5 Regulation of Melanoblast Migration and Differentiation

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Summary

1 The discovery of the relationship between the neural crest and pigment cells is briefly reviewed.
2 In all animals studied thus far, neural crest cells are known to migrate along specified pathways and to differentiate into a variety of cell types. These phenomena are directed by a variety of determining factors.
3 With regard to understanding pigment cell migration and differentiation, fish have recently contributed in a significant way primarily because of the large number of mutants that have been isolated in zebrafish and other species. Moreover, the clear embryos are amenable to experimental manipulation and visualization.
4 Amphibians have provided extensive opportunities to examine the movement and differentiation of a variety of pigment cell types, and in this case a number of mutants have been studied in some detail. However, this system has not been exploited to its full potential.
5 Although many reports of pigment variants exist in reptiles, little work has been done on developmental aspects of reptilian chromatophores.
6 Our understanding of the cellular and biochemical controls of pigment cell development has been greatly enhanced by work done in avian systems. Recently the development of methods to up- and downregulate gene expression in the avian embryo has contributed importantly to the understanding of melanogenesis in this system.
7 The many coat color mutants of mice provide extensive opportunities for better understanding the motive forces in pigment cell development.
8 A comparative evolutionary approach to studye pigment cell development will continue to be essential for understanding how such discrete cells (pigment cells) from a well-defined progenitor cell population (the neural crest) have differentiated and evolved.

Historical Background

Embryologists have long been interested in the mechanisms that produce the correct arrangement of cells and tissues in the developing embryo. Two of the processes that drive this event, called pattern formation, are morphogenesis and differentiation. Morphogenesis refers to the generation of biologic structure by the rearrangement of cells or tissues. In many cases this is achieved by the active migration of cells from one location in the embryo to another. Differentiation, on the other hand, is the process by which cells acquire morphologic and biochemical characteristics distinct from other cells. Although morphogenesis and differentiation usually are studied as independent developmental events, in fact these two processes are highly interconnected. The development of the neural crest exemplifies this complex interplay between morphogenesis and differentiation.

Neural crest cells are a population of cells found in all vertebrate embryos. Excellent introductions to the older literature on neural crest development can be found in Hörstadius (1950) and Le Douarin and Kalcheim (1999). The early studies demonstrated three important aspects of neural crest development. First, neural crest cells detach from the neuroepithelium shortly before or soon after fusion of the neural folds. Second, neural crest cells migrate extensively throughout the developing embryo. Third, the neural crest is not a terminally differentiated population of cells, but instead can give rise to a wide range of cell and tissue types. These include neurons and glial cells of the peripheral nervous system, the secretory cells of the adrenal medulla, certain cardiac structures, enteric ganglia, and connective tissues of the craniofacial skeleton.

Pigment cells are another derivative of the neural crest. There are several types of pigment cell, also called chromatophores, in vertebrate ectotherms (fishes, amphibians, reptiles) including: black, melanin-containing melanophores; yellow, pteridine-containing xanthophores; and silvery, purine-containing iridophores. In contrast, endotherms (birds and mammals) have a single type of skin pigment cell, the melanin-containing melanocyte. The study of pigment cell development in vertebrates has a rich historical tradition, and many older reviews provide access to these classical studies (e.g., Bagnara and Hadley, 1973; Le Douarin, 1982).

The purpose of this chapter is to present an overview of the current state of knowledge on the development of neural crest-derived pigment cells in vertebrate embryos. We begin with a discussion of themes in neural crest and pigment cell development common to all vertebrates. Because this textbook is organized around the concept of taxonomy, we have similarly organized this chapter by taxon. Thus, we summarize what is

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known about pigment cell development specifically within the different classes of vertebrates: fishes, amphibians, reptiles, birds and mammals. The reader is encouraged to note the similarities and differences in the control of pigment cell development across these major phylogenetic groups. Although mathematical modeling is one technique that has been employed to study the generation of pigment patterns in vertebrates (reviewed in Murray, 1990), we approach the topic from a molecular and cellular perspective, and thus do not discuss any of these models specifically. Instead, our discussion is framed around the subtopics of morphogenesis and differentiation. Morphogenesis and differentiation are treated separately because most empirical studies to date have focused principally on one process or the other. Nevertheless, there is clear evidence that these two events are closely linked during pigment cell development (reviewed by Erickson and Reedy, 1998). Additionally, we discuss the role that pigmentation mutants have played in elucidating the molecular control of pigment cell development. In some animal models, such as birds and amphibians, pigment mutants have been useful but not the primary means of understanding melanogenesis. In the case of fishes and to a lesser extent mammals, most of what we know about pigment patterns has been derived from mutants and these are discussed extensively throughout the topics of morphogenesis and differentiation. Finally, we discuss major unresolved questions and suggest future research directions in the field.

Current Concepts: Overview of Neural Crest and Pigment Cell Development

An understanding of the mechanisms that control neural crest and pigment cell morphogenesis is predicated on knowledge of the precise pathways these cells follow as they migrate. Several methods have been used to trace the migratory pathways of neural crest cells. One approach is to employ vital labeling of premigratory neural crest cells, for example with lysinated rhodamine dextran (LRD) or the lipophilic dye, DiI, and subsequently following migrating cells in living embryos over time. Alternatively, immunohistochemical markers and molecular probes specific for neural crest cells often are used to assess the distributions of cells in fixed tissues.

