

Melanocytes in Development, Regeneration, and Cancer

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The genes required for stem cell specification and lineage restriction during embryogenesis also play fundamental roles in adult tissue regeneration and cancer. This “development-regeneration-cancer” axis is exemplified by the vertebrate pigmentation system. Melanocytes exhibit almost unlimited self-renewal capacity during regenerative processes such as mammalian hair recoloration and zebrafish fin regeneration. Melanoma utilizes many regulatory signals and pathways required during ontogeny and regeneration. A discussion of these interconnections highlights how studies of stem cell function in embryonic and regenerative contexts can yield insights into melanoma biology.

The pigmentation of the vertebrate skin has been well studied in part due to the great variety of mutations that affect diverse species from human to zebrafish. Many of these mutations are not fatal and have allowed for a detailed examination of the involved genes. These pigment genes have been classically studied in the context of developmental aberrations, but more recent work has focused on the remarkable regenerative capacity of the vertebrate pigmentation unit, including the adult melanocyte. Several of the genetic requirements of embryonic pigmentation are strongly shared with the adult regenerative unit. These same genetic pathways have been demonstrated to be important in the development of melanoma, a deadly cancer that originates in the melanocyte. All three of these processes—embryonic development, tissue regeneration, and cancer formation—have a great deal of cellular and genetic events in common. The initial events involved in embryonic development of the melanocyte lineage depend upon neural crest stem cell specification, followed by migration/epithelial-mesenchymal transitions, niche localization, and maintenance of a balance between multipotency and differentiation. The adult melanocyte regenerative unit (composed of adult stem cells and progenitor cells) likely becomes specified during these critical embryonic periods, and the cellular processes it demonstrates are similar to those of the embryonic unit. The regenerative unit, however, differs from embryogenesis in the need for unlimited, but tightly regulated, self-renewal throughout the lifetime of the organism. Many of the same processes that we have come to associate with the cancer phenotype, such as self-renewal and migration capacity, are shared with both the embryonic and regenerative cells. The purpose of this review is to highlight recent advances in understanding these commonalities, using the melanocyte system as a prototypical example.

Function of Pigmentation

One of the more striking aspects of vertebrate pigmentation is the interspecies variety of coloration, patterning, and function. In some species such as birds, pigmentation serves an important role in identification of mating behaviors (Chaîne and Lyon,

2008). In zebrafish, a variety of subspecies, each with different pigmentation patterns (i.e., *Danio rerio* with horizontal stripes, *Danio nigrofasciatus* with vertical stripes, etc.), indicates that a combination of genetic and environmental cues play a role in the adult pigment pattern. Pigmentation plays a role not only in behavioral choices, but strongly protects against ultraviolet light induced changes in DNA structure, since the skin is almost constantly exposed to high levels of this potential mutagen (Meredith and Sarna, 2006). Melanin, the principal component of the melanocyte, is one of the most potent free radicals in humans and helps to protect from the high levels of reactive oxygen species that would otherwise damage DNA in skin cells.

The Developmental Biology of the Melanocyte Early Events in Neural Crest Specification

All pigmented melanocytes are derived from a group of migratory embryonic cells referred to as the neural crest. During gastrulation, the NC is induced at the edge of the neural plate on the border between the neural and nonneural ectoderm (Erickson and Reedy, 1998). The derivatives of the neural crest are extensive, indicating that the stem cells which emerge during embryogenesis are truly multipotent but undergo gradual lineage restriction. The anatomic location of the crest cell determines, in part, their developmental potential. Cells in the mid-/hindbrain region form the cranial neural crest whose derivatives include jaw cartilage and glia. The vagal and sacral crest gives rise to the neurons of the enteric nervous system. Derivatives such as adrenal cells and sensory ganglia come from the trunk neural crest. Although pigmented melanocytes are typically considered to emerge from the trunk neural crest, studies in avians have indicated considerable developmental plasticity such that cranial neural crest may give rise to melanocytes as well (Baker et al., 1997).

The early induction of the neural crest is at least in part dependent upon intact BMP signaling, as the BMP2 knockout mouse shows an almost complete absence of cranial and migratory neural crest cells (Kanzler et al., 2000). In the chick, BMP4/7 is induced downstream of Notch signaling, the latter of which is required for early neural crest development in the zebrafish as well

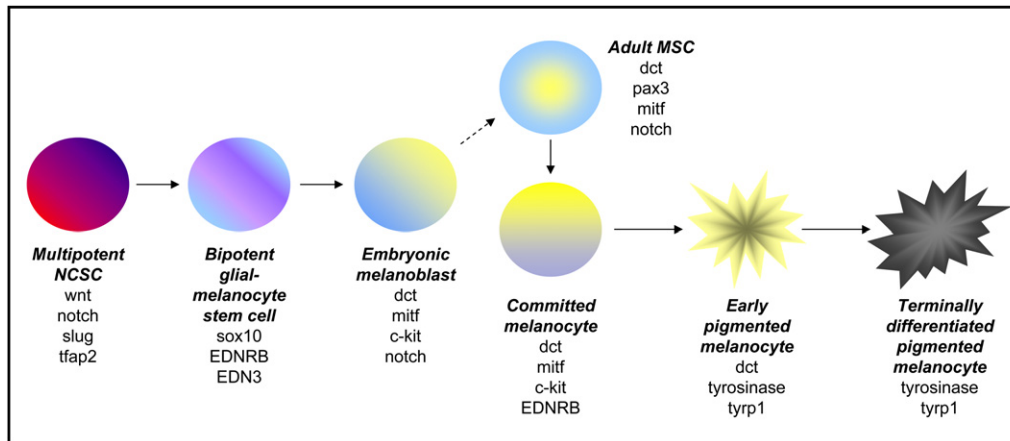


Figure 1. Melanocyte Development from the Neural Crest

Early neural crest specification is dependent upon interactions between wnt, notch, and bmp signaling. Slug transcription marks an early specified neural crest cell and is coincident with migration. Gradual lineage restriction toward the melanoblast fate is dependent upon mitf, EDNRB, and c-kit signaling. The embryonic melanoblast and adult melanocyte stem cell (MSC) share some overlapping molecular markers, particularly *dct*. It is unclear if there are some adult MSCs which arise independently of the embryonic melanoblast.

(Cornell and Eisen, 2005). The period after induction but before migration requires the transformation of the neural crest stem or progenitor cell to undergo an epithelial-to-mesenchymal transition (EMT) in which downregulation of cell adhesion molecules such as E-cadherin allow for widespread and stereotyped cellular movements. One important family of genes involved here are *snail/slug* members, which transcriptionally repress E-cadherin expression and allow for EMT to occur (Cano et al., 2000). Onset of *snail* transcription is coincident with migration of neural crest precursors from the neural plate.

The notch family of proteins play at least 3 distinct roles in neural crest development. First, it induces cranial neural crest development in the frog (Glavic et al., 2004) and chick (Endo et al., 2003). Second, notch activation plays a crucial role in diversification of neural crest progenitors, including the melanocytes. In the trunk premigratory NC cells, notch signaling prevents receiving cells from adopting a neuronal fate, instead becoming unspecified neural crest precursors (Cornell and Eisen, 2002). Support for a role of notch in the generation of melanocyte precursors is further supported by the zebrafish *mindbomb* or *whitetail* mutant. This homozygous lethal mutant encodes a defective E3-ubiquitin ligase that is necessary for adequate cleavage of the notch ligands on the presenting cell (Itoh et al., 2003). Loss of this cleavage leads to a deficiency of notch signaling in the receiving cell. The two names for this mutant adequately reflect the variety of phenotypes observed, which include a bias toward a neuronal lineage (thus, *mindbomb*) but also an almost complete lack of neural crest derivatives, including *crestin*-positive premigratory cells and differentiated melanocytes beyond the cranial region (Kelsh et al., 1996). Whether notch plays a role in proliferation or survival of these neural crest progenitors is unclear and awaits further analysis. The third and final important role of notch signaling is in the maintenance of the adult melanocyte stem cell, which will be more fully discussed below.

Establishment of the Multipotent Neural Crest Stem Cell

Multipotent neural crest stem cells (NCSCs) that give rise to a large number of lineages typical of this cell (Sieber-Blum and

Cohen, 1980) can be isolated flow cytometrically using an antibody to p75, and these cells demonstrate self-renewal capacity (Morrison et al., 1999). Lineage tracing experiments of trunk neural crest cells supports differentiation into at least four derivatives (melanocytes, glia, sensory neurons, and adrenal cells) (Bronner-Fraser and Fraser, 1988). Whether all species have neural crest stem cells with true multipotency remains unclear, as zebrafish appear to determine fate restriction prior to overt migration, but this may be an artifact of the rapid development of the zebrafish embryo. The mechanisms by which a NCSC becomes lineage restricted into the melanocyte fate has been well studied, and data support the notion that there is a bipotent glial-melanocyte lineage progenitor (Dupin et al., 2000), which then becomes further restricted to an unpigmented, but committed, melanocyte lineage cell referred to as the melanoblast. This commitment occurs either within the neural tube or after migration has commenced. The specific genes involved in each state of melanocyte development from the neural crest, as is understood from genetic model organisms and human patients, are discussed below and outlined in Figure 1.

Restricting Neural Crest Stem Cells toward a Melanoblast Fate

Initial data on the importance of Wnt signaling on neural crest-derived melanoblasts came from observations of Wnt1;Wnt3a knockout mice. In the Wnt-deficient animals, almost no melanoblasts, marked by positivity for *dct*, were seen (Ikeya et al., 1997). Studies in the fish have suggested that Wnt signaling, through activated beta-catenin, promotes a fate decision between melanogenesis and gliogenesis, since overexpression of beta-catenin leads to an expansion of melanocytes but a loss of glial derivatives (Dorsky et al., 1998). Using a transgenic approach in which Wnt1 was activated in a neural precursor line, Pavan demonstrated that Wnt1 is sufficient for inducing an increase in melanized (i.e., terminally differentiated) melanocytes, demonstrating that Wnt1 could influence fate decisions in vivo. Importantly, beta catenin appears to also expand the population of neural crest-derived melanoblasts, an effect that is at least partly

dependent upon endothelin-3 (Dunn et al., 2000). Finally, Wnt signaling is also important for terminal differentiation of melanoblasts in vitro, and it thus appears that Wnt plays a multifactorial role in melanocyte lineage decisions.

But in what ways does Wnt/beta-catenin drive the melanocyte lineage? The most direct evidence comes from observations that beta-catenin could directly activate the melanocyte-specific *mitf* promoter (Takeda et al., 2000). *Mitf* itself directly interacts with LEF1, which is part of the transcriptional complex that includes beta-catenin. *Mitf* and LEF1 act synergistically to increase transcription of *dct* (Yasumoto et al., 2002), which sets up a feedback loop in which *mitf* is a direct transcriptional target of beta-catenin, and then interacts with the beta-catenin pathway to induce markers of the restricted melanoblast fate.

Melanocyte Specifiers Drive the Production of Pigmented Derivatives

The importance of *mitf* in melanocyte development is widely seen in both model organisms, such as mice and zebrafish, but also in human patients with the Waardenburg syndrome Type IIa (Widlund and Fisher, 2003). The *mitf* phenotype was initially identified in a mouse mutant in 1942 (Hertwig, 1942), notable primarily for its lack of melanocytes rather than a defect in melanin synthesis. This was really the initial observation that *mitf* was required for either specification or survival of the melanocyte lineage in mammals. *Mitf* encodes a transcription factor that belongs to the myc family of bHLH-Zip factors (Moore, 1995). *Mitf-M*, the melanocyte-specific isoform in humans, is well conserved among vertebrate species, and closely related genes also appear in the genomes of invertebrates such as *C. elegans* (Rehli et al., 1999). In the mouse, 24 alleles of *mitf* have been described, which lead to a wide variety of pigmentation defects, ranging from white coats to spotted coats. The zebrafish nacre mutant, which harbors a mutation in the *mitfa* gene, is completely devoid of both embryonic and adult melanocytes (Lister et al., 1999). *Mitf* regulates the melanocyte lineage in part by acting as a transcriptional activator of several pigment cell-related genes, including *dct*, *tyrosinase* (Yasumoto et al., 1994), *tyrp1* (Yasumoto et al., 1997), *c-kit* (Tsujiyama et al., 1996), *AIM1* (Du and Fisher, 2002), and *MC1r* (Aoki and Moro, 2002). Fate-mapping studies suggest that *mitf* is required for melanoblast survival. This occurs prior to migration from the neural tube (Hornyak et al., 2001), an effect that is in part related to up-regulation of the *bcl2* gene (McGill et al., 2002). *Bcl2* and *mitf* interact to promote the survival of the melanocyte lineage, which likely explains why *mitf* mutants have defects in melanocyte numbers and not generally just defects in melanin synthesis.

Data from humans, mice, and zebrafish have provided a robust literature regarding the role of *c-kit* in melanocyte development. In humans, heterozygous mutations of *c-kit* lead to the pigmentation disorder referred to as piebaldism (Giebel and Spritz, 1991). Mouse mutants for the *c-kit* receptor tyrosine kinase (Geissler et al., 1988) or its ligand, steel/stem cell factor (Williams et al., 1990), exhibit various degrees of pigmentation defects as well as hematopoietic and germ cell deficiencies. *Kit* and the *kit* ligand play a complex role in melanocyte development, survival, and migration, which appears to depend in part upon the specific species. *Kit* mutants never develop the normal number of melanocytes; this may be due to a failure of melanoblast migration in the mouse (Wehrle-Haller and Weston, 1995) or due to melano-

blast survival in the fish (Parichy et al., 1999). *Kit* is also required for mammalian melanoblast differentiation in part due to its cooperation with *mitf* signaling (Hemesath et al., 1998; Wu et al., 2000). Analysis of the zebrafish-sparse mutant, which corresponds to a *kit* mutation, reveals that embryonic melanocytes are formed (albeit to a lesser degree than normal) but that these melanocytes die by 11 days postfertilization, strongly indicating a role for *kit* in melanocyte survival. The zebrafish mutant also supports a role for *kit* in migration of melanocyte precursors, since the mutants exhibit a greater proportion of mutant melanocytes that are found close to their site of origin than in wild-type embryos. Finally, the development of the adult pigment pattern of the zebrafish is abnormal in *kit* mutants, as they never develop a population of early adult melanocytes referred to as early stripe melanocytes. Because these cells are likely derived from a latent pool of melanocyte stem cells, this data (along with a complete lack of melanocytes in mouse *kit* mutants) suggests that *kit* plays a role in the development of at least some melanocyte stem cells in both mice and fish.

The role of the endothelins in neural crest and melanocyte development was recognized over a decade ago, when knockout mice for either the endothelin-B receptor (*EDNRB*) or its ligand, endothelin-3 (*ET3*), were demonstrated to have an almost complete lack of melanocytes (Baynash et al., 1994). In the mouse, *EDNRB* is expressed in a subset of premigratory and migrating neural crest cells and, in vitro, is coexpressed with the melanoblast marker *dct* (Pavan and Tilghman, 1994). *EDNRB* is only required during a critical period of mouse melanoblast dispersal; its absence during this period leads to an almost complete loss of melanocytes in the offspring. (Lee et al., 2003) This would indicate that the endothelin system is necessary not for initial specification of a mammalian neural crest-melanocyte lineage but, rather, for dispersal and survival of the melanoblasts. There appear to be species-specific functions of the endothelins in pigment pattern formation, as the zebrafish expresses *EDNRB* early in development in migratory neural crest cells in the dorsolateral and ventromedial pathways, but the phenotypic defect of *EDNRB* mutation (as seen in the rose mutant fish) does not manifest until the metamorphic period, when the adult pigment pattern emerges (Parichy et al., 2000). This indicates that *EDNRB* likely plays a role in neural crest migration in cells destined to become melanocytes in most vertebrates, but that its requirement for differentiation of latent precursors may be species specific. In vitro, *ET3* causes a marked expansion of melanocytes from a quail neural crest culture, due primarily to a large increase in proliferation (Lahav et al., 1996). *ET3* can expand the population of bipotent neural crest precursors that can give rise to both glial and melanocytic lineages, and addition of *ET3* can revert differentiated melanocytes toward a more bipotent state (Lahav et al., 1998).

Melanocyte Regeneration

Given the extensive knowledge of melanocyte development in the embryo, it is rational to ask whether some of the same genetic pathways maintain their importance in the adult melanocyte system. Along with the hematopoietic system, the adult skin represents one of the most regenerative organs in vertebrate biology. On any given day, we generate millions of new epidermal skin cells as well as hair, both of which are repigmented in an identical fashion each day by the melanocytes. The coloration

process of hair and skin differs somewhat, although the principles are largely the same. In humans, hair typically becomes repigmented due to the transfer of melanin from a differentiated melanocyte at the base of the hair follicle into the developing new hair shaft. In the skin itself, melanocytes transfer melanin to the surrounding keratinocytes, forming the “pigmentation unit.” In mice, the hair shaft is pigmented in a manner analogous to humans, but the skin is often (although not invariably) unpigmented. Zebrafish, another important model for melanocyte regeneration, do not have “hair” in this sense, but they offer one of the most genetically powerful systems for studying melanocyte regeneration, which is the fin regeneration model. Although the epidermal component likely plays an important supportive role in melanocyte regeneration, this review will focus on the role of adult melanocyte stem and progenitor cells, since the keratinocyte system has recently been reviewed (Fuchs, 2007). The incredible longevity of hair pigmentation throughout the adult life cycle strongly suggested the presence of a cell that would have the characteristics of a stem cell: slowly cycling, harbored in a protective niche, and unlimited self-renewal capacity.

Melanocyte Stem Cells in Mammalian Skin

Regeneration of the hair shaft itself, at early anagen, is known to initiate in the bulge area of the hair follicle, where the keratinocyte stem cells had been previously localized (Oshima et al., 2001). Because individual hair follicles must be melanized during each hair cycle, Nishimura used a *dct-lacZ* transgenic mouse to follow the fate of individual melanoblasts during the hair recoloration cycle (Nishimura et al., 2002). When neonatal mice were treated with a neutralizing anti-kit antibody, the initial hair cycle of the live animals were largely unpigmented. However, subsequent hair cycles showed recoloration of the hair, which demonstrated that there was a kit-independent population of melanocyte precursors. This was consistent with the work described above, indicating that although kit is important for survival and migration of melanocyte precursors and perhaps some stem cells, there are large numbers of kit-independent melanocytes in the zebrafish mutants. When the anti-kit-treated hair was examined, there was a residual population of kit-independent, *dct+* melanoblasts located in the bulge area of the follicle. Using a combination of BrdU labeling and transplantation assays, these *dct+* melanoblasts fulfilled all the criteria of a stem cell compartment, including very high rates of self-renewal. Because mouse skin is typically unpigmented in contrast to humans, the authors generated K14-SLF mice (in which kit ligand or steel factor is expressed in skin keratinocytes). These animals develop pigmented skin similar to humans, and the follicle bulge melanocyte stem cells appear to migrate toward epidermal niches to allow for skin pigmentation. Although this does not definitively prove the location of the human epidermal melanocyte stem cell, it provides compelling reason to determine whether the hair follicle bulge may be a common source of all pigment stem cells in mammals. These data suggest that, much like the hematopoietic system, there are populations of cells with stem cell/self renewal capacity as well as those that would be considered “transient amplifying” compartment when out of the primary niche.

Molecular Profiling of the Mammalian Melanocyte Stem Cell

It is unlikely that *dct* alone identifies the melanocyte stem cell, since it is a marker of virtually all melanocyte lineage cells, sub-

suming both stem cell and differentiated melanocytes. To better define the nature of the stem cell, Osawa used the *dct-Cre/CAG-CAT-GFP* transgenic system, in which all *dct+* cells are identified by GFP positivity (Osawa et al., 2005). After dissecting single hair follicles from postnatal day 6 skin, two populations of *dct+* cells were isolated—those from the bulb matrix (differentiated melanocytes) or the bulge region (melanocyte stem cells). These single cells underwent transcriptional profiling using qRT-PCR to compare the levels of a panel of melanocyte-related genes. Surprisingly, the postnatal bulge area hair follicles were only positive for *dct* and *pax3* and virtually negative for the markers *tyrosinase*, *silver*, *tyrp1*, *kit*, *mitf*, *sox10*, *EDNRB*, *mc1r*, *oa*, and *lef1* (the latter by IHC). Some of these markers were also decreased in embryonic E16.5 melanoblasts (i.e., *mc1r*, *oa*, and *mitf*) but were positive for *ednrb*, *kit*, and *sox10*. Thus, although the melanocyte stem cells likely arise from the same neural crest-derived population as the embryonic melanoblast, it is clear that once in the adult niche, the molecular profile of the stem cell is quite distinct from that of either embryonic melanoblasts or differentiated melanocytes in the hair matrix. The precise extent to which *mitf* is expressed in the bulge melanocyte stem cell remains unclear and warrants further investigation.

The molecular mechanism regulating self-renewal versus differentiation of the stem cell remained unknown, but a recent elegant analysis of the interaction of *pax3* with Wnt and *mitf* provides some of the answers (Lang et al., 2005). *Mitf* acts in concert with *sox10* to regulate the *dct* promoter, but what regulates *mitf* activity? Again using the murine hair follicle system, Lang noted a population of cells within the hair follicle bulge that expressed Pax3, consistent with the data above. Although Pax3 strongly upregulates *mitf* expression itself, it also causes a strong repression of *dct* transcription under similar conditions. *Mitf*, *pax3*, and *sox10* all bind to an enhancer element in the *dct* promoter, which is located nearby to a previously described LEF1-binding site. Pax3 and *mitf* thus act as “competitors” on the *dct* promoter, and this competition is relieved by beta-catenin binding to the LEF1 site of the *dct* promoter. This explains how the Wnt/beta-catenin pathway regulates the melanocyte lineage, since low levels of beta-catenin will maintain the stem cell in an undifferentiated state, but upregulation of beta-catenin will then relieve the *pax3*-mediated repression of the *dct* promoter and promote terminal differentiation into a mature melanocyte.

Notch in Embryonic Melanoblasts and Postnatal Melanocyte Stem Cells

Nishikawa’s group noted that the E16.5 embryonic melanoblasts abundantly express several members of the notch family, including the notch-1 receptor and the jagged2 ligand, as well as the notch target genes *hes1*, *hes5*, and *hey1* (Moriyama et al., 2006). To address the function of notch in adult melanocytes, they performed conditional ablation of the RBP-J gene (a mediator of notch signaling in the nucleus) in the melanocyte lineage using a tyrosinase-Cre driver line. This led to a loss of hair pigmentation by the second postnatal hair cycle. However, because the promoter used in this system was tyrosinase (a differentiated melanocyte marker), it was difficult to directly ascribe a role for notch to maintenance of the stem cell itself. Therefore, they examined notch-related genes in postnatal hair follicles and found that the bulge melanoblasts (the putative melanocyte stem cell) were positive for both NICD1 and *hes1* and that these were

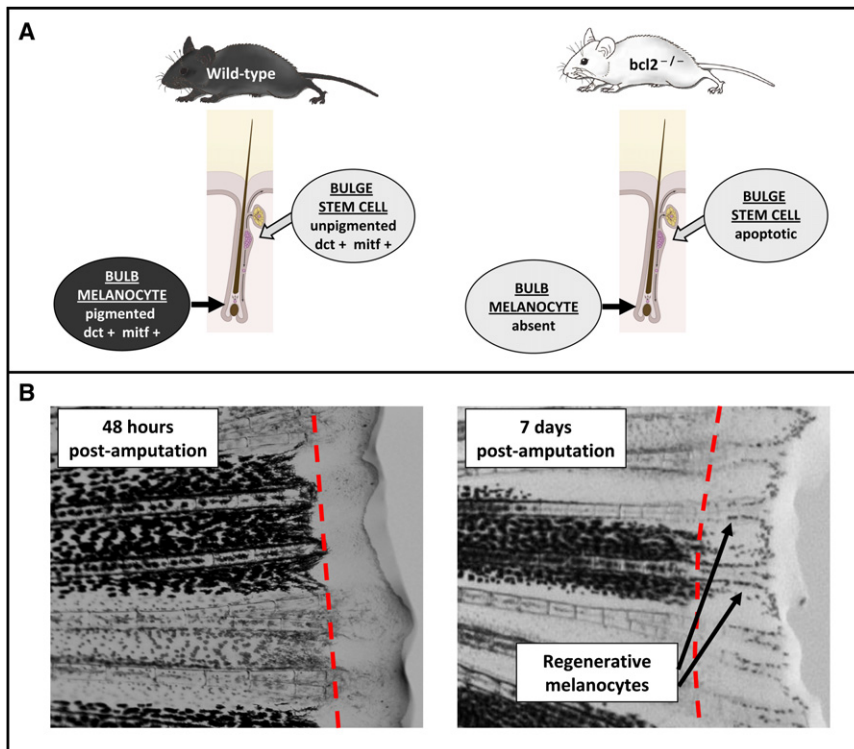


Figure 2. Examples of Melanocyte Regeneration

Repigmentation of mammalian hair requires an intact melanocyte stem cell. In the *bcl2*^{-/-} mouse (A), hair graying is related to failure to maintain the melanocyte stem cell in the follicle bulge niche. A similar mechanism is involved in human hair graying. In (B), the highly organized repigmentation of the amputated zebrafish fin is demonstrated. Whereas little repigmentation is seen in the first 2 days following amputation (left), a progressive increase in melanocytes are seen over the next 7 days (right) such that the original pigmentation pattern is fully recapitulated by 2 weeks.

mouse strains that showed evidence of premature graying, but without affecting other cell lineages (Nishimura et al., 2005). The *bcl2*^{-/-} mouse is born with essentially normal hair pigmentation, but the hair grays after the first hair cycle. Examination of the hair follicles during the second hair cycle revealed a complete lack of *dct*⁺ melanoblasts in the bulge area as well as differentiated melanocytes in the hair matrix, or bulb, area, as shown in Figure 2A. This strongly suggests that the melanocyte stem cell is

downregulated in the differentiated melanocytes in the hair matrix. Further supporting a role for notch in melanocyte stem cell specification and/or maintenance, compound heterozygous *notch1*^{+/-}; *notch2*^{+/-} mice are born with a normal pigment pattern but become increasingly gray starting at the second hair cycle (Kumano et al., 2008). To functionally assess whether notch was required in the stem cell compartment, the authors then treated 8-week-old mice with an oral gamma secretase inhibitor (GSI), which blocks notch activity. These animals began to develop gray spots on their hair at 2 weeks after treatment, and these defects in hair pigmentation were maintained for at least 6 months after discontinuation of the GSI. Examination of the bulge region of the hair follicles after GSI using the *dct*-LacZ system revealed a marked decrease in the number of *dct*⁺ cells in the bulge region, supporting at least a supportive if not essential role for notch signaling in melanocyte stem cell maintenance. Taken together, these data support a role for the notch pathway in both embryonic melanoblasts as well as adult melanocyte stem cells. The precise mechanism by which notch helps to maintain melanocyte stem cells in a quiescent state and whether notch directly interacts with *pax3*, *mitf*, or *dct* all await further characterization.

Mammalian Hair Graying as a Tool for Understanding Molecular Control of the Melanocyte Stem Cell

The observation that alterations which reduce notch signaling in the melanocyte stem cell often lead to premature graying highlights the utility of hair graying for studying the melanocyte stem cell. Since hair undergoes essentially constant cycling/repigmentation throughout life, the gradual loss of hair coloration with age (i.e., “physiologic aging”) in both mouse and humans suggested the possibility that defects in melanocyte stem cells could play a role. To examine this, Nishimura used two mutant

lost in these animals, since the stem cells are known to exclusively give rise to the differentiated cells in the hair matrix. The loss of *bcl2* in the bulge leads to apoptosis of the stem cell. In contrast, examination of the *mitf*^{vit/vit} mouse, which carries a mild hypomorphic allele of *mitf*, demonstrates a slower graying phenotype that correlates with a gradual loss of melanocyte stem cells from the bulge niche. At early to midanagen of the third hair cycle, the *dct*⁺ cells in the bulge region were seen to be pigmented and appeared dendritic, both of which are characteristics of premature or ectopic differentiation. In young humans with colored hair (age 20–30), unpigmented *mitf*⁺ cells were seen in the bulge area, whereas in 40–60 year olds with mild graying, pigmented *mitf*⁺ cells were seen in the bulge area, similar to the *mitf*^{vit/vit} mice. By age 70–90, the *mitf*⁺ cells were completely lost. Because pigmented melanocytes should never be seen in the bulge stem cell niche, the process of hair graying is likely due to a gradual failure to maintain the melanocyte stem cell self-renewal program is poorly understood, but one mechanism might relate to its role in cell-cycle entry. Overexpression of *mitf* activates p16Ink4a expression, which causes cell-cycle arrest and a morphology consistent with melanocyte differentiation (Loercher et al., 2005). *Mitf* may also act to promote cell-cycle arrest through upregulation of p21Cip1, which may act together with p16Ink4a to promote the effect of *mitf* on the cell cycle (Carreira et al., 2005).

A Different Spin on Melanocyte Stem Cells: Fin Regeneration in the Zebrafish

The regenerative capacity of the teleost fin has been well recognized for decades (for a review, see Poss et al., 2003). In the zebrafish, the caudal (tail) fin is the most commonly studied system, primarily because it is easy to perform a precise

amputation, and the stages of regeneration in this region can be precisely well defined. Following initial amputation of the distal fin, epithelial cells migrate over the wound and cover the surface. The next stage, essential for normal regrowth, consists of blastema formation, a proliferative mass of mesenchymal cells that will ultimately form the basis of the new fin structures (Poleo et al., 2001). It is unclear precisely what these cells are composed of in terms of their multipotency and whether they represent dedifferentiated cells present in the fin or whether there is a latent pool of stem cells that then migrate to the blastema. Following blastema formation is the regenerative growth phase, marked by a much more rapid cell turnover (i.e., the G2 cycle length shrinks from 6 hr in blastema formation to 1 hr during outgrowth) (Nechiporuk and Keating, 2002). During zebrafish fin regeneration, the pigment cells completely regrow so that by 1 to 2 weeks postamputation (Figure 2B), the melanocyte pattern is essentially undistinguishable from normal fins.

The first indication that regenerative pigmentation was derived from a melanocyte stem cell and not simply migration came from observations of fin regenerates in the presence of the melanin-synthesis inhibitor PTU (Rawls and Johnson, 2000). Since PTU will inhibit melanogenesis only in newly formed melanocytes (i.e., those that differentiated from an unpigmented precursor), Rawls amputated fins and allowed them to regenerate in the presence or absence of PTU. PTU almost completely blocked visible melanocyte regeneration, but upon removal of the drug, pigmentation of the regenerate commenced within hours. This strongly indicated that the cells that form the melanocyte regenerate in the fin had previously differentiated from an unpigmented, latent precursor. That this precursor cell was likely a stem cell with unlimited self-renewal was suggested by the fact that the fin melanocytes would regenerate through at least 10 successive rounds of amputation and regeneration. To get at the molecular regulation of this potential stem cell, fin regeneration was examined in the zebrafish *kit* mutant (Rawls and Johnson, 2001). This revealed an almost complete lack of melanocyte regeneration by day 7, and examination of the melanoblast marker *dct* in the regenerating fin revealed a complete lack of expression in the mutant animals. This indicates that *kit* is required for at least a subset of regenerating melanocytes in the fin, but *kit* mutants eventually do recover the melanocyte pattern in the fin regenerate (Figure 2B), albeit at a much slower rate than wild-type. This is consistent with the existence of a *kit*-independent population of unpigmented melanocyte precursors in the zebrafish fin, although the molecular identity of these cells remains obscure.

Small Molecules Reveal Novel Pathways in Melanocyte Stem/Progenitor Function

One of the major advantages of studying regeneration in the zebrafish is the opportunity to perform forward genetic screens. This allows for unbiased identification of genes that may play a role in melanocyte regeneration. Recently, Yang et al. used a clever approach to studying melanocyte regeneration in zebrafish larvae (Yang et al., 2007). Previous studies had demonstrated that the small molecule MoTP could ablate melanocytes or melanoblasts in the zebrafish larvae and that regeneration of new melanocytes occurred from proliferation of an undifferentiated precursor (Yang and Johnson, 2006). Taking this as a starting point, Yang performed a parthenogenesis (early-pressure) ENU

mutagenesis screen in which they searched for mutants that had normal ontogenetic melanocyte development during embryogenesis but failed to regenerate larval melanocytes after MoTP treatment. This approach holds significant promise for dissecting the genetic pathways that separate the developmental requirement of embryonic melanocyte formation from those of regenerative melanocytes. Two mutants isolated from that screen, named *eartha* and *julie*, encode the *gfpt1* (glutamine:fructose-6-phosphate aminotransferase 1) and *skiv2l2* (superkiller viralicidic activity 2-like 2) genes, respectively. These two genes appear to affect different aspects of melanocyte regeneration. *Gfpt1* mutants develop melanoblasts up to the *dct*+ stage but fail to melanize beyond that, suggesting that the gene is required for the final steps in melanocyte development. In contrast, the *skiv2l2* mutants fail to develop *dct*+ melanoblasts after MoTP treatment, suggesting that this gene is necessary for early proliferation of melanocyte precursors prior to the *dct*+ stage. It is evident that both of the genetic pathways identified by this screen will require further investigation in the future to understand their specific role in melanocyte regeneration beyond the zebrafish.

Developmental Pathways Implicated in Melanoma

Melanoma is the deadliest form of skin cancer (Miller and Mihm, 2006). The incidence of melanoma has been increasing more rapidly than any other solid tumor, in part likely due to enhanced detection. There are no particularly effective therapies once the disease is metastatic, highlighting the need for new approaches to identifying genetic events that initiate and maintain the tumor. The past decade has seen a dramatic increase in our knowledge of melanoma biology, and identification of sentinel events such as mutations of the *B-raf* oncogene (Davies et al., 2002) and loss of the *CDKN2A* locus (Yang et al., 2005) have been well documented. For many years, it has been well recognized that melanomas express a number of markers, discussed above, that are typical of the embryonic and regenerating melanocyte lineage (i.e., endothelin and *c-kit*). However, one challenge in interpreting this observation has been to understand whether these markers are simply “bystanders” or whether they serve specific oncogenic functions. Emerging evidence would suggest that “lineage specific” markers in melanomas play important pathological roles, several examples of which will be discussed below.

Mitf

The concept of “lineage addiction” emphasizes that particular oncogenic events are specific to the cellular lineage from which the cancer arises and may only act to promote cancer when in the correct cellular context. The *mitf* gene itself is perhaps the most typical example of this model. *Mitf* had been known to be expressed on melanoma cells for several years, although its precise role in tumorigenesis remained obscure (King et al., 2001). Garraway et al. used an integrative genomic approach with high-density SNP arrays to identify areas of copy number gains or losses in a panel of human melanomas and found that *mitf* itself was amplified in 15%–20% of human melanomas (Garraway et al., 2005). *Mitf* demonstrated oncogenic activity, but only in the presence of aberrant MAP kinase activity induced by *B-raf* mutations, suggesting that contextual cues are required for full oncogenic transformation (i.e., a lineage-specific gene such as *mitf* plus a driver mutation such as *B-raf*). But how does *mitf*

act as an oncogene? One potential mechanism involves dysregulation of the cell-cycle arrest that is typically induced by *mitf* in melanocytes. This likely involves either *p16* (*CDKN2A*) and/or *Rb*. Indeed, loss of *p16* (either through deletion or silencing) is commonly seen in melanomas (Bardeesy et al., 2001), which suggests one mechanism by which cell-cycle arrest can be avoided in melanoma cells that express *mitf*. Beyond its effects on the cell cycle, *mitf* exerts broad transcriptional effects on other genes in the melanocyte lineage such as *dct* and *tyrp1*, as discussed above. Supporting the important function of *mitf* as a transcriptional regulator in melanoma, Fisher et al. recently utilized a ChIP-CHIP approach to understand the promoter regions where *mitf* binds in the MALME melanoma cell line and found that *mitf* binding sites generally correlate with nucleosome-free sites, as expected (Ozsolak et al., 2007). Importantly, though, the frequency of nucleosome-free promoters was strongly dependent upon lineage. In melanocytes and melanoma cells, expression of both *mitf* and the melanocyte-lineage gene *SILV* correlate, and the *SILV* promoter is nucleosome-free in these cell types. In contrast, *SILV* and *mitf* are nearly absent in breast epithelial and breast cancer cell lines, and the *SILV* promoter was occupied by a positional nucleosome in these nonmelanocyte cell lines. Thus, it would appear that regulation of lineage-specific oncogenes is in part related to the chromatin accessibility of the promoters in question.

Endothelins

Lahav et al. examined the role of endothelin signaling on melanoma growth nearly a decade ago (Lahav et al., 1999). EDNRB was found to be highly expressed on nearly all melanoma cell lines tested and appeared to correlate with the differentiation state of the cell. Treatment of melanoma cells with the specific EDNRB antagonist BQ788 caused the cells to differentiate and cease proliferating, in part due to an increase in apoptosis. Inhibition of EDNRB also inhibited tumor growth in a nude mouse transplant model. The molecular mechanism by which endothelins promote growth of melanoma cells has remained somewhat obscure, but more recent data indicates that endothelins are able to transcriptionally upregulate expression of *snail*, which allows for downregulation of E-cadherin and upregulation of N-cadherin, likely contributing to melanoma cell invasiveness (Bagnato et al., 2004). Perhaps even more strikingly, the endothelins signal through the MAP kinase pathway (Simonson et al., 1992), providing a significant cooperation with the dysregulated MAPK signaling induced by oncogenic B-raf. Knockdown of mutant B-raf V600E in melanoma cells (with shRNA) led to decreased proliferation and reduced colony formation by 80%, an effect that could be partly overcome by addition of endothelin-1 (Christensen and Guldborg, 2005). Finally, the endothelins, particularly ET-1, are known to be hypoxia-responsive genes, and ET-1 acts to stabilize the HIF1- α monomer, leading to increased production of angiogenic factors such as VEGF and PGE2 (Spinella et al., 2007). Blockade of EDNRB using the small molecule A192621 suppresses HIF1 α and VEGF-associated neovascularization in a xenograft model, suggesting that this pathway is an important mechanism of angiogenesis in melanoma (Grimshaw, 2007). Based in part upon these observations, a small phase II trial of bosentan (a mixed EDNRA/EDNRB antagonist) in advanced melanoma was recently completed, which resulted in "disease stabilization" in 6/32 patients (Kefford et al.,

2007), which is not atypical for small molecule based therapeutics currently in clinical trials.

c-kit

The role of kit in human melanoma has undergone significant evolution over the past several years. C-kit expression is typically lost with melanoma progression, suggesting that its loss is associated with invasion and/or metastasis (Natali et al., 1992). Nevertheless, several clinical trials have examined the efficacy of c-kit inhibition, using the multikinase inhibitor imatinib mesylate, in melanoma. All three of these trials yielded disappointing and essentially negative results (Eton et al., 2004; Ugurel et al., 2005; Wyman et al., 2006). However, it was recently reported that a significant proportion of melanomas arising from atypical areas, such as mucosal surfaces, palms, soles, and nailbeds, harbor activating mutations of the c-kit gene. In one report, 21% of mucosal melanomas had activating kit mutations (Curtin et al., 2006) and up to 15% of anal melanomas had similar mutations (Antonescu et al., 2007). Most of these mutations occurred in the juxtamembrane region of the gene, which is known to be predictive of clinical responses to the kit-inhibitor imatinib mesylate. In a preliminary report of a single patient (from a larger phase II trial) with anal melanoma and a kit mutation, a dramatic response to imatinib was seen, with over 50% tumor size reduction at 4 weeks and an almost complete absence of metabolic activity seen by FDG-PET (Hodi et al., 2008). This new data indicates that well-selected patients, with acral or mucosal melanoma and activating kit mutations, are likely to benefit from this therapy. Given the paucity of any effective therapy for metastatic melanoma, this may represent a significant advance in the treatment of this subset of patients. The true efficacy of c-kit inhibitors in melanoma await proper clinical trials using patients selected for mutation or overexpression.

Notch

Several studies have implicated dysregulated notch signaling in patients with melanoma. One of the earliest observations by Hoek et al. was the finding that *notch2* and *hey1* (a downstream target) was upregulated by expression microarrays in several human melanoma cell lines (Hoek et al., 2004). Around the same time, Qin noted that the gamma-secretase inhibitor DAPT potentially induced apoptosis in 9/9 tested melanoma cell lines, but not in normal melanocytes, suggesting a particularly pathogenic function of notch in melanoma (Qin et al., 2004). Interestingly, the apoptosis was, in part, related to an upregulation of the BH3 family member, NOXA, and was essentially p53 independent. Activation of notch in vertical growth phase (VGP) melanoma cells, using an activated NICD construct, led to increases in growth and metastatic potential that was in part related to activation of the MAP kinase and AKT pathway (Liu et al., 2006). Consistent with this, a dominant-negative form of MAML1 (a component of the notch transcriptional complex) resulted in a decrease proliferation rate in primary, although not metastatic, melanoma cells, indicating a stage-specific requirement for notch signaling (Balint et al., 2005). This pathway has begun to come under intense scrutiny as a therapeutic target, with new trials in breast cancer and likely in melanoma in the near future.

Wnt5a

Although mutations in beta-catenin, the central activator of Wnt signaling, are seen in melanoma, their frequency is quite low and

Table 1. Genes Demonstrated to Have Important Functions in Embryonic Development of the Melanocyte Lineage, and Corresponding Roles in Regeneration and Melanoma

	Development	Regeneration	Cancer
<i>mitf</i>	Required for segregation of multipotent neural crest stem cells toward a melanocyte fate in zebrafish and mammals	Expressed in mouse hair follicle during hair cycling, and lost in <i>bcl2</i> ^{-/-} -mediated hair graying (mice, humans)	Amplified and/or overexpressed in a subset of human melanomas, represents a lineage specific oncogene
<i>EDNRB</i>	Multiple roles, including early induction of melanocyte precursors (mammals, fish) as well as fate restriction of bipotent glial-melanocyte toward pigment fate (mouse, humans)	unknown	<i>EDNRB</i> widely expressed on many human melanomas. Inhibition of this pathway leads to apoptosis and may have clinical benefit
<i>C-kit</i>	Required for a subset of embryonic melanocytes in zebrafish, and differentiation of melanoblasts in concert with <i>mitf</i> in mice. Loss leads to human piebaldism	Mutations in <i>kit</i> lead to defects in melanocyte regeneration in the zebrafish fin, although there is <i>kit</i> -independent regeneration.	Expression typically lost with human melanoma progression. A subset of mucosal melanomas harbor activating <i>kit</i> mutations which may derive clinical benefit from imatinib
<i>slug</i>	Early marker of the neural crest (mouse, fish, <i>Xenopus</i>), required for epithelial-mesenchymal transition of migratory neural crest cells	unknown	Expression of <i>slug</i> is associated with metastatic potential of melanoma (mouse)
<i>dct</i>	Central marker of melanoblasts and melanocyte stem cells (mice), also directly involved in pigmentation of the terminally differentiated melanocyte (mammals, fish)	Expression increases during larval regeneration in zebrafish and in regenerating hair follicles of mouse	Expressed in a subset of human melanomas, but function unclear
<i>notch</i>	Notch mutants fail to develop neural crest in zebrafish. Required for cranial neural crest induction in frog and chick	Dominant-negative notch in mouse melanocyte lineage leads to hair graying	Inhibition of gamma-secretase leads to apoptosis of many human melanoma cells lines. Inhibitors are in clinical development
<i>wnt</i>	Required for early fate decisions toward a melanocyte fate (mice), and directly activates the <i>mitf</i> promoter (mammals and fish)	Inhibition of <i>wnt</i> abrogates entire zebrafish fin regenerate; specific effect on melanocytes unclear	<i>Wnt5a</i> overexpressed in a subset of human melanoma, and associated with increased invasiveness

the role of canonical Wnt in melanoma awaits further refinement (Pollock and Hayward, 2002). In contrast, overexpression of *wnt5a*, a member of the “noncanonical” Wnt pathway, may play an important role in melanoma invasion and metastasis. Using a hierarchical clustering algorithm, *wnt5a* defined a subclass of cutaneous melanoma that exhibited invasive and motile properties as determined by scratch assay and collagen gels (Bittner et al., 2000). *Wnt5a* may mediate this process via induction of *vimentin* and *snail*, factors known to induce the epithelial-mesenchymal transitions necessary for melanoma metastasis (Dissanayake et al., 2007).

Slug/Snail

The migratory nature of neural crest derived melanocytes led Weinberg’s group to question whether these developmental programs were recapitulated during melanoma metastasis (Gupta, et al., 2005). Using an identical set of oncogenic insults, including Ras activation, in three different cell lines, only the transformed melanocytes gave rise to clinically evident metastasis. This was due in part to expression of *slug*, a transcription factor known to be associated with melanocyte migration during development. Whether all cancers have metastatic programs that are specific to the cell of origin remains an unanswered question, and awaits further investigation.

dct

Given the central role of *dct* as a marker of the embryonic melanoblast and the melanocyte stem cell, it is reasonable to question whether this enzyme plays a pathogenic role in melanoma. This question has received little attention thus far, but several studies indicate that this gene may have an important role in this disease. One of the most interesting observations came from a study that used a retroviral insertional mutagenesis screen to isolate melanoma cells that were resistant to the chemotherapeutic agent cisplatin (Pak et al., 2004). Using gene expression analysis, the chemoresistant cell line was found to overexpress *dct*, and that *dct* expression correlated well with chemoresistance in a variety of melanoma cell lines. This correlation seemed to also hold for radiation resistance, as induced by UV-B. The molecular mechanism of this remains unclear, but one suggested possibility is that UV-B, which is known to upregulate *dct*, activates the ERK/MAPK pathway, which may in part explain the chemo and radiation resistance of these cells. *Dct* may also play a role in progenitor proliferation. Using the *dct*-LacZ mouse system, Jiao et al. demonstrated that in the developing central nervous system, *dct*+ cells are localized to the ventricular zone, where neuronal stem cells reside. siRNA mediated knockdown of *dct* decreases neural progenitor proliferation

by 48% (Jiao et al., 2006). Although this effect has not yet been tested in neural crest progenitors and the mechanism remains obscure, future experiments aimed to elucidate the role of this melanoblast marker in melanoma seem warranted.

Remaining Questions

It is abundantly clear that human cancers share important characteristics with both development and tissue regeneration (Table 1). This occurs not only at the cellular level, but also on genetic, genomic, and likely epigenetic bases. One important question left unanswered by these studies is the relationship of the embryonic and adult melanocyte stem cell to the melanoma stem cell. Recent work by Frank (Schatten et al., 2008) has demonstrated that the ABCB5 transporter can be utilized to selectively isolate a subpopulation of melanoma cells that fulfill the criteria of a tumor stem cell, including self-renewal and differentiation capacity. Importantly, a novel anti-ABCB5 antibody could significantly inhibit tumor growth after transplantation, highlighting the potential of cancer stem cell based therapeutics in humans with this disease. What is the overlap between the melanoma stem cell and normal tissue melanocyte stem cell in terms of signaling pathways? Several models exist for the generation of a tumor stem cell: a transformation of an otherwise normal melanocyte stem/progenitor cell or acquisition of self-renewal capacity in a more differentiated cell type. In the former case, it is expected that inhibition of a signaling pathway common to both the tumor and normal stem cell may have significant antitumor effect but at the cost of severe inhibition of normal melanocyte regeneration. This would have significant biological repercussions, since a continued supply of melanocytes is required for protection against UV-induced DNA damage. Further molecular characterization of the stem cells present in each period of life—embryonic, adult, and cancerous—are required to define the differences between these cell types and capitalize on these for the design of rational therapeutic targets in melanoma.

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