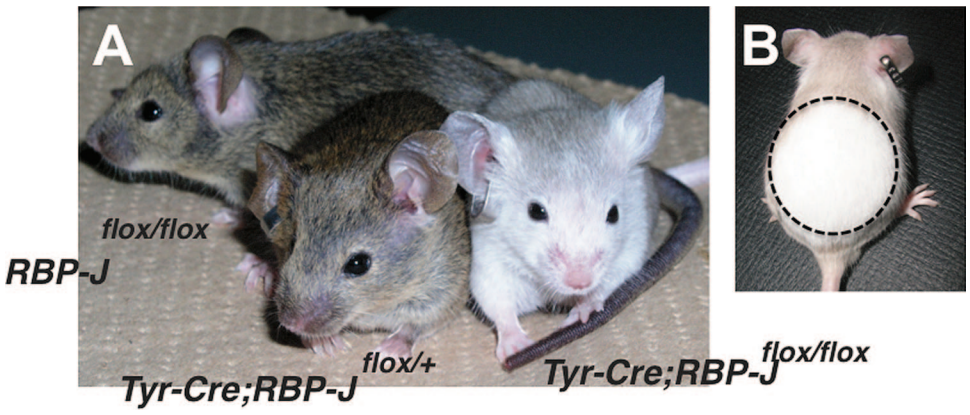

Figure 6. Notch signaling plays a crucial role in melanocyte development. Melanocyte-lineage specific ablation of *RBP-J*, an essential transcriptional mediator for Notch signaling, results in severe dilution of hair pigmentation (**A**). This is due to apoptotic elimination of melanoblasts during development. To test whether Notch signaling plays a role in melanocyte stem cells, the first hairs were shaved to induce the second hair cycling (**B**, circled area). The mice also exhibit premature hair graying from the second postnatal hair cycle (**B**), indicating an indispensable role of Notch signaling in the maintenance of melanocyte stem cells.

hair follicle (Delmas et al., 2003). Therefore, this system allows investigating the Notch functions in the melanocyte lineage. Conditional ablation of Notch signaling results in a dramatic reduction of embryonic melanoblasts (via apoptosis), which is evident from the dilution of initial hair pigmentation (Moriyama et al., 2006) (Figure 6). These animals also exhibit premature hair graying in hairs grown from subsequent hair cycles, suggesting a role of Notch signaling in the regulation of melanocyte stem cells (Moriyama et al., 2006; Schouwey et al., 2007) (Figure 6). In fact, histological analyses of postnatal follicles revealed a progressive loss of the bulge melanoblasts during the first hair cycle (Moriyama et al., 2006; Schouwey et al., 2007). Hence these data suggest the indispensable role of Notch signaling in the establishment and/or maintenance of melanocyte stem cells. Despite the obvious phenotype of the stem cell loss, the question about whether Notch signaling is required for stem cell specification or maintenance is not easy to be addressed. This is mainly because of lack of a definitive maker that allows detection of melanocyte stem cells. Nevertheless, a series of recent studies support the latter possibility that Notch signaling play a role in the maintenance of the stem cells (Kumano et al., 2008). *Notch1*$^{+/-}$*;Notch2*$^{+/-}$ compound heterozygous mice display normal hair pigmentation at the birth but exhibit gradual hair graying from the second hair cycle (Kumano et al., 2008) (Figure 7). Since the stem cell establishment is completed during the initial hair follicle morphogenesis, this result suggests that