In all species studied thus far, neural crest cells migrate along stereotyped routes. For reasons of space and simplicity, we focus on the migration of neural crest cells in the trunk. Discussions of cranial neural crest migration are available elsewhere (e.g., Köntges and Lumsden, 1996; Kulesa, 2004; Kulesa *et al.*, 2004; Lumsden *et al.*, 1991; Noden, 1991; Trainor *et al.*, 2003). There are two principal migratory pathways for neural crest cells in the trunk (reviewed in Erickson, 2003; Erickson and Perris, 1993). One subpopulation of neural crest cells follows a *ventromedial*, or *medial*, pathway between the somite and neural tube. Glial and neurogenic precursors of the peripheral nervous system are among the neural crest cells that differentiate along this path, although in fishes and amphibians some of the neural crest cells in this path can also differentiate as pigment cells. A second subpopulation of neural crest cells migrates along a *dorsolateral*, or *lateral*, path between the somites and the overlying epidermis. Neural crest cells in this path give rise to pigment cells (reviewed in Erickson, 1993). In fishes and amphibians, some neural crest cells take a third pathway and migrate dorsally into the developing fin.

In birds and mammals, neural crest cells in the dorsolateral path differentiate exclusively as pigment cells. Similarly in ectotherms, there is no definitive evidence that neural crest cells in the dorsolateral path contribute to any neural crest derivative other than pigment cells. Conversely, there is a bias toward nonpigment cell phenotypes in ventromedially migrating neural crest. This correlation between migratory path and differentiated phenotype is well documented, but its developmental basis is controversial. There is evidence not only to support the notion that environmental factors encountered within a particular migratory path dictate neural crest fate, but also conversely, that prior specification dictates the choice of migratory path (discussed in Erickson, 2003; Le Douarin *et al.*, 1993).

The developmental potential of neural crest cells has been studied by a variety of means, including clonal analysis of neural crest differentiation and grafting neural crest cells or neural tube segments to ectopic axial levels and determining the differentiated phenotypes of the transplanted cells. Taken together, these studies suggest that most early neural crest cells are multipotent but become fate-restricted gradually over time (reviewed in Weston, 1991; Stemple and Anderson, 1993; Le Douarin *et al.*, 1993, 2004; see below). Defining the precise lineage segregation of pigment cells has been difficult for two reasons. First, pigment cells differentiate from neural crest cells at most, if not all, axial levels of the trunk. As a result, heterotopic transplantation studies of the sort used to study the differentiation of other crest subpopulations in ectopic locations are not useful for studying the commitment of neural crest cells to a pigment cell fate. Second, markers for pigment cell precursors at the earliest stages of differentiation were not available until relatively recently. Consequently, early studies inferred that a particular cell was a pigment cell precursor simply by virtue of it being in the dorsolateral path but could not further evaluate the cell's phenotype. Now, however, there is a substantial arsenal of lineage-specific markers for pigment cell precursors in most taxa. As detailed below, these markers have proven critical in studying the factors involved in pigment cell development.

Fishes

Fishes provide three advantages for studies of pigmentation. First, the embryos of many species are oviparous and transparent so that neural crest cells and chromatophores can be observed throughout early development. Second, some fishes have short generation times and can be bred and reared in the laboratory, permitting genetic approaches to studies of pigment cells and pigment pattern development. Third,

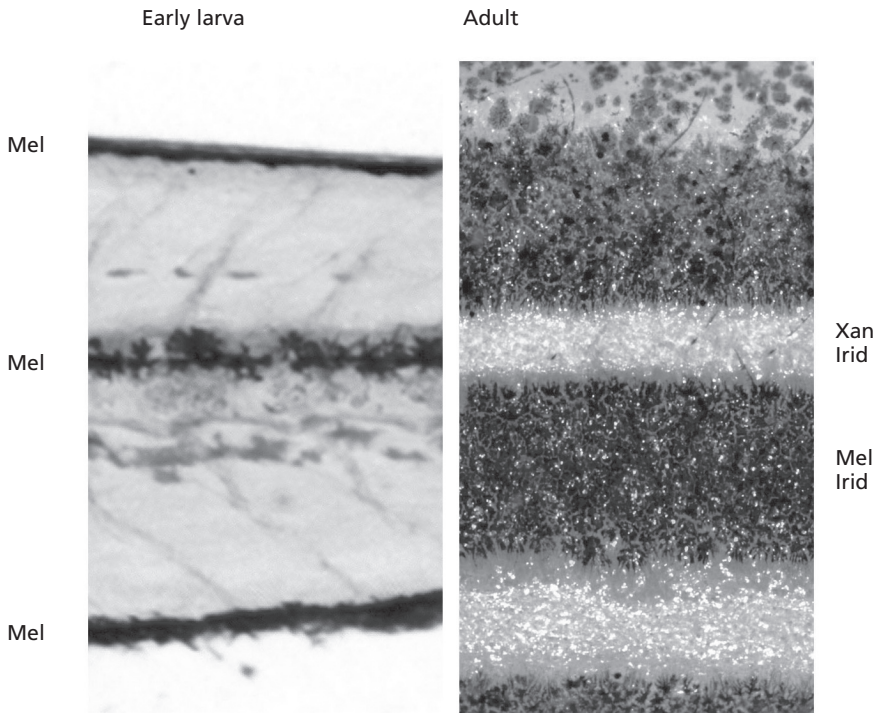

Fig. 5.1. Different pigment patterns of early larval and adult zebrafish (see also Plate 5.1, pp. 494–495). Mel, melanophore stripes; Xan, xanthophores; Irid, iridophores.

