CHAPTER 9

The Genetic Regulation of Pigment Cell Development

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Abstract

Pigment cells in developing vertebrates are derived from a transient and pluripotent population of cells called neural crest. The neural crest delaminates from the developing neural tube and overlying ectoderm early in development. The pigment cells are the only derivative to migrate along the dorso-lateral pathway. As they migrate, the precursor pigment cell population differentiates and expands through proliferation and pro-survival processes, ultimately contributing to the coloration of organisms. The types of pigment cells that develop, timing of these processes, and final destination can vary between organisms. Studies from mice, chick, *Xenopus*, zebrafish, and medaka have led to the identification of many genes that regulate pigment cell development. These include several classes of proteins: transcription factors, transmembrane receptors, and extracellular ligands. This chapter discusses an overview of pigment cell development and the genes that regulate this important process.

Introduction

The pigment cell is one of the most well characterized derivatives of neural crest. Early in development, the pluripotent neural crest population develops from the border between the neural tube and overlying ectoderm. The neural crest then migrates along two paths relative to the neural tube. The cells that migrate dorso-laterally become the pigment cells while those that migrate ventrally become various cell types including the entire peripheral and enteric nervous system. Variations in pigment cell development and function are easily identifiable and apparent as unique colorations and patterns amongst vertebrates. These and additional differences between pigment cells and other neural crest derivatives have made them an excellent model to dissect the molecular mechanisms underlying neural crest lineage specification, migration, differentiation, and related human disorders.

Vertebrate pigment cells can be divided into two types based upon their embryonic origins. One type, the retinal pigment epithelium, is found only in an outer layer of the eye and is derived from an outpocketing of the developing forebrain, the optic cup. The second type, the neural crest derived pigment cells, encompass the pigment cells of the integuement and the inner ear, the iris, and the internal organs. The primary function of these cells is to produce pigment for coloration of skin and appendages (hair, feather, scale). Depending on the organism, the type of pigment produced, and the developmental stage, neural crest-derived pigment cells are defined by different nomenclature such as melanoblasts, melanocytes and chromatophores (including melanophores, iridipohores, xanthophores, and erythophores).

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Neural Crest Induction and Differentiation, edited by Jean-Pierre Saint-Jeannet.

Since pigment cells are not essential for viability, mutations that affect their development are frequently not lethal yet cause obvious alterations in the coloring of adult vertebrates. This has led in the availability of many mutants to dissect developmental pathways. Studies using rodents, chick, quail, *Xenopus*, and zebrafish, have provided both complementary and overlapping information on pigment cell development. Each organism lends itself to unique experimental studies and the specific details of pigment cell development can vary amongst them. Thus pigment cell number, timing of migration and differentiation, and type of pigmentation produced varies across the vertebrates. Comparisons of studies with different organisms have revealed that the genetic regulation of pigment cell development is highly conserved.

Model Organisms

Work in amphibians such as *Xenopus* has been instrumental in understanding early aspects of pigment cell development, in particular pigment cell fate specification and the genetic hierarchies regulating it. While they are not genetically amenable, *Xenopus* are suitable for both loss and gain-of-function studies using anti-sense oligos and morpholinos, which block protein translation. In contrast to mammals, differentiated pigment cells are observed early during development which has facilitated developmental analyses.[1]

Zebrafish, and more recently a distant relative, medaka, have emerged as excellent model systems for studying pigment cell development primarily because this process can be visualized in live embryos. Zebrafish have three pigment cell types (melanophores, xanthophores, and iridophores) and medaka have a fourth type.[2,3] The distribution of the different types of chromatophores is the major determinant of the coloration pattern in adult fish. Similar to *Xenopus*, pigment is produced in the chromatophores before the completion of migration. The melanophores, which are similar to melanocytes and produce black pigment, appear around 24 hr laterally and then along the trunk. The xanthophores appear next followed by the iridophores, which produce yellow pigment and silvery pigment, respectively. Also unique to fish, their pigment cells migrate both dorso-laterally and ventrally, and do not cross the basement membrane into the epidermis.[3]

Zebrafish and medaka are amenable to both genetic and embryological experiments. Over 90 pigmentation mutants in zebrafish have been identified from large-scale ENU mutagenesis screens, and additional mutants have been created from insertional mutagenesis.[3,4] In medaka, over 40 spontaneous mutants exist.[2] Similar to *Xenopus*, zebrafish are also well suited to loss and gain of function analyses using genes and morpholinos, respectively.