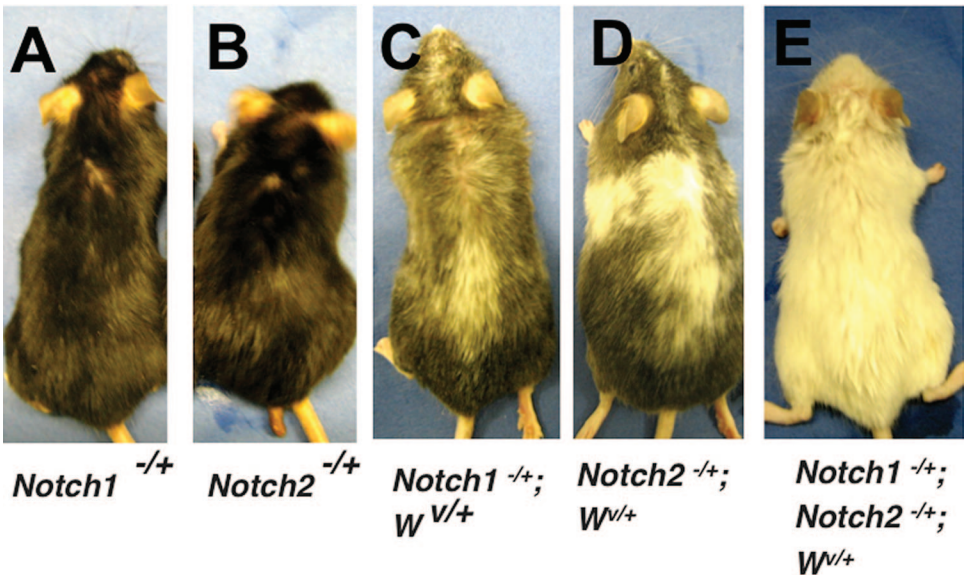

Figure 7. Genetic links between Notch signaling and c-Kit signaling. Both *Notch1*$^{-/+}$ (**A**) and *Notch2*$^{-/+}$ (**B**) mice show no obvious defect in hair pigmentation, while combinatory attenuation of Notch and c-Kit signaling enhances the spotting phenotype of W $^{v/+}$ mice, suggesting a link between Notch signaling and c-Kit signaling. (**C**), *Notch1*$^{-/+}$*;* $W^{v/+}$; (**D**), *Notch2*$^{-/+}$*;* $W^{v/+}$; (**E**), *Notch1*$^{-/+}$*; Notch2*$^{-/+}$*;* $W^{v/+}$. Pictures are provided courtesy of Dr. Keiki Kumano and Dr. Shigeru Chiba.

Notch signaling is critical for the maintenance of melanocyte stem cells rather than their establishment. Indispensable role of Notch signaling in the maintenance of melanocyte stem cells has been further confirmed by pharmacological inhibition of Notch signaling in adult mice (Kumano et al., 2008). Administration of a γ-secretase inhibitor into adult mice causes gray spots on their hair, which is associated with a significant reduction of the bulge melanocyte stem cells. Hence, taken together, these findings suggest the multiple role of Notch signaling in: the promotion of melanoblasts survival; and the maintenance of melanocyte stem cells (Aubin-Houzelstein et al., 2008; Osawa and Fisher, 2008).

These studies indicate that Notch signaling compromises an essential component of epidermal–melanocyte interactions; but what is the underlying mechanism? One possible clue to address this question is provided by Kuman *et al*, who suggested the potential synergism between Notch signaling and c-Kit signaling pathway (Kumano et al., 2008). It has been shown that, whereas both *Notch1*$^{+/-}$*;Notch2*$^{+/-}$ mice and $W^{+/v}$ mice show subtle coat phenotypes, their combinatory mutants in *Notch1*$^{+/-}$*;Notch2*$^{+/-}$*;*$W^{+/v}$ compound mice result in dramatic enhancement of white spotting and premature hair graying phenotypes (Kumano et al., 2008) (Figure 7). Thus, these results clearly indicate a synergistic cooperation of these signaling pathways in the regulation of the melanocyte lineage (Kumano et al., 2008). Given the fact that both Notch and c-Kit signaling pathways are essential components of keratinocyte-melanocyte interactions, it would be tempting to speculate that the collaboration between these signaling pathways may function as a safety lock mechanism, by which melanocytes are allowed to survive only under the strict control of keratinocytes, while the exact molecular interactions between Notch and c-Kit signaling pathways in the melanocyte lineage remains open question.

It has been recognized that dysregulation of normal homeostatic regulation may cause neoplasmic conversion of the cells. Because melanomas emerge primarily within epidermal melanoblasts, it is reasonable to speculate that both normal and malignant melanoblasts may share key pathways that regulate biological homeostasis and maintenance. Indeed, it has been recently demonstrated that Notch signaling participates in melanoma progression (Balint et al., 2005; Liu et al., 2006). Thus, the elucidation of molecular mechanisms downstream of Notch is important not only for melanoblast development but also for understanding the molecular mechanisms of melanomagenesis.

5. Hair graying: loss of melanocyte stem cells

Although hair graying represents the most common phenotype of human aging, it has remained largely unknown how aging impacts on the melanocyte system in the hair follicle. One classical hypothesis that might explain the age-associated hair graying is “free radical theory of graying”, in which reactive radicals produced through melanin biosynthesis reactions might be responsible for the induction of oxidative stress and the subsequent cytotoxic elimination of mature melanocytes in the hair matrix (Arck et al., 2006). However, a series of recent observation of premature hair graying mutant animals have provided alternative evidence that hair graying is due to loss of melanocyte stem cells rather than the cytotoxic damage of mature melanocytes in the hair matrix (Mak et al., 2006; Moriyama et al., 2006; Nishimura et al., 2005).

Bcl2-deficient mice exhibit normal hair pigmentation in the initial hairs, but loose hair pigmentation from the second postnatal hair cycle. Histologically, in *Bcl2*-deficient follicles, melanocyte stem cell population is absence due to accelerated apoptosis during the first anagen, while mature melanocytes in the hair matrix appear to be intact and remain until the end of the first anagen (Mak et al., 2006; Nishimura et al., 2005). The loss of melanocyte stem cells is also observed in those in albino strain, neglecting the possibility for the cytotoxic effect of melanin biosynthesis reaction (Nishimura et al., 2005). Hence, it is clearly shown from these observations that hair-graying phenotype of Bcl2-deficient mice appears due to selective loss of melanocyte stem cells during hair follicle morphogenesis. These data suggest that Bcl2 may be required for the stem cell specification and/or maintenance, while the exact role of Bcl2 in melanocyte stem cells awaits further investigation.

Analysis of *Bcl2*-deficient mice suggests that hair graying may result from loss of melanocyte stem cells in the hair follicle. The next question is how the stem cells are lost during hair graying with physiologic aging. *Mitf*$^{vit/vit}$ mouse, which encodes a mild hypomorphic mutation of *Mitf*, would provide another model to address this question. In contract to *Bcl2*-deficient mice, *Mitf*$^{vit/vit}$ mice exhibit a gradual hair graying phenotype (Figure 2D), which is reminiscent of hair graying with physiologic aging. Histological analysis of the hair follicle indicates a gradual loss of melanocyte stem cells from the bulge region, but instead pigmented melanocytes are frequently seen at the same region (Nishimura et al., 2005). More detailed analysis demonstrate that loss of the stem cells is closely associated with ectopic differentiation of melanocytes (Nishimura et al., 2005), suggesting the notion that the stem cells may be lost due to precocious terminal differentiation resulted from their defective self-replicative activity. Similarly, the

ectopic localization of differentiated melanocytes is also observed in the follicles with physiologic aging in both mice and humans (Nishimura et al., 2005). Hence, it is likely that hair graying with aging may be due to a gradual failure of self-renewal activity of the stem cells at the niche. Although function of Mitf in melanocyte stem cells is largely unknown, one potential role of Mitf might be to determine cell fate by regulating the cell cycle status. Mitf appears to be downregulated in the quiescent stem cells (Osawa et al., 2005), but its expression is transiently upregulated during early anagen when the stem cells should be activated (Osawa et al. unpublished data). It has been shown that upregulation of *Mitf* induces cell cycle arrest and terminal differentiation through the induction of $p21^{Cip}$ (Carreira et al., 2005), whereas *Mitf* downregulation results in the $p27^{kip}$-mediated cell cycle exit associated with stem cell-like morphological change (Carreira et al., 2006). These data indicate a possibility that the balance between differentiated and undifferentiated states is tightly regulated by Mitf activity in melanocytes. Hence, subtle defect of $Mitf^{vit}$ protein might result in defective self-renewal capacity, which finally leads to premature differentiation of melanocyte stem cells. Further studies are needed to define the exact function of Mitf in the regulation of melanocyte stem cells.

The fact that loss of melanocyte stem cells exhibits a premature hair graying phenotype would provide an invaluable opportunity to identify key molecules required for the stem cell regulation by a phenotype-driven mutagenesis approach. Hence, by integrating a melanocyte-specific conditional gene knockout approach and phenotype-base screening to select the mutants that exhibit premature hair graying, one can easily identify critical genes for melanocyte stem cell regulation. Validity of this approach has been already proven in several conditional gene knockout mice including *RBP-J* (Figure 6B) and *ATR* genes, whose conditional ablations have been shown to cause premature hair graying (Moriyama et al., 2006; Ruzankina et al., 2007; Schouwey et al., 2007). Despite a large number of coat color mutants, only a few mutants exhibit the hair graying phenotype (Bennett and Lamoreux, 2003). Given the existence of potential "stemness" genes (Ramalho-Santos et al., 2002), this may be reflecting a possibility that the genes required for melanocyte stem cell regulation may share certain critical roles in other stem cell systems, and spontaneous mutations in such genes may cause tissue malfunction during embryogenesis resulting in embryonic lethality. Thus, the availability of melanocyte-specific gene ablation system would afford a clue to identify such stemness genes.

6. Melanocyte stem cells: a model for the study of stem cell specification

One of the key questions of stem cell biology is how stem cells are specified during tissue development. However, elucidation of the underlying mechanism has been largely hampered because of the difficulty in characterizing the process of stem cell specification during tissue development. Taking advantage of *Dct-LacZ* transgenic mice (Mackenzie et al., 1997), in which individual melanoblasts/melanocytes are traceable during the hair follicle development, now investigators are bingeing to address this question.

Despite the absence of melanocyte stem cells, the *Bcl2*-deficient mice exhibit normal hair pigmentation in the initial hairs. This clearly indicates that the first wave of melanogenesis is not dependent on melanocyte stem cells, but rather derived from the melanoblasts which directly migrate into the hair matrix region (Mak et al., 2006). More detailed analysis using *Dct-LacZ* follicles has demonstrated that the earliest stem cells (i.e. the anti-c-Kit antibody-resistant bulge melanoblasts) appear in the stage 7–8 guard hair follicles, which is corresponding to the latest stages of hair follicle development (Nishimura et al., 2002). As the initial melanogenesis becomes detectable during the stage 5, it is likely that the stem cell specification is occurred much later than the initiation of the melanogenesis (Mak et al., 2006). The fact that melanocyte stem cells are generated later and independent on the first wave of melanogenesis suggests the idea that the specification of the stem cells is determined by developmentally distinct molecular events that are diverged from the differentiation program. Remarkably, this mode of the stem cell specification is likely to be commonly conserved among diverse stem cell systems. In fact, it has been shown in testicular development that the first round of spermatogenesis is directly derived from gonocytes (precursors for spermatogonia cells) rather than from undifferentiated spermatogonia, while subsequent cycles of spermatogenesis is derived from Ngn3-positive spermatogonial stem cells (Yoshida et al., 2006). In muscular development, Pax3/Pax7-double positive myogenic precursors give rise to skeletal muscle prior to appearance of muscle satellite cells (muscle stem cells; Relaix et al., 2005). Precursors for hematopoietic cells first appear in embryonic yolk sac and constitute the primitive hematopoiesis, while definitive hematopoietic stem cells are generated at the much later stage of embryogenesis (Samokhvalov et al., 2007). While the question of which emerges first, stem cells or the niche, is matter of critical debate of stem cell biology, the fact that the stem cell emerges later after tissue development may support the idea that the differentiated progenies create the niche first, and then the tissue precursors that colonize at the niche become stem cells. Because of critical importance, more detailed studies are obviously needed to determine how stem cells are generated during tissue development.

Elucidation of the molecular mechanism underlying the stem cell specification and maintenance is of great importance for the application of stem cells in regenerative medicine. A decade of intensive studies of embryonic stem (ES) cells have now enabled us to generate diverse ranges of tissue precursor cells from ES cells *in vitro*, yet generation of tissue stem cells is still challenging. By combining melanocyte-specific gene manipulation *in vivo* and a phenotype-driven screen to identify hair-graying animals, melanocyte system should provide an irresistible opportunity to clarify the molecular basis of stem cell specification and maintenance during follicular development. As the core molecular mechanism appears to be conserved in discrete stem cell systems, these efforts should reveal fundamental molecular pathways required for the induction of tissue stem cells, which would ultimately provide a clue to establish novel strategies for the generation of tissue stem cells *in vitro*.

7. Concluding remarks

As described in this review, melanocyte stem cells provide an advantageous model for the study of stem cell biology. Since genetic alternations in the melanocyte lineage don't affect the animal's viability, the resulting pigmentary phenotypes serve as valuable probes to define gene functions in the melanocyte regulation. While recent advances in genetic modification technology make it feasible to generate conditional knockout mice, it still requires laborious and labor intensive work. Thus, in order to perform this screening in mice, more convenient and rapid genetic modification system is needed to be established. One candidate is to apply lentivirus-mediated gene-knockdown transgenesis system (Szulc et al., 2006) in mice. The robustness of this gene knockdown system has been already demonstrated elsewhere (Park, 2007; Szulc et al., 2006). While it requires further optimizations for its practical application in the melanocyte lineage, the integration of such approach would dramatically accelerate number of genes to be tested, and thus, increase the chance to identify stem cell genes.

As discussed in this review, zebrafish offers an alternative animal model to study the molecular basis of melanocyte regeneration. One advantage of zebrafish is the feasibility in performing large-scale random mutagenesis screens. Such large-scale approaches permit genome-wide functional screening, which would be hard to be achieved in higher model animals like mice. Thus, in this regard, continuation and expansion of these screens would increase the chance to discover previously unrecognized genes. As the fundamental molecular mechanisms implicated in stem cell regulation appear to be conserved across the animal species, such genes may have broad implications in various stem cell systems in humans.

It is widely accepted that dysfunctions of normal homeostatic regulation of stem cells may result in neoplasmic conversion into cancer stem cells (Beachy et al., 2004; Reya et al., 2001). In supporting this notion, it has been shown that, as in normal melanocyte stem cell maintenance, Notch signaling is also implicated in melanoma progression. The existence of melanoma initiating cells or melanoma stem cells has been recently suggested in human melanomas (Schatton et al., 2008), while it remain controversial whether melanoma stem cells exit or not (Quintana et al., 2008). Therefore, the elucidation of molecular mechanism underlying the regulation of melanocyte stem cells is important not only for the understanding of stem cell biology, but also the development of novel therapeutic strategies for melanocyte malignancy.

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