pigment patterns of fishes are exceptionally diverse across taxa (e.g., Orton, 1953; Quigley *et al.*, 2005; Riehl, 1991), allowing for comparative analyses of pigment cell development.

To date, the advantages of fish are being exploited most fully in the tropical, freshwater zebrafish *Danio rerio*, as well as the freshwater medaka *Oryzias latipes*. Several sources provide information on staging, husbandry, experimental techniques, as well as the histories of these species as research models [zebrafish: Grunwald and Eisen (2002), Kimmel *et al.* (1995), Westerfield (2000), Zebrafish Information Network (http://zfin.org); medaka: Iwamatsu (2004), Shima and Mitani (2004), Medaka Homepage (http://biol1.bio.nagoya-u.ac.jp:8000/)]. Studies of these species have revealed numerous similarities in the genetic and cellular bases for neural crest and pigment cell development between teleosts and amniotes (see below). Recently several more extensive reviews of teleost pigment pattern formation have appeared (Kelsh, 2004; Parichy, 2003; Quigley and Parichy, 2002; Rawls *et al.*, 2001).

Like other vertebrates, a neural crest origin for fish pigment cells was long suspected (Borcea 1909), though not verified until considerably later (Humm and Young, 1956; Kajishima, 1958; Lopashov, 1944; for experiments on lampreys, see Newth, 1951, 1956). Early light microscopic studies of neural crest cells and chromatophores in teleosts identified these cells by location, shape, or externally visible pigmentation (e.g., Milos and Dingle, 1978a; Orton, 1953; Shephard, 1961). Grafting of [3H]thymidine-labeled cells also has been employed to examine neural crest migration (Lamers *et al.*, 1981). Similarly, the HNK-1 antibody, which recognizes migrating neural crest cells of avian, rat, and turtle embryos (see below), has been used to identify neural crest cells in some species (Sadaghiani and Vielkind, 1989, 1990a; Sadaghiani *et al.*, 1994). Finally, vital dyes have been used to label neural crest cells and their derivatives in zebrafish (e.g., Dutton *et al.*, 2001; Raible and Eisen, 1994; Smith *et al.*, 1994; Vaglia and Hall, 2000).

Teleost chromatophores retain their pigments intracellularly and pigment patterns thus reflect the numbers and arrangements of the various chromatophore classes (black melanophores, yellow xanthophores, iridescent iridophores, white leukophores, and red erythrophores). The precise complements of these chromatophores differ among species. For example, erythrophores are present in several *Danio* species, though not in zebrafish (McClure, 1999; Quigley *et al.*, 2004, 2005).

Adding to the complexity of teleost pigment cell development, pigment patterns of many species differ between early larval and adult stages (Fig. 5.1). Early larval patterns of many species (including zebrafish and medaka) comprise stripes of melanophores at the dorsal and ventral edges of the myotomes, along the horizontal myoseptum, and over the dorsal and ventral surfaces of the yolk (Kimmel *et al.*, 1995; Kirschbaum, 1975; Lamoreux *et al.*, 2005; Milos and Dingle, 1978a, b; Milos *et al.*, 1983; Orton, 1953; Quigley *et al.*, 2004). Iridophores or leukophores are found within the melanophore stripes, whereas xanthophores are widely dispersed outside of the stripes. This early larval pigment pattern arises during embryogenesis and is largely completed by hatching.

After several days to weeks depending on the species, early larval pigment patterns are transformed into adult pigment patterns that are exceptionally diverse among species (Booth,

1990; Kirschbaum, 1975; Johnson *et al.*, 1995; Mabee, 1995; Parichy and Johnson, 2001). For example, even within the genus *Danio*, different species exhibit horizontal stripes of varying number, width, and distinctiveness, vertical bars, uniform patterns, and more ornate patterns (Quigley *et al.*, 2004, 2005). Pigment patterns are even more diverse among other groups including cichlids and coral reef fishes (Allender *et al.*, 2003; Lorenz, 1962; Streelman *et al.*, 2003).