Much of our understanding of the cell biology of pigment cell development has come from studies of the avian species. Although chick and quail are not well suited for genetic studies, neural tubes can be dissected from chick embryos, cultured, and loss and gain of function analyses can be carried out. Since quail cells can be distinguished from chick cells by the presence of a large amount of heterochromatin in their nuclei, cell transplantation experiments have been used to understand pigment cell fate and migration. These techniques provided the first evidence that pigment cells migrate dorso-laterally and that pigment cell fate, like other neural crest derivatives, is dependent upon the environment through which they migrate.[5-7] DiI analysis in chick also revealed that pigment cell precursors migrate a day after other neural crest-derived lineages have begun to emigrate from the neural tube.[8]

The rodent is another classical system that has been instrumental to the identification of genes required for pigment cell development. Mice and rats with black and white coat coloration patterns have been favorites of mouse fanciers for centuries. In mammals, the pigment cell precursor is called the melanoblast and the mature pigment cell is called the melanocyte. A major determinant of skin and hair color results from the regulation of the two types of pigment produced by melanocytes, eumelanin (brown or black) and pheomelanin (yellow/red). In many mammals including mice, the Agouti signaling pathway regulates this switching of melanin type within a cell. Genetic analyses of over 100 spontaneous coat color mutants in small rodents have led to the identification of at least ten loci essential for pigment cell development.[9,10]

Defects in pigment cell development in small rodents are often evident as white spots in an otherwise dark animal.

Melanoblast development in mice is well characterized by expression of molecular markers (Fig. 1A). The precursors to melanoblasts develop around embryonic day 8.5 (E8.5), concurrent with the migration of other neural crest, and in a cranial to caudal order.[11] These neural crest cells express Wingless/INT-related 1 (Wnt1), Paired homeodomain transcription factor 3 (Pax3), and SRY box containing transcription factor (Sox10).[12-15] The precise time that neural crest are specified to melanoblasts in not known, but at E10.0 - E10.5 a subset of crest begin to express the earliest known melanoblast precursor markers Kit and microphthalmia-associated transcription factor (Mitf).[16-20] A few hours later these cells express a melanogenic enzyme marker gene, *Dopachrome tautomerase* (*Dct*), and localize to the migration staging area, dorsal to the neural tube.[21] From this region they continue to divide while migrating through the dermis, and by E13.5 a large proportj3é of melanoblasts migrate into the epidermis. By E15.5 a subset of melanoblasts enter the developing hair follicle, where they express markers downstream of Mitf, including *Tyrosinase* (*Tyr*) and *Tyrosinase related protein 1* (*Tyrp1*). Between E15.5 and birth, once the melanoblasts begin to produce pigment, they become melanocytes.

The controlled differentiation of melanocytes from melanoblasts and their precursors does not end during development, as adult hair follicles require repopulation of melanocytes at each cycle (Fig. 1B). These pigment cells originate from a *Dct* positive melanocyte stem cell pool located in the bulge area of the hair follicle.[22] Precise regulation of cell division and differentiation is needed to maintain the stem cell population and instruct differentiation into melanocytes in repopulated follicles. Interestingly, defective maintenance of this population may be responsible for hair graying.[23] Mitf, Pax3, and Wnt signaling, which are all required for pigment cell development also regulate stem cell maintenance.[23-25]

Studies of neural crest in these model organisms have identified several genes that regulate pigment cell development (Figs. 1, 2). Some genes are required for early aspects of neural crest development, including their induction and epithelial to mesenchymal transition, yet also appear to be essential for later aspects of pigment cell development. Many genes are needed for development of multiple neural crest derivatives, including pigment cells. These include the *Wnt* signaling pathway, *Snail/Slug*, *Sox10*, *Pax3*, *AP-2*, and *Ednrb*. Other genes may be more specific for pigment cells, including *Kit* and *Mitf*. In this book chapter we will review the important genes that regulate the specification, cell survival, differentiation, and migration of pigment cells. Although we do not discuss uncloned mutants, it is important to note that many uncharacterized mutants generated in the above model organisms will continue to advance our understanding of pigment cell development.

