Cell, Vol. 121, 9–12, April 8, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.03.021

Melanocyte Stem Cell Maintenance and Hair Graying

Minireview

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Hair graying is an obvious sign of human aging, yet little was known about its causes. Two recent papers provide compelling evidence that hair graying is due to incomplete melanocyte stem cell maintenance and identify Pax3 and Mitf as key molecules that help regulate the balance between melanocyte stem cell maintenance and differentiation.

The neural crest origin of pigment-producing melanocytes has been carefully traced and documented. Melanoblasts, the precursors to melanocytes, originate in the neural crest and travel to their destinations, including the hair follicle, where they differentiate into melanocytes that produce the pigment melanin. In the hair follicle, the life of a melanocyte is tightly integrated with the cyclical nature of the follicle. The hair follicle cycle consists of a finite period of hair fiber production and growth (anagen), followed by a brief regression period (catagen) and a resting period of little activity (telogen). In mice and most other mammals, the anagen growth phase occurs in a wave-like pattern across the skin surface, whereas, in humans, each follicle cycles independently of its neighbors. The activity of melanocytes in the hair matrix is also under cyclical control where melanogenesis and anagen are tightly coupled; melanocytes in the hair bulb are terminally differentiated and die in early catagen by an unknown mechanism.

Hair follicles are known to contain a reservoir of epithelial stem cells in the bulge region (Figure 1), which can regenerate new follicles upon each hair cycle (Blanpain et al., 2004). The existence of a pool of undifferentiated melanocyte stem cells (MSCs) in the hair that can also replenish the pool of differentiated melanocytes has long been suspected. Nishimura et al. (2002) used a simple yet clever technique that made it possible to identify and localize MSCs in the hair follicle. In these studies, *Dct-LacZ* transgenic mice were used as a marker for early melanoblast development (Mackenzie et al., 1997); *Dct* (sometimes referred to as *Trp2*) codes for dopachrome tautomerase. In addition, an anti-Kit antibody was used to selectively deplete amplifying melanoblasts from neonatal mice, while at the same time leaving resting cells intact (Nishikawa et al., 1991; Okura et al., 1995). When newborn mice were injected subcutaneously with the anti-Kit antibody, they had unpigmented first hairs. In subsequent hair cycles, however, pigmentation was restored in the developing dorsal overhairs and ventral sensory hairs (Nishimura et al., 2002). This suggested that the hair follicle contains a Kit-independent (resting) melanocyte precursor population that can restore pigment to the hair in later hair cycles. In antibody-treated *Dct-LacZ* transgenic mice, LacZ-positive cells were seen in the bulge area (Figure 1A) and sometimes also in the subbulge region of the hair follicles. This is consistent with previous antibody staining experiments, which documented the existence of "amelanotic melanocytes" within human hair follicles (Commo and Bernard, 2000) and with more recent studies showing that Dct-positive cells are present in the bulge region of the mouse hair follicle (Botchkareva et al., 2003). These LacZ-positive cells are the ones most likely to produce mature melanocytes, since, during subsequent hair cycles, pigmented cells were only found in the hair matrix of cells whose bulge area retained LacZ-positive cells (Nishimura et al., 2002). This raised the interesting possibility that LacZ-positive cells in the bulge area are MSCs, capable of both self renewal and differentiation into fully fledged pigmented melanocytes.

The LacZ-positive cells have a low proliferation rate, consistent with stem cell properties. To investigate the regenerative potential of these cells, vibrissal hair follicles from Rosa26 mice, which express LacZ in all tissues, were divided into three fragments and each fragment implanted into the skin of newborn albino pups. This experiment clearly demonstrated the existence of MSCs in the lower portion of the hair follicle and showed that there are Dct-positive melanoblasts in the bulge. Although this confirms the presence of MSCs in the bulge area, it is not yet clear if Dct-positive or -negative MSCs also exist outside the bulge area in the hair follicle.

The LacZ-positive cells in the bulge area are small and oval shaped and are considered immature since they do not contain melanin; this is considered the dormant state of the cells. As the hair cycle progresses from telogen to anagen, expression of LacZ, as well as the size of the cells, increases. Subsequently, the cells divide, and at least one LacZ-positive cell remains in the bulge while others extend their processes toward the follicular papillae and eventually localize to the hair matrix where they undergo further cell divisions and differentiate into pigment-producing cells. Thus, the LacZ-positive cells in the bulge region have all the hallmarks of stem cells; they are slow cycling, self maintaining, and able to generate differentiated pigment cells.