Morphogenesis of Fish Chromatophores

Prospective neural crest cells in most vertebrates are found within the neural folds, which fuse to form a neural tube. In fishes, the neural tube arises instead from a ventral thickening of the dorsomedial ectoderm termed the "neural keel." Subsequently, the neural keel delaminates from surrounding epidermal ectoderm and a central canal develops to generate the definitive neural tube (Lowery and Sive, 2004; Schmitz *et al.*, 1993). Neural crest cells first become apparent along the dorsal surface of the developing neural tube as the neural keel separates from the epidermis (Lamers *et al.*, 1981; Lowery and Sive, 2004; Raible *et al.*, 1992; Sadaghiani and Vielkind, 1989, 1990a; Sadaghiani *et al.*, 1994; Schmitz *et al.*, 1993).

The details of neural crest migration in fishes are best documented in zebrafish. After segregating from the neural keel, zebrafish neural crest cells migrate along the two major pathways discussed previously: ventromedially between the somites and neural tube, and dorsolaterally between the somites and epidermis (Raible and Eisen, 1994; Raible *et al.*, 1992; Vaglia and Hall 2000; Wood *et al.*, 1992). In addition, some neural crest cells migrate dorsally and localize in the fins. Zebrafish neural crest cells in all pathways give rise to pigment cells (Raible and Eisen, 1994). The mechanisms underlying early neural crest cell dispersal and migration remain unknown in teleosts, though transient roles for somitic mesoderm in blocking early migration are likely (Jesuthasan, 1996).

Some insights into early larval pigment cell morphogenesis have come from analyses of *sparse* mutant zebrafish. In contrast to wild-type, *sparse* mutants have fewer melanophores and these cells are largely absent from the anterior head as well as ventral regions of the flank. Molecular cloning identified *sparse* as a teleost ortholog of *Kit* (formerly c-*kit*; Parichy *et al.*, 1999), long-studied for its role in mouse melanocyte development (see Mammals, below). *kit* encodes a type III receptor tyrosine kinase containing five extracellular immunoglobulin repeats and a split kinase domain. The ligand of Kit is Steel factor (SLF; also known as stem cell factor, mast cell growth factor, and kit ligand). Like other receptor tyrosine kinases, dimerization of Kit upon ligand binding is necessary for activation of the kinase domain (Blume-Jensen *et al.*, 1991). Downstream signal transduction events initiated by Kit signaling in melanoblasts are largely unknown, but evidence suggests that the phospholipase C and phosphatidylinositol-3′-kinase pathways are activated (reviewed in Galli *et al.*, 1994). Zebrafish *kit* is expressed by melanophores and their precursors. In null alleles, melanophores undergo apoptosis and are extruded from the skin (Parichy *et al.*, 1999). More recent analyses of an allelic series including conditional, temperature-sensitive alleles reveal genetically and temporally separable roles for *kit* in melanocyte migration (at early stages) and melanocyte survival (at later stages) (Rawls and Johnson, 2003; see also, Rawls and Johnson, 2000, 2001). Interestingly, the *kit*-related gene *colony stimulating factor-1 receptor* (*csf1r*; formerly, *fms*) is required for the survival and migration of zebrafish xanthophores (Parichy *et al.*, 2000b), suggesting that similar requirements for receptor tyrosine kinase activity are fulfilled by *kit* and *csf1r* in melanophore and xanthophore lineages, respectively.

Recent studies have identified a second locus required for melanophore survival during early larval pigment pattern formation. *Danio rerio touchtone* mutants exhibit normal migration and specification of melanophores, but melanoblasts and melanophores subsequently undergo apoptosis. Genetic mosaic analyses reveal that *touchtone* acts autonomously to the melanophore lineage (Arduini and Henion, 2004; Cornell *et al.*, 2004). The *touchtone* mutant phenotype results from lesions in *trpm7*, which encodes a member of the transient receptor potential (TRP) family (Elizondo *et al.*, 2005). The transmembrane trpm7 protein serves as both a cation channel and as a kinase (Wolf, 2004), and mutants have additional defects in kidney function and skeletal development. A role for trpm7 channel activity during melanophore development is demonstrated by the partial rescue of trpm7 mutant melanophores by simple calcium or magnesium supplementation to the medium. While trpm7 appears to act independently of the *kit* pathway during melanophore development (Cornell *et al.*, 2004), the upstream regulators and downstream targets of trpm7 remain unknown.

Beyond permissive roles for cell migration, survival, and proliferation, of particular interest are the instructive factors that generate specific arrangements of chromatophores. From several recent studies we now have a clearer notion of what these factors are both in early larval and adult pigment pattern formation.

The arrangement of early larval melanophores depends in part on the stromal-derived factor 1a (sdf1a) chemokine. sdf1a is a chemoattractant that contributes to migration of primordial germ cells (PGCs) as well as the lateral lines, a bilateral sensory system that develops by the directed migration of a bullet-shaped "primordium" of cells within the epidermis. Both PGCs and lateral line primordia express cxcr4b, a G-protein-coupled receptor for sdf1a, and migrate towards sources of sdf1a in the prospective gonads and along the horizontal myoseptum, respectively (David *et al.*, 2002; Doitsidou *et al.*, 2002; Li *et al.*, 2004). A role for sdf1a in melanophore patterning is revealed by *choker* mutant zebrafish (Kelsh *et al.*, 1996). By early larval stages, these mutants have a "collar" of melanophores that covers the normally melanophore-free regions of the lateral myotomes. Myotome development is severely disrupted in *choker* mutants and *sdf1a* is expressed ectopically along the myotomes where melanophores accumulate. Morpholino knockdown of *sdf1a*