Initial Induction and Expansion of the Pigment Cell Population

Components of the Wnt/β-catenin pathway are among the earliest proteins expressed during neural crest development, supporting their role in neural crest and pigment cell induction, specification and expansion. The canonical Wnt signaling pathway is activated when an extracellular Wnt binds to a coreceptor complex including Frizzled, resulting in the accumulation of cytoplasmic β-catenin and the subsequent shuttling of β-catenin into the nucleus.[26] Nuclear β-catenin then interacts with members of the Lef1/Tcf transcription factor family and regulates transcription of target genes. Wnts 1,3, and 3A are expressed in the dorsal portion of the neural tube in mice, chick, and zebrafish embryos prior to and during neural crest specification.[27-30] *β-catenin* is expressed in both premigratory and migratory neural crest in chick.[31]

Insight into the role of WNT signaling in pigment cell development has come from studies in multiple organisms. In *Xenopus* and zebrafish overexpression of canonical Wnt signaling causes an expansion of neural crest markers and inhibition of this pathway leads to a reduced neural crest population.[1,32-34] In mice, individual knockouts of either Wnt1 or Wnt3a do not display overt neural crest defects, however Wnt1 and Wnt3a double knockout embryos have reduced numbers of *Dct*-positive melanoblasts.[35] Since these mutant embryos do not exhibit

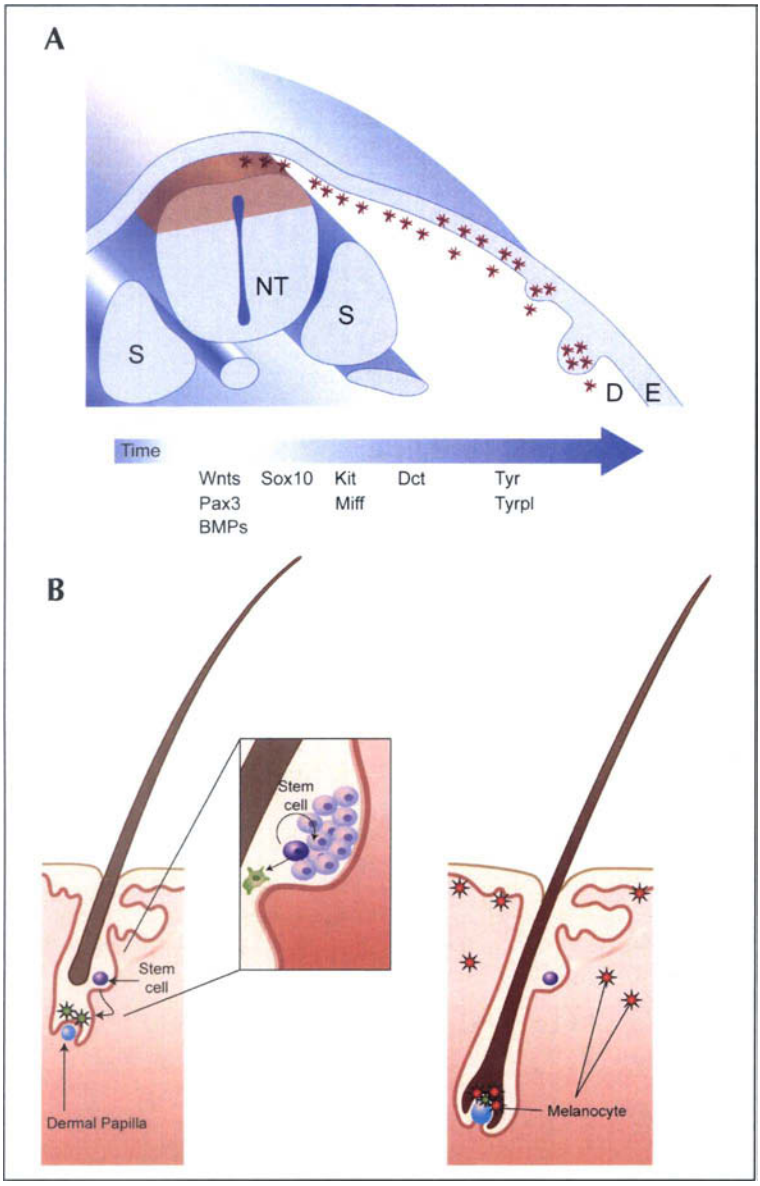

Figure 1. Cartoon depicting pigment cell specification and migration, and melanocyte stem cells. A,B) Pigment cell development and stem cell regeneration of pigment cells are based primarily on work done in mice. A) The neural crest population is induced from the dorsal edge of the neural tube (dark shading). The pigment cell precursor cells (asterisks) arise from a subset of the neural crest and migrate dorso-laterally beneath the ectoderm. As the cells migrate they divide and express more specialized markers of pigment cells, as indicated. The pigment cells migrate from the dermis (D) into the epidermis (E) and then a subset of these cells enters the hair follicle. B, left) Melanocyte stem cells are found in the bulge region of the mature hair follicle. B, middle) During the growth phase of the hair follicle, these cells divide generating a pluripotent stem cell and a population of melanocyte precursors. B, right) As the hair follicle cycle reaches resting phase the precursors become mature melanocytes and populate the hair follicle to produce pigment. Modified from Steingrimsson et al.[140]

increased cell death or reduced cell proliferation, this phenotype is likely to be due to defective melanoblast specification.

Neural crest-specific ablation of *β-catenin* in mouse blocks the formation of both melanoblasts and sensory neurons.[36] Surprisingly, over-expression of an activated form of *β-catenin* in descendents of Wnt1-cre neural crest cells induces sensory neurons but at the expense of melanoblasts.[37] These apparently contradictory results suggest that different neural crest derivatives may respond differently to Wnt signaling at various times in development. Deletion of *β-catenin* function specifically in melanoblasts will be necessary to assess its role in pigment cell development at later stages. In Zebrafish, over-expression of *β-catenin* in migratory neural crest cells promotes pigment cell formation at the expense of neurons and glia.[27] Moreover over-expression of a dominant-negative form of *tcf/lef* significantly reduces expression of *mitf*, without globally affecting neural crest populations.[32] Wnt signaling is proposed to specify neural crest to a pigment cell fate by direct regulation of the melanocyte transcription factor, Mitf. In support of this, the *Mitf* promoter contains Lef binding sites that are highly conserved between zebrafish and mice.[38,39]

In vitro studies using neural crest cultures also support a role for Wnt1 and 3a in promoting pigment cell differentiation and expansion at the expense of other neural crest derivatives. Addition of Wnt3a into neural crest cultures from mouse and chick induces pigment cell differentiation and an expansion in the number of pigment cells, probably at the expense of other neural crest derivatives.[28,40] Although the Wnt1 and 3a knockout mice suggest that these Wnts act redundantly, this might be temporally regulated as studies in mouse neural crest cultures indicate that Wnt3a but not Wnt1 can signal melanoblast differentiation.[41]

Members of the Bone Morphogenetic Protein (BMP) signaling family are also involved in neural crest and pigment cell formation. In mice, BMP2 expression has been reported in the surface ectoderm of E8.5 embryos.[42] Two independent studies have shown that treatment of chick neural crest cultures with BMP4, or with FGF2 and BMP2 together, reduces the overall number of pigment cells while expanding the population of glia and neurons.[28,43] These results suggest that the BMPs may inhibit pigment cell specification and/or expansion. At later stages of pigment cell development, BMPs may have a different role as BMP2 treatment of chick neural crest cultures increases synthesis of melanin but not expression of *Dct, Tyrp1*, or *Mitf*.[44]

In addition to the Wnts and BMPs, *Pax3* is also expressed very early in the dorsal neural tube and neural crest[13,15] and is required for development of several neural crest derivatives. *Pax3^{sp}*/+ animals have belly spots and *Pax3^{sp}* homozygous mouse embryos are embryonic lethal and exhibit reduced numbers of melanoblasts suggesting that Pax3 may be important for early expansion, survival, and/or migration of melanoblasts.[45] Pax3 may be required both cell and noncell autonomously, since transplantation of *Pax3^{Sp}*/*Pax3^{Sp}* neural tubes onto neural tubes of chick embryos results in normal neural crest cell migration.[46]

Similar to Wnt signaling, Pax3 also promotes pigment cell differentiation. Pax3 transactivates *Tyrp1* and acts synergistically with Sox10 to activate *Mitf*.[24,47-49] In addition, a recent study detailed Pax3 expression in the bulge region of hair follicles, and identified dual roles for Pax3 in the activation and repression of pigment genes throughout their development.[24] Lang et al found that in addition to its role in transactivating pigment genes, such as *Mitf*, Pax3 can also inhibit Mitf-mediated expression of *Dct*. This Pax3-mediated repression may be abolished by expression of β-catenin. Together these results suggest that Pax3 may function in both developing pigment cells and in the regulation of pigment stem cell maintenance and differentiation.

Pigment Cell Specification, Differentiation and Survival

Several transcription factors regulate pigment cell specification and differentiation. These include Foxd3, Slug/Snail, Sox9, Sox10, Ap-2, and Mitf. Work from a number of organisms has helped to elucidate the complex relationships among these proteins. Although a genetic hierarchy is apparent, it is not linear and almost certainly involves positive and negative feedback loops to promote development of the pigment cells.

Foxd3 may function to inhibit pigment cell development. Expression studies in chick, mice, *Xenopus*, and zebrafish have shown that Foxd3 is expressed at high levels in the dorsal neural tubes and in premigratory and migratory neural crest.[50-54] In addition, Dottori et. al. also showed that Foxd3 is expressed at lower levels in pigment cells. Consistent with a role in inhibiting pigment cell development, Foxd3 depletion from chick neural tubes causes formation of ectopic pigment cells, while over-expression inhibits pigment cell formation and migration along the dorso-lateral pathway.[52] Additional studies will be necessary to delineate the specific role of Foxd3 in regulating pigment cell development.

Members of the Snail family of transcription factors, which include Snail and Slug, regulate the early specification and differentiation of pigment cells. Depending upon the organism, different members of the family are present and are expressed in premigratory neural crest of zebrafish (*snail2*), *Xenopus* (*snail* and *slug*), chick (*slug*) and mouse (*Snail*).[55-58] Over-expression studies in *Xenopus* have indicated that *slug* is sufficient to promote pigment cell formation.[59] In addition, expression of a dominant negative form of *slug* in *Xenopus* reduces expression of *sox10*, which marks neural crest and pigment precursor cells.[1] Since these studies also showed that over-expression of *sox10* induces ectopic *slug* expression, in *Xenopus slug* and *sox10* regulate each other's expression. In support of this complex relationship, Sox10 transactivates *Mitf*, which in turn transactivates the *SLUG* promotor in vitro.[48,60] Since some *snail2* homozygous mutant mice exhibit belly spots, it seems that the function of Snail and Slug may be conserved.[55,57]

Sox9 is another transcription factor that is thought to act early in pigment cell development.[61] Sox9 is a member of a family of SRY box-containing transcription factors that is expressed in the pre and post-migratory neural crest.[62,63] Studies in chick, *Xenopus*, and mice suggest that *Sox9* acts upstream of the neural crest marker, *sox10*. In chick, *Sox9* can induce *Sox10* expression.[1,63,64] In zebrafish it has been shown that *sox9* acts upstream of *foxd3* and *snail2b*.[65] In support of a role of sox9 in pigment cell specification and/or migration, electroporation of *sox9*-GFP into neural crest cells of chick embryo explants induces GFP positive cells that only migrate along the dorso-lateral pathway.[62] Consistent with this, Zebrafish harboring mutations in both orthologs of *sox9*, *sox9a* and *b*, have reduced numbers of iridophores but not other pigment cell types.[65] Taken together, these data suggest that Sox9 family members may promote pigment cell differentiation and possibly their migration.

Sox10 is another SRY containing transcription factor that is essential for pigment cell differentiation and survival. Sox10 is expressed in early premigratory and migratory neural crest in humans, mice, chick, zebrafish, and *Xenopus* and is detectable in developing pigment cells of mice.[1,14,66-72]

The first studies to indicate a requirement for Sox10 in pigment cell development came from analysis of *Sox10^{Dom}* heterozygous mouse mutants that have a premature truncation of the protein.[72,73] Heterozygous mice have belly spots and embryos contain fewer melanoblasts than wild-type embryos and younger embryos exhibit reduced *Dct* expression.[74] Homozygous mutant embryos also exhibit increased apoptosis of neural crest as assessed by TUNEL staining, suggesting that *Sox10* is essential for neural crest cell survival.[72,75] These mice also have defects in enteric ganglia resulting in megacolon, a condition associated with Hirschsprung's disease in man. Studies in mouse neural crest cultures confirmed that Sox10 acts intrinsically to the melanoblasts.[76]

Studies in zebrafish and *Xenopus* have corroborated those in mice and additionally helped to elucidate the relationship between Sox10 and other transcription factors. As mentioned previously, studies from *Xenopus* have shown that *sox10* acts downstream of *wnt1*, *sox9, and slug*.[1,77] These studies also demonstrated that over-expression of *sox10* induced ectopic pigment cells, while depletion of *sox10* in *Xenopus* led to a decrease in cell proliferation and an increase in apoptosis. *Sox10* zebrafish mutants and morpholinos treated embryos exhibit increased apoptosis and failure to differentiate, as assessed by reduced *mitf* expression.[69] Together these studies suggest a requirement for Sox10 in pigment cell differentiation and

survival. In support of this, in vitro studies have shown that Sox10 transactivates expression of *Mitf* and *Dct*.[48,74,78-81]

Downstream of Sox10 is another transcription factor, AP-2α, that promotes pigment cell development. In mice, AP-2α is expressed in the premigratory and migratory neural crest around E8.5 (although its expression is thought to be lost in differentiating neural crest).[61,82] A conditional knockout of *Ap-2α* in neural crest, using *Wnt1*-Cre mediated recombination, results in mice with belly spots and demonstrates a requirement for AP-2α in pigment cell development.[12] Similarly, zebrafish *ap-2α* mutants, called *lockjaw*/ *mont blanc*, and embryos injected with *ap-2α* morpholinos have reduced numbers of early melanophores and iridophores.[50,83-86] These studies demonstrate a role for AP-2α neural crest development that may be confined to pigment cells since the zebrafish embryos exhibited normal expression of the pan-neural crest markers *snail2*, *foxd3* and *sox10*, but reduced expression of *dct*, *mitf*, and *kit*.[50,84,86]

As mentioned above, Sox10 activates expression of another transcription factor, *Mitf*, that itself has been referred to as the pigment cell master regulatory gene.[87,88] *Mitf* directly regulates the expression of the melanogenic genes *Pmel17*, *Melan-a*, and *melastatin* (TRPM1), and the enzymes, *Tyr*, *Tyrp1*, and *Dct*[63,89-92,93] In vitro studies show Mitf and Sox10 act synergistically to activate Dct expression.[94,114] There are nine isoforms of *Mitf* with Mitf-M being the main isoform thought to regulate pigment cell development. Murine *Mitf-M* is expressed in developing neural crest-derived pigment cells and in the neuroepithelium-derived retinal pigment epithelium (RPE) of the eye beginning at E10.[17] At least twenty-three different murine mutant alleles of *Mitf* have been identified, displaying phenotypes ranging from moderate spotting to complete absence of mature pigment cells.[25,95]

Mitf homologs also regulate pigment cell development in zebrafish and *Xenopus*. A zebrafish *mitf* ortholog, *nacre* or *mitfa*, is required for melanophore development, and misexpression of *nacre* produces ectopic melanophores and abnormal eye development.[33] Recent work has demonstrated the existence of two *Xenopus mitf* isoforms, *XIMitfα* and *XIMitfβ*, which appear homologous to Mitf-M and Mitf-A, respectively. *XIMitfα* is expressed in presumptive pigment cells and the RPE.[96] In addition to a role for MITF in regulating pigment cell differentiation, *Mitf* also functions in cell survival by directly regulating *Bcl2* in pigment cells and osteoclasts.[97] Recent studies have shown that *Mitf* also activates INK4A and p21-Cip1 expression, suggesting *Mitf* coregulates differentiation and cell cycle exit in pigment cell precursors.[98-100] Thus Mitf may act as the pigment cell master gene by a combination of controlling differentiation and cell cycle progression.

Pigment Cell Migration and Development: Cell Adhesion and Signaling

Concurrent with induction, expansion and differentiation of the pigment cell population is their migration. Specified pigment cells are the only known neural crest derivative to migrate along the dorso-lateral pathway, in contrast to other neural crest derivatives that migrate ventrally relative to the neural tube and somites.[101,102] As mentioned, the timing of their migration differs amongst organisms. There is evidence that a long distance cue from emerging dermis could stimulate their dorso-lateral migration since dermal grafts in chick neural tube cultures can induce migration from a distance.[103] One possible candidate is Kit ligand, to be discussed below, since it is expressed in the dermamyotome.[104,105]

Questions remain concerning the timing of migration and specification. Neural crest cells may need to be initially specified to the pigment cell lineage thereby allowing their migration along the dorso-lateral pathway, or alternatively only those cells that migrate along the dorso-lateral pathway are subsequently specified as pigment cells. In support of the first theory, Erickson and Goens showed that specified cultured pigment cells transplanted back into chick neural tube explants, migrated only along the dorso-lateral pathway while non pigment cells failed to migrate along this pathway.[101] The expression of pigment cell specific markers, *Kit*,

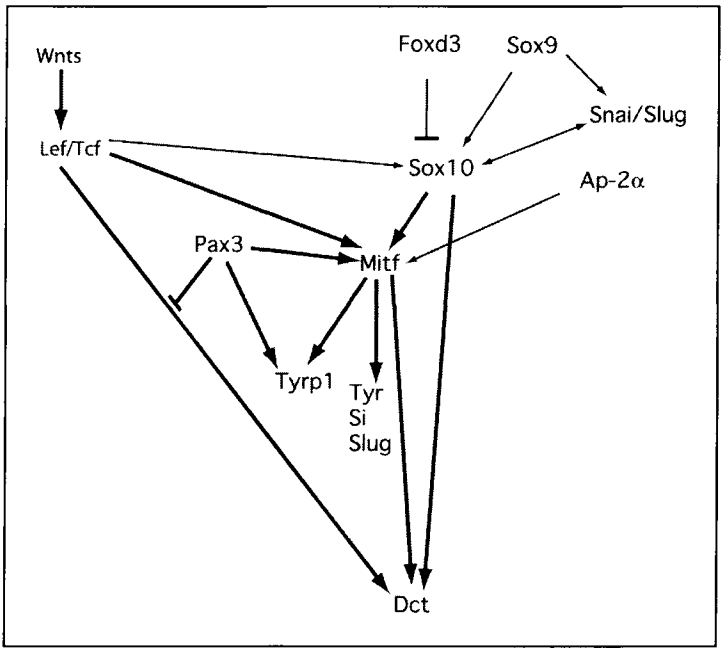

Figure 2. A schematic diagram that represents the transcription factor hierarchy of pigment cell development. Shown are the genetic relationships amongst transcription factors that regulate pigment cell development, based upon work in many vertebrates. Dark lines indicate direct relationships that have been characterized biochemically.

Mitf, and *Dct* adjacent to the neural tube is also consistent with this theory (Fig. 2). However, some other neural crest derivatives have been observed along the dorso-lateral migration pathway but may be eliminated by apoptosis.[102]

Inhibitory proteins within the dermamyotome may prevent nonpigment cells from migrating along the dorso-lateral pathway. In support of this, removal of dermamyotome in chick causes neural crest to migrate precociously along the dorso-lateral pathway.[106] Inhibitory factors may include components of the extracellular matrix. For example, proteins such as F-spondin, are required to block early neural crest migration along the dorso-lateral pathway.[107] Peanut agglutinin lectin-binding activity and chrondroitin-6-sulfate are also good candidates for inhibition of neural crest migration since they are expressed in dermamyotome.[108]

Not surprisingly, specific cell adhesion molecules Integrins and Cadherins are expressed in neural crest derived pigment cells and contribute to defining appropriate migration pathways. Integrins α-2, 3, and 5, which interact with extracellular matrix proteins are expressed in pigment cells.[109] Evidence supporting *Integrin β-1* involvement in dorso-lateral migration comes from analysis of null ES cells transplanted onto avian neural tubes.[110] These *Integrin β-1* deficient ES cells fail to migrate along the dorso-lateral pathway but do migrate ventrally. Conditional knockout studies in mice are necessary to examine the roles of the various integrins in melanoblast development.

Cadherins, which exhibit homophilic interactions between cells, also exhibit a dynamic expression pattern during pigment cell development and are involved in regulating the timing and path of migration. Immunohistochemical studies in mice have revealed that as neural crest initiate migration, E-cadherin and P-cadherin expression is high in these and surrounding cells.[111] As the melanoblast population migrates from the dermis to the epidermis to the hair

follicle, the expression pattern changes. Epidermal melanoblasts express primarily E-cadherin, while those in the dermis and hair follicles express primarily N and P cadherins, respectively. Interestingly, over-expression of *N-cadherin* and *Cadherin 7* in chick inhibits migration of melanocytes out of the neural tube.[31] This suggests that the expression level and type of cadherin expressed regulates pigment cell migration.

Santiago and Erickson also showed using chick explants that Ephrin B, a receptor tyrosine kinase, has dual requirements in regulating pigment cell migration.[112] In Chick, Ephrin B receptors are expressed in melanoblasts and the ligands are expressed along the dorso-lateral pathway throughout melanoblast migration.[113] Blocking ephrin signaling in stage 12 chick explants leads to increased neural crest migration but inhibits migration in stage 18 explants, when pigment cells normally initiate migration. While this analysis revealed an important function for ephrins in chick neural crest, there may be redundant genes in mice since the knockout of murine *ephrin B2* and 3 does not affect neural crest migration.[113,114]

Another receptor tyrosine kinase pathway that is required for both pigment cell survival and migration is Kit. Kit is expressed coincident with Mitf in both premigratory and migratory pigment cells throughout their development.[16,19,20] It has been reported that Kit expressing neural crest cells give rise to only pigment cells,[20,115] however in mouse neural crest cultures not all Kit positive cells are committed to the melanocyte lineage.[41] The ligand for Kit (Kitl) is expressed in a complementary pattern in the dermamyotome, dermis and hair follicles.[116,117] There are two alternatively spliced isoforms of Kit ligand. One generates a protein that is proteolytically cleaved to make a soluble protein while the other generates a protein thought to be primarily membrane bound.

The requirement for Kit signaling in pigment cell development was first identified from analysis of spontaneous mouse mutants, Kit^{W} and $Kitl^{Sl}$. Heterozygous mice have belly spots and in the case of $Kitl^{Sl}$ are hypopigmented on their ventrum. The temporal requirement for Kit in pigment cell development was explored by injection of Kit antibody into pregnant mice at various stages of gestation.[118,119] These studies showed that there are Kit dependent and independent times during development. Kit is required at the times coincident with migration in the dermis, proliferation and survival in the epidermis, however not for proliferation in the hair follicles during development.

Embryonic analyses of Kit mutants support a role for this pathway in multiple steps of melanoblast development. Kit^{h}/Kit^{h} and Kit^{Wv}/Kit^{Wv} embryos have reduced numbers of melanoblasts by E12.5, suggesting a requirement in melanoblast survival but not for migration and differentiation.[120,121] Similarly, $Kitl^{SD}$ mutant embryos, which lack the membrane form of Kitl, also have reduced numbers of melanoblasts; however, they are more evenly distributed across the embryo as compared to Kit mutants. This suggests that the membrane form is required for melanoblast survival and perhaps less so for melanoblast migration.[105] *Sparse* zebrafish mutants that lack Kit have increased numbers of dorsal melanophores but decreased pigment cells ventrally, demonstrating that Kit function is conserved.[122]

The specific requirement of Kit in migration was examined using *Kit* embryos that contain a mutation in *Nf1*, a gene that blocks apoptosis of melanoblasts (and other cells).[123] These embryos exhibit migration defects suggesting that the requirement for Kit in migration is distinct from its requirement in survival. It is worth noting that addition of exogenous Kitl bound to a bead in embryonic skin cultures is sufficient to promote migration of pigment cells into hair follicles but does not act as a chemoattractant.[124] In addition, analysis of various *kit* mutant alleles in zebrafish that distinctly affect pigment cell survival and migration suggests that Kit signaling promotes both processes but that they are separable.[125]

In vitro studies also support a role for Kit in pigment cell differentiation. Addition of Kitl to neural crest cultures increases the number of Dct-positive cells.[18] Kit^{lacZ} mutant neural crest cultures retain expression of some genes, including *Mitf*, but not all pigment cell markers.[126] Kit may promote pigment cell differentiation by post-translational modifications, since Kit signaling can lead to Mitf phosphorylation.[127]

The seven transmembrane domain, G protein-coupled endothelin receptor-B (*Ednrb*) is also required for normal development of pigment cells.[128] Ednrb is expressed in developing neural crest cells, pigment cells, and enteric ganglia in mouse embryos, while the ligand, Edn3, is expressed in tissues associated with *Ednrb*-expressing cells.[128-132] Two orthologs of *Ednrb*, *Ednrb1* (classically referred to as Ednrb) and *Ednrb2*, are present in chick and quail. The two Edrnb homologues in chick may have different functions since at the onset of pigment cell migration, Ednrb1 expression decreases and Ednrb2 expression increases.[133] This function may be conserved, since Ednrc, which is very similar to quail Ednrb2, was isolated from Xenopus melanophores.[134]

Studies in mice have established that *Ednrb* signaling is not necessary for the specification of pigment cells but is involved in the proliferation and migration of pigment cells.[21,128,132,135] Experiments using a conditional *Ednrb* knockout with inducible expression and repression demonstrated Ednrb is required between E10 and E12.5, when melanoblasts migrate and proliferate along the dorso-lateral pathway.[136] In zebrafish, mutation of *ednrb1* causes the *rose* mutant. Zebrafish *ednrb1* is expressed in embryonic melanophore, iridophore and xanthophore precursors. It continues to be expressed after melanophore and iridophore differentiation but its expression decreases during xanthophore differentiation. While *rose* mutants display normal larval pigmentation patterns, adults are missing a subset of pigment cells, including late stripe melanophores, and also display reduced iridophores.[137]

In addition to migration and proliferation, in vitro studies indicate that the Ednrb pathway also promotes pigment cell differentiation and survival.[18,131,132,138,139] Tissue recombination experiments using *Ednrb*-null neural crest cell cultures showed that *Ednrb* performs sequential cell-autonomous and nonautonomous actions during pigment cell differentiation.[139] Ednrb is not required for the generation of early neural crest-derived pigment cells but is required for the later expression of *tyrosinase*, via noncell autonomous production of Kitl. Taken together, these studies suggest that both Ednrb and Kit function during multiple steps of pigment cell development.

Concluding Thoughts

This chapter reveals that the genetic pathways that regulate how pigment cells form, differentiate, and migrate are highly conserved. While we have some understanding of how the various transcription factors, receptors and signaling proteins are related, there are still many questions. What are the downstream targets of these pathways? How are the genes regulated so that they can function at several steps during neural crest and pigment cell development? And ultimately, how do mutation in these genes cause human disease? Continued work of many laboratories in the above model organisms will provide additional insight into the complex and intriguing process of pigment cell development.

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