The bulge region also has the properties expected for a MSC niche. In mice, melanocytes are generally confined to the hair follicles in the epidermis (with the exception of ears, footpads, and tail), whereas, in humans, they are dispersed among epidermal keratinocytes. When Kitl, the ligand for Kit, is expressed in the skin of transgenic mice under the control of the keratinocyte K14 promoter, the mice develop pigmented skin, much like human skin (Kunisada et al., 1998). By making mice that carry both the *K14-Kitl* and *Dct-LacZ*

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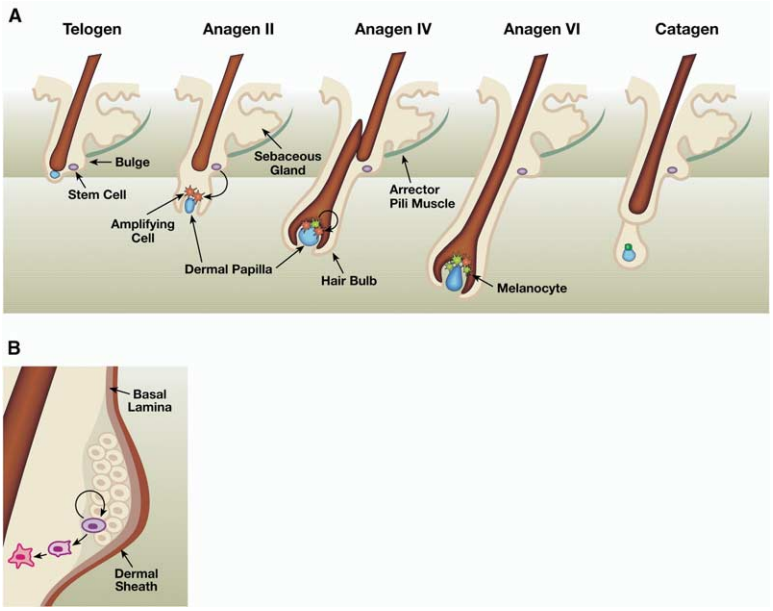

Figure 1. The Melanocyte Stem Cell during the Hair Cycle

(A) Several stages of the hair cycle are shown, with the MSCs (purple), amplifying cells (red), and melanocytes (green) indicated. Modified from Nishimura et al. (2002).
(B) The MSCs in the bulge area divide to produce daughter cells that self renew (purple) and cells that differentiate (red).

transgenes and then using the anti-Kit antibody to deplete amplifying melanoblasts from neonates, Nishimura et al. (2002) showed that, as expected, LacZ-positive cells are exclusively maintained in the bulge area during the first hair cycle when Kitl is ectopically expressed in the skin. In the next hair cycle, however, many follicles had no functional melanoblasts, suggesting vacant niches and that not all the MSCs are Kit independent. The follicles that did contain functional melanocytes produced pigmented hair and were able to drive repigmentation of the epidermis. LacZ-positive melanoblasts were observed in the bulge region as well as the surrounding epidermis during the repigmentation process. This indicates that bulge MSCs are a source of melanocytes in the epidermis and that Kitl expression in the epidermis provides a migration route for the differentiating melanoblasts. Some of the migrating melanoblasts were also seen to repopulate empty niches where they returned to a quiescent state with small round cells, eventually generating more melanocytes. The melanocyte niche therefore seems to have the ability to attract more MSCs, similar to the homing mechanism described for hematopoietic stem cells (Whetton and Graham, 1999). Interestingly, Tumbar et al. (2004) have shown that bulge epidermal cells express high levels of Kitl, which may help in the recruitment of MSCs to their niche. In summary, MSCs reside in the niche and can produce transiently amplifying cells, which can differentiate into melanocytes or migrate into empty niches and return to a stem cell fate. Currently, it is not known whether the MSCs are restricted in potential and can only give rise to melanocytes or whether they are multipotent and able to produce other cell types as well.

Along with David Fisher, Nishimura set out to examine the relationship between MSC maintenance and hair graying. Previous investigators had proposed that the oxidative nature of melanin biosynthesis is cytotoxic, leading to the degeneration of pigmented melanocytes over time and to hair graying (Johnson and Jackson, 1992; Veis et al., 1993). However, Fisher, Nishimura, and their colleagues show that this is not the case and instead provide compelling evidence that hair graying results from incomplete MSC maintenance (Nishimura et al., 2005). Using the *Dct-LacZ* transgenic mice, Nishimura and associates mapped the fate of MSCs in *Bcl2* and *Mitf* mutant mice, two mouse models of hair graying. Mice carrying a knockout mutation in the antiapoptotic *Bcl2* gene turn gray during the second hair follicle cycle, suggesting that Bcl2 protects melanocytes from apoptosis (Veis et al., 1993). Melanocyte distribution and morphology are normal during early embryonic development in these mice, consistent with the initial normal phenotype of $Bcl2^{-/-}$ mice (Nishimura et al., 2005). However, in 39-day-old $Bcl2^{-/-}$ mice, differentiated melanocytes are nearly absent from both the body and whisker hair follicles. The follicles are missing differentiated melanocytes from the hair bulb as well as undifferentiated Dct-LacZ-positive melanoblasts from the bulge area. $Bcl2^{+/-}$ heterozygote and wild-type controls have normal pigmentation, and Dct-LacZ-positive cells are present in both cases. The same effect was observed in $Bcl2^{-/-}$ mice on the albino background, showing that melanin pigment is not necessary for melanocyte loss in these mice.

Both differentiated melanocytes and Dct-LacZ-positive cells in the bulge area were present and appeared normal in 6.5-day-old $Bcl2^{-/-}$ mice. However, 2 days later, nearly all Dct-LacZ-positive cells were gone from the bulge area, whereas differentiated melanocytes could still be seen in the hair bulb in normal numbers. Staining with Kit antibodies showed that, at this stage in wild-type mice and then cyclically during subsequent hair cycles, the bulge MSCs change from dendritic to a slender, oval shape, and their nuclear-to-cytoplasmic ratio increases. This represents the dormant state of the MSCs (Nishimura et al., 2002; Nishimura et al., 2005). TUNEL staining of MSCs in $Bcl2^{-/-}$ mice showed that these cells undergo apoptosis at this critical stage of melanocyte development. Thus, Bcl2 appears to be

necessary for protecting the MSCs from succumbing to apoptosis during entry into the dormant state.

Another model of hair graying is provided by the *vitiligo* (*Mitf*^{vit}) mutation in the melanocyte master transcriptional regulator *Mitf*. Homozygous *Mitf*^{vit} mice are born pigmented with dorsal and ventral white spots and then develop progressive depigmentation of pigmented hair with each hair cycle or after plucking (see Steingrímsson et al. [2004]). MSCs are initially present in these mutant mice but are gradually lost with each hair cycle. Paradoxically, the LacZ-positive cells left in the niche of third hair cycle follicles of *Mitf*$^{vit/vit}$ mice often contain melanin pigment and show dendritic morphology, a sign of differentiation. These cells are not found in age-matched controls, suggesting that they represent MSCs that have differentiated prematurely while residing in the niche or have lost the ability to migrate to their destination in the matrix.

Hair graying is also observed in mice during normal aging. When the fate of LacZ-positive niche stem cells was traced during normal aging in *Dct-LacZ* transgenic mice, they were seen to be gradually lost with age (Nishimura et al., 2005). Furthermore, pigmented melanocytes were found in the niche of 8-month-old wild-type follicles, just like in *Mitf*^{vit}/*Mitf*^{vit} mice. The appearance of the pigmented cells in the niche happened as the LacZ-positive cells disappeared and both processes correlated with aging. Moreover, by staining human hair follicles with antibodies to the MSC marker Mitf, Nishimura et al. (2005) showed the existence of Mitf-positive cells in the bulge area of human hairs, similar to what was observed in mice. "Amelanotic melanocytes" had been previously observed in human follicles using another melanocyte marker, pMel17 (Commo and Bernard, 2000). The Mitf-positive cells were numerous in 20- to 30-year-olds, whereas they were absent in 70- to 90-year-olds. Remarkably, in 40- to 60-year-old individuals, just like in *Mitf*^{vit}/*Mitf*^{vit} and normal aging mice, pigmented Mitf-positive cells were observed in the bulge area. This suggests that hair graying involves a failure in MSC maintenance, resulting in the gradual loss of pigmented progeny upon aging. Furthermore, the MSCs seem to differentiate prematurely within the niche, a process enhanced by the *Mitf*^{vit} mutation. Thus, Mitf may be important for MSC self renewal. Mitf has been shown to regulate *Bcl2* expression (McGill et al., 2002). Since the effect of the *Mitf*^{vit} mutation on stem cell fate is less severe than the *Bcl2* mutation, the *Mitf*^{vit} mutation might not eliminate *Bcl2* expression, and other transcriptional targets might be involved in the resulting phenotype.

What, then, are the genes that control MSC maintenance and hair graying? In a recent paper, Jonathan Epstein and colleagues show that the transcription factor Pax3 is critically important for MSC maintenance (Lang et al., 2005). The *PAX3* gene is mutated in a subgroup of patients with Waardenburg Syndrome, a pigmentary and deafness disorder. Mutations in the *MITF* and *SOX10* genes also result in Waardenburg Syndrome, and several studies have shown that the PAX3 and SOX10 proteins regulate MITF expression (reviewed in Steingrímsson et al. [2004]). Lang and associates now show that Pax3 is expressed in MSCs in the bulge region where its expression overlaps *Dct-LacZ* transgene expression in most cases. *Dct-LacZ*-expressing cells are neural crest derived and express Mitf and Sox10 in addition to Pax3. Transfection studies in 293T cells showed that Sox10 and Mitf activate the *Dct* enhancer synergistically, whereas Pax3 represses Mitf/Sox10-mediated activation of *Dct*. Binding sites for all three transcription factors are present in the *Dct* enhancer, and chromatin immunoprecipitation studies showed that all three proteins are able to bind this enhancer region. In parallel studies using identical conditions, Lang et al. (2005) showed that Pax3 activates *Mitf* expression, while, at the same concentration, it represses expression from the *Dct* enhancer. Pax3 binds the enhancer with low affinity. Interestingly, however, chromatin immunoprecipitation studies using different ratios of the Pax3 and Mitf proteins showed that Pax3 binding is preferred when the proteins are at equal ratios, whereas Pax3 is displaced from the enhancer at higher concentrations of Mitf. Thus, Pax3 seems to act as a competitive inhibitor of Mitf on the *Dct* enhancer.

In addition to Pax3 and Mitf, β-catenin and its interacting transcription factor Tcf/Lef, as well as the Groucho-related corepressor Grg4, also seem to be involved in this process. A binding site for the *Lef/Tcf* transcription factor is located immediately adjacent to the Mitf binding site in the *Dct* enhancer (Lang et al., 2005). By cotransfecting activated β-catenin, a cofactor for Tcf/Lef proteins, Pax3-mediated repression was relieved and the Pax3 protein displaced from the *Dct* enhancer. Pax3 also interacts with Grg4 on the *Dct* enhancer. Activated β-catenin removes Grg4 from the *Dct* enhancer, resulting in the loss of Pax3-mediated repression. This suggests that β-catenin causes the realignment of the protein complex at the *Dct* enhancer. Consistent with this, immunoprecipitation studies showed that Lef1, Pax3, and Grg4 form a complex in solution, and, when activated β-catenin is added, the Pax3 and Grg4 proteins can no longer complex with Lef1. Further support for this complex formation comes from studies on mouse B16 melanoma cells. Upon transfection of a dominant-negative Lef1 protein into these cells, Pax3, which is not normally located at the *Dct* enhancer in these cells, and Grg4 bind to the enhancer and repress *Dct* expression. Interestingly, by creating mouse embryos that lacked β-catenin in Pax3-expressing cells, Lang and associates were able to show that Dct was no longer expressed in the skin, while Mitf- and Pax3-expressing cells could still be found. Thus, β-catenin is required in Pax3-expressing melanocyte precursors for the proper induction of *Dct* expression.

The model of Lang and associates proposes that Pax3 and Sox10 activate Mitf expression while, at the same time, Pax3 inhibits Mitf from activating downstream genes (Lang et al., 2005). Thus, the Mitf protein accumulates and is ready to rapidly activate downstream genes as soon as Pax3-mediated repression is lifted upon β-catenin signaling. This is an interesting model with implications for stem cells in general. However, the interactions are probably not as simple as proposed. First, the three pigmentation enzymes Dct, Tyrp1, and tyrosinase (all Mitf targets) are expressed in a temporally regulated manner, suggesting that the cellular environment may have a major effect. While expression of Dct is increased during anagen (Nishimura et al., 2005), it is expressed in MSCs before they differentiate into pigment-producing cells, as evidenced by in situ hybridization (Nishimura et al., 2002), immunostaining (Lang et al., 2005), and *Dct-LacZ* transgenic expression (Nishimura et al., 2002). Detailed expression analysis of Dct, Tyrp1, and tyrosinase proteins

during hair follicle morphogenesis in C57BL/6 mice shows that Dct is expressed in early hair follicles and stays on in the differentiating melanocytes as well as in nonproliferating cells in the bulge area (Botchkareva et al., 2003). Tyrp1 protein expression comes on later as the cells near the dermal papilla; melanin synthesis becomes apparent as the cells become tyrosinase positive (Botchkareva et al., 2003). Tyrp1 gene expression is itself regulated by Pax3 (Galibert et al., 1999). Second, the premature pigmentation seen in niche MSCs in $Mitf^{vit}/Mitf^{vit}$ mice is also not consistent with the model, since, according to the model, reduced function of Mitf should lead to reduced pigmentation. Rather, the premature pigmentation of MSCs suggests that Mitf might play a role in MSC self renewal and may itself provide a nodal point in the process of deciding whether to maintain an undifferentiated state or to differentiate. This may initiate with a decision of whether to enter or exit the "dormant state" in the niche, and, ultimately, this decision may be directly related to cell cycle decisions.

Interestingly, several laboratories have recently shown that Mitf regulates the expression of genes involved in controlling cell cycle exit. Loercher et al. (2005) showed that Mitf activates the expression of $p16^{Ink4a}$ resulting in cell cycle arrest and reduced phosporylation of the Rb protein. They also showed that Mitf-mediated cell cycle arrest and differentiation are interconnected processes. Using mouse embryonic fibroblasts from wild-type and *Ink4a* homozygous mutant mice, they showed that transfecting Mitf-expressing constructs into these cells induces cell cycle arrest in wild-type cells but not in mutant cells. Similarly, when assessed for morphologic changes, the Mitf-transfected cells showed a morphology consistent with melanocyte differentiation, whereas *Ink4a* mutant cells did not, suggesting that $p16^{Ink4a}$ is necessary for the Mitf-mediated differentiation program. In another recent paper, Carreira et al. (2005) showed that Mitf regulates the expression of $p21^{Cip1}$, inducing cell cycle arrest; the arrest does not take place in $p21^{Cip1}$-deficient cells. While they point out that mice lacking $p21^{Cip1}$ have normal pigmentation, as do mice lacking *Ink4a*, it is possible that a certain degree of redundancy is built into the system, with Mitf activating both $p21^{Cip1}$ and $p16^{Ink4a}$ to guarantee cell cycle exit. Carreira and associates propose that Mitf acts to integrate different extracellular signals with cell division and differentiation and propose a model involving a positive feedback loop.

Similar mechanisms might also be at work to regulate MSC fate in the niche. During the hair cycle, quiescent MSCs in bulge regions of hair follicles are likely to receive signals instructing entry into the cell cycle. One of the resulting daughter cells may partially exit the niche microenvironment and thereby receive additional differentiation signals (Figure 1B), while the daughter cell remaining in the niche is instructed to exit the cell cycle and at the same time is protected from apoptosis. Mitf is known to regulate all of these processes, and, since multiple different signaling mechanisms can affect the expression, transcriptional activity, or stability of the Mitf protein (reviewed in Steingrímsson et al. [2004]), it is entirely possible that the different signals received by the daughter cell are translated into different effects on Mitf activity, with subsequent effects on transcription regulation. Although the exact signals involved in each case are not known at present, Okura et al. (1995) have shown that Kit mediates a survival signal in melanocytes. In support of this, Kitl is expressed in bulge epidermal cells (Tumbar et al., 2004). Additional signals may include β-catenin as well as other signaling mechanisms known to be important in the bulge stem cell niche (Fuchs et al., 2004).

In summary, melanocytes and pigment mutations in the mouse have yet again proven their worth for dissecting the inner workings of complex eukaryotic cell processes.

Acknowledgments

We thank Colin Goding and Heinz Arnheiter for comments on the manuscript and Allen Kane for the artwork.

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