in the *choker* mutant background reduces the numbers of collar melanophores, whereas ectopic sources of human sdf1a attract melanophores in wild-type embryos (V. Svetic and R. N. Kelsh, personal communication). During normal development then, *sdf1a* plausibly contributes to melanophore localization. Whether sdf1a acts directly on melanophores or through an intermediary signaling pathway remains unclear, particularly since melanophores do not express detectable levels of cxcr4b by *in situ* hybridization. Intriguingly, *choker* mutants lack xanthophores in the collar region. Genetic analyses show this defect is independent of the defect in melanophore patterning, whereas the defect in melanophore patterning is independent of the defect in xanthophore distribution.

Development of the zebrafish adult pigment pattern depends on a complex interplay between directed chromatophore migration and death, as well as differentiation (see below; Parichy and Turner, 2003b; Parichy *et al.*, 2000b; Quigley *et al.*, 2004, 2005). During metamorphosis, new xanthophores arise just ventral to the horizontal myoseptum and at the dorsal and ventral margins of the flank, whereas new melanophores arise widely dispersed over the flank and outside of the early larval stripes. Some of these "metamorphic" melanophores migrate short distances to join the first two adult melanophore stripes, immediately dorsal and ventral to the horizontal myoseptum. Simultaneously, additional metamorphic melanophores develop already within the adult stripes. Many early larval melanophores initially within the lateral stripe along the horizontal myoseptum die, though some migrate to join the developing adult stripes, thereby producing a melanophore-free "interstripe region" that is, however, populated by xanthophores. As metamorphosis proceeds, the adult stripe borders become increasingly distinct. During juvenile and subsequent adult development, additional melanophore stripes are added dorsally and ventrally as the fish grows. In the mature adult pigment pattern of zebrafish, dark stripes comprise melanophores and iridophores with occasional xanthophores, whereas light stripes comprise xanthophores and iridophores (Hirata *et al.*, 2003; Parichy and Turner, 2003a). Dermal and epidermal chromatophores also are found outside of stripes; for example, scale melanophores contribute an overall dark cast to the dorsum of the fish.

The morphogenetic bases for the zebrafish adult striped pattern have been examined in several recent studies that have identified a critical role for interactions within and among chromatophore classes. The notion that chromatophore interactions could be important comes initially from the *csf1r* mutant phenotype (Parichy *et al.*, 2000b; Fig. 5.2). As compared to wild-type, *csf1r* mutants have fewer adult melanophores and these cells fail to organize into normal stripes. Beyond the melanophore defect, *csf1r* mutants lack embryonic xanthophores (as mentioned above) as well as adult xanthophores. The correlated adult melanophore and xanthophore defects raised the possibility that interactions between these lineages might underlie melanophore stripe development. Support for this notion comes from two experimental paradigms (Parichy and Turner, 2003a). First, organized melanophore stripes are recovered in genetic mosaics in which melanophores and xanthophores are experimentally juxtaposed: when cells are transplanted between *csf1r* mutants (which lack xanthophores but retain melanophores) and *nacre* (*mitfa*) mutants [which lack melanophores but retain xanthophores (Fig. 5.2 and see below)] the resulting chimeras develop stripes. Second, if csf1r is conditionally activated or inactivated using a temperature-sensitive allele, xanthophores are gained or lost, respectively, and the number of xanthophores present correlates with the degree of melanophore stripe organization. Indeed, xanthophores and melanophore stripes can be gained or lost in a *csf1r*-dependent manner throughout development, revealing roles for *csf1r* in both initiating and maintaining stripe formation. Since *csf1r* is not detectably expressed by melanophores but is expressed by the xanthophore lineage, these results suggest that interactions between melanophores and *csf1r*-dependent cells of the xanthophore lineage are required for stripe organization. Chromatophore interactions probably also contribute to development of stripes on the fins (Goodrich and Greene, 1959; Goodrich and Nichols, 1931; Goodrich *et al.*, 1954; Parichy and Turner, 2003a). Analyses of other *Danio* species suggest that evolutionary changes in chromatophore interactions may underlie interspecific variation in adult pigment patterns (Parichy and Johnson, 2001; Quigley *et al.*, 2005).

Further indications that chromatophore interactions underlie stripe development come from studies of *obelix* (*jaguar*) and *leopard* mutant zebrafish (Haffter *et al.*, 1996; Johnson *et al.*, 1995; Fig. 5.2). *obelix* mutants exhibit fewer and broader stripes than wild-type and xanthophores are intermingled with melanophores in the melanophore stripes (Maderspacher and Nüsslein-Volhard, 2003). *leopard* mutants often develop spots, but alleles of differing severity range in phenotype from irregular stripes to nearly uniformly distributed, intermingled melanophores and xanthophores (Asai *et al.*, 1999; Maderspacher and Nüsslein-Volhard, 2003). Thus, mutant phenotypes may be interpretable in the context of defective boundary formation between chromatophore classes. Cell transplantation and genetic analyses indicate that *obelix* acts within melanophores to promote homotypic interactions between these cells, whereas *leopard* acts within melanophores and xanthophores to promote both homotypic and heterotypic interactions (Maderspacher and Nüsslein-Volhard, 2003). The *obelix* mutant phenotype results from mutations in *kir7.1*, encoding an inwardly rectifying potassium channel (S. L. Johnson and S. Kondo, personal communication); the cellular basis for translating channel activity into cellular pattern remains to be elucidated. *leopard* mutant phenotypes result from mutations in the gap junction gene, *connexin-40*, suggesting that direct cell–cell contacts and cytoplasmic communication may underlie *leopard*-dependent chromatophore interactions and boundary formation (S.L. Johnson, personal communication). Interestingly, N-CAM and N-cadherin had previously been associated with xanthophores but not other chromatophore classes in *O. latipes* (Fukuzawa and Obika, 1995), consistent with roles for dif-

Fig. 5.2. Mutational analyses allow a genetic dissection of adult pigment pattern development in zebrafish. Shown are wild-type zebrafish as well as single and double mutant zebrafish with corresponding pigment pattern defects (see also Plate 5.2, pp. 494–495). See text for details.

ferential adhesion in the sorting-out of specific chromatophore classes, although this model has yet to be tested directly.

Besides the spatial arrangements of chromatophore classes across the flank, different chromatophores types occupy different strata within the skin itself (Hawkes, 1974a, b). In zebrafish, light stripes comprise xanthophores superficial to iridophores, whereas dark stripes comprise occasional xanthophores superficially, followed by iridophores, then melanophores, and additional iridophores more internally. Moreover, the organization of iridophore reflecting platelets differs depending on whether or not these cells are associated with xanthophores or melanophores (Hirata *et al.*, 2003). The molecular signals associated with these arrangements are not known.

Differentiation of Fish Chromatophores

Factors controlling the specification and differentiation of teleost chromatophores are beginning to be elucidated. An early step in neural crest specification towards chromatophore fates requires the transcription factor sox10, as revealed by analyses of *colourless* mutant zebrafish in which the *sox10* gene is disrupted (Dutton *et al.*, 2001; Kelsh and Eisen, 2000). *sox10* (*colourless*) mutants have severe defects in chromatophores, glia, and neurons: precursors to these lineages fail to leave the neural crest and instead undergo apoptosis. In contrast, ectomesenchymal neural crest derivatives (e.g., craniofacial skeleton and fin mesenchyme) are unaffected.

Nonectomesenchymal *sox10*-dependent neural crest cells often are fate-restricted even prior to leaving the neural crest, as revealed by elegant single cell lineage analyses (Dutton *et al.*, 2001; Raible and Eisen, 1994). Such analyses do not yet provide a clear picture of lineage relationships between chromatophores and other nonectomesenchymal lineages, or even among chromatophore classes themselves. (For a fuller discussion of neural crest specification, see Birds, below.) Nevertheless considerable progress has been made in defining the mechanisms of fate specification and differentiation in the melanophore lineage.

An important advance came from the isolation of *nacre* mutant zebrafish that lack melanophores. This mutant phenotype results from mutations in *microphthalmia-a* (*mitfa*) (Lister *et al.*, 1999), encoding a member of the basic-loop-helix-leucine-zipper (bLH-ZIP) family of transcription factors (Hodgkinson *et al.*, 1993; see Steingrimsson *et al.*, 2004 for a recent review). Mammalian orthologs of *mitfa* have long been studied and are known to directly upregulate genes required for melanin synthesis (see Mammals, below). Additional studies of zebrafish have provided insights into how

mitfa is itself regulated, with important roles identified for both intrinsic and extrinsic factors. An intrinsic factor relative to the melanophore lineage is the sox10 transcription factor. *mitfa* expression is curtailed in *sox10* (*colourless*) mutants (Dutton *et al.*, 2001). The *sox10*-dependence of melanophore development lies entirely in sox10-dependent regulation of *mitfa*: sox10 binds and activates the *mitfa* promoter, *sox10* overexpression upregulates *mitfa* expression in vivo, and forced *mitfa* expression rescues melanophore development in *sox10* mutant backgrounds (Elworthy *et al.*, 2003).

An extrinsic factor relative to the melanophore lineage lies in the Wnt signaling pathway. *Wnt1* and *Wnt3a* are expressed in the dorsal neural keel. This expression domain has functional significance in promoting chromatophore over non-chromatophore fates as revealed by injections of single premigratory neural crest cells with activating or inhibitory mRNAs for the Wnt signal transduction pathway (Dorsky *et al.*, 1998). This effect is mediated, at least in part, by the binding of Wnt-responsive Lef1/T-cell factor (TCF) transcription factors within the *mitfa* promoter, which upregulate *mitfa* transcription and thus melanophore fate specification (Dorsky *et al.*, 2000a, b). More recent studies using a heat shock inducible transgene for a dominant negative form of TCF demonstrate that Wnt signals are required at several distinct steps in the neural crest–melanophore lineage (Lewis *et al.*, 2004; see also Birds, below).

Beyond the critical roles for *mitfa*, *sox10*, and Wnts in melanophore specification, a large number of mutants have been isolated in zebrafish and medaka that identify genes likely to function primarily in melanophore differentiation (Kelsh *et al.*, 1996, 2000, 2004). Many of these probably affect melanin synthesis or melanosome biogenesis (e.g., Fukamachi *et al.*, 2001; Schonthaler *et al.*, 2005). One gene likely to regulate these processes encodes transcription factor activating protein-2a (tfap2a). The zebrafish *lockjaw* and *mont blanc* mutant phenotypes result from mutations in *tfap2a* and have severe defects in melanophore as well as iridophore development, in addition to defects in craniofacial, glial, and neural derivatives of the neural crest (Barrallo-Gimeno *et al.*, 2004; Knight *et al.*, 2003, 2004). The failure of melanophore development results at least in part from a *tfap2a*-dependence of *kit* expression, and epistasis analyses combined with morpholino knockdown support a model in which *tfap2a* and *kit* act in a common pathway (O'Brien *et al.*, 2004). As melanoblasts express early markers of the melanophore lineage, and some melanophores develop but are lightly pigmented, it appears *tfap2a* allows the terminal differentiation of this cell type.

While considerable effort is directed towards understanding specification and differentiation of chromatophores during embryonic stages, mutational analyses have also started to reveal genetic requirements of metamorphic melanophores that contribute to adult pigment patterns. For example, *puma* mutant zebrafish have a severe deficit of metamorphic melanophores despite having a normal complement of early larval melanophores (Parichy and Turner, 2003b; Parichy *et al.*, 2003; Fig. 5.2). *puma* is required during metamorphosis for promoting melanoblast and melanophore development and acts autonomously to the melanophore lineage. A superficially similar phenotype is observed for *picasso* mutants (Quigley *et al.*, 2004; Fig. 5.2), which result from mutations in the receptor tyrosine kinase gene *erbb3* (Lyons *et al.*, 2005; E. Budi and D. Parichy, unpublished data). Nevertheless, it has yet to be determined whether *puma* and *picasso* (*erbb3*) function primarily to promote metamorphic melanophore specification and differentiation, or survival and proliferation. Regardless of the precise mechanism, comparative analyses demonstrate that most adult pigment patterns among danios arise from metamorphic melanophores that differentiate from latent precursors during the larval-to-adult transformation (Quigley *et al.*, 2004); understanding the factors that promote the establishment, maintenance, and recruitment of such latent precursors is thus critical to elucidating the mechanisms underlying naturally occurring pigment pattern variation in this group. As an extreme example of this notion, the zebrafish sister species, *D. nigrofasciatus*, mostly lacks metamorphic melanophores and their precursors owing to evolutionary changes that are extrinsic to this melanophore lineage and likely to involve the *puma* pathway (Quigley *et al.*, 2004).

Despite their apparent uniformity, metamorphic melanophores actually comprise two genetically and temporally distinct populations, as revealed by analyses of *kit*, *csf1r*, and *endothelin receptor b1* (*ednrb1*) mutants (Johnson *et al.*, 1995; Parichy *et al.*, 1999, 2000a, b). *kit* mutants not only lose their early larval melanophores, but lack early-appearing, dispersed metamorphic melanophores; nevertheless, these mutants ultimately recover metamorphic melanophores, already situated in stripes, during late metamorphosis and juvenile development. In contrast, *csf1r* and *ednrb1* mutants have normal numbers of early-appearing, initially dispersed metamorphic melanophores and instead are deficient for late-appearing metamorphic melanophores at sites of stripe formation. That early *kit*-dependent and late *csf1r*- and *ednrb1*-dependent melanophores are distinct populations is supported by epistasis analyses: in contrast to single mutants, fish doubly mutant for *kit* and *csf1r*, or *kit* and *ednrb1*, lack nearly all body melanophores (Johnson *et al.*, 1995; Parichy *et al.*, 2000b; Fig. 5.2). Additional discussion of endothelin receptors is provided in sections on Birds and Mammals, below.

In contrast to melanophores, relatively less is known about the specification and differentiation of xanthophores, iridophores, or other chromatophore classes. Early larval iridophores are reduced in *tfap2* mutants whereas adult iridophores are absent in *ednrb1* mutants. Molecular identification of additional mutants affecting iridophore development [e.g., *shady* and *parade* (Kelsh *et al.*, 1996)] will surely provide valuable new insights.

Virtually nothing is known about xanthophore fate specification, though precursors of these cells and xanthophore numbers are somewhat reduced in *mitfa* mutants (Lister *et al.*, 1999; Parichy *et al.*, 2000b). Xanthophore pigments are

derived, in part, from the pteridine pathway (Ziegler, 2003; Ziegler *et al.*, 2000) and enzymes within this pathway are expressed by xanthophore precursors, although also in some other lineages, including melanophores (Parichy *et al.*, 2000b; Parichy, unpublished data), suggesting caution in their use as lineage markers. Mutational analyses reveal that xanthophore differentiation requires PAM (protein associated with c-myc), which regulates pteridine synthesis enzymes in a cell autonomous manner (Le Guyader *et al.*, 2005), as well as somatolactin, which acts as an endocrine factor (Fukamachi *et al.*, 2004).

Mutants

Besides those discussed above, a very large number of other mutants affecting chromatophore development have been isolated, particularly in zebrafish (Golling *et al.*, 2002; Haffter *et al.*, 1996; Kelsh *et al.*, 1996; Odenthal *et al.*, 1996; Rawls *et al.*, 1993) and O. *latipes* (Kelsh *et al.*, 2004). These mutants, high-throughput screens for chemically-induced phenotypes (Peterson *et al.*, 2000), and genomic resources (Pickart *et al.*, 2004) offer outstanding opportunities for dissecting morphogenesis and differentiation, as well as the evolution of these processes. Descriptions of mutant screens and on-line resources for these mutants can be found at:

- http://zfin.org
- http://biol1.bio.nagoya-u.ac.jp:8000/
- http://protist.biology.washington.edu/dparichy/ImagesMutants.htm

Amphibians

Amphibians offer several advantages for studies of pigment cells and pigment pattern formation. For example, embryos of many species are quite large and develop oviparously. Moreover, development rate often can be controlled by incubating embryos at a wide range of temperatures. This makes amphibian embryos highly accessible to both observation and experimentation. Finally, amphibians offer a wide range of patterns to study: not only do different taxa exhibit an extraordinary diversity of larval and postmetamorphic patterns [e.g., urodeles (Bishop, 1941; Epperlein and Löfberg, 1990; Olsson, 1993, 1994; Pfingsten and Downs, 1989; Twitty, 1936, 1966), reviewed in Parichy, 1996b; anurans (Altig and Johnston, 1989; Andres, 1963; Bagnara, 1982; Elias, 1941; Ellinger, 1979; Frost and Robinson, 1984; Gosner and Black, 1957; Smith-Gill, 1974), reviewed in Frost-Mason *et al.* (1995)], but mutations have been identified in some species that specifically affect chromatophores or pigment pattern formation (see below).

Salamander embryos were used by DuShane (1934, 1935, 1938) in his pioneering studies that first revealed a neural crest origin for chromatophores. These findings were later extended to anurans (Stevens, 1954) and inspired numerous analyses of pigment pattern development in amphibians. Several reviews provide an introduction to these classical studies (Bagnara, 1983; DuShane, 1943; Epperlein and Löfberg, 1993; Erickson, 1993; Frost and Malacinski, 1980; Hall and Hörstadius, 1988; Lehman and Youngs, 1959; Parichy, 1996b; Rawles, 1948; Twitty, 1949, 1953, 1966). Amphibian chromatophores in the integument can localize in both the dermis and the epidermis, though dermal chromatophores are primarily responsible for distinctive pigment patterns in most taxa (e.g., Bagnara *et al.*, 1968; DuShane, 1943; Stearner, 1946).

Morphogenesis of Amphibian Chromatophores

In salamanders, rounded, presumptive neural crest cells are first detectable within the neural folds (Hirano and Shirai, 1984; Raven, 1931; Spieth and Keller, 1984). As the neural folds fuse at the midline, prospective neural crest cells form a wedge within the neuroepithelium and finally emerge as a distinctive but transient cord or "crest" running anteroposteriorly along the dorsal aspect of the completed neural tube (Fig. 5.3). Presumptive neural crest cells also have been identified in the neural folds of the anuran *Xenopus laevis* (Mayor *et al.*, 1995; Sadaghiani and Thiébaud, 1987; Schroeder, 1970).

Many techniques have been used to identify neural crest cells in amphibian embryos, including light and electron microscopy (Hirano and Shirai, 1984; Löfberg and Ahlfors, 1978; Löfberg *et al.*, 1980, 1985; Spieth and Keller, 1984), vital labeling (Collazo *et al.*, 1993; Detwiler, 1937), and intrinsic characteristics or labeling of transplanted cells in chimeric embryos (Chibon, 1967; Krotoski *et al.*, 1988; MacMillan, 1976; Moury and Jacobson, 1989, 1990; Sadaghiani and Thiébaud, 1987; Raven, 1931, 1936). Finally, amphibian—like teleost—chromatophores often produce pigment prior to reaching their final destination and can be observed directly (Epperlein and Claviez, 1982; Epperlein and Löfberg, 1990; Keller and Spieth, 1984; Tucker and Erickson, 1986a; Twitty, 1945).

Amphibian neural crest cells disperse along dorsolateral and ventromedial pathways and also are found within the expanding fin. In salamanders, most or all neural crest cells that contribute to pigment patterns on the dorsal flank of young larvae migrate dorsolaterally between the somites and epidermis (Epperlein and Löfberg, 1990; Olsson and Löfberg, 1992). Neural crest cells traveling dorsolaterally are probably the major contributors to externally visible pigment patterns in most frog tadpoles as well (Andres, 1963; Tucker, 1986). In contrast, most *X. laevis* neural crest cells and melanophores fail to migrate subepidermally in the anterior trunk and instead remain near the dorsal apex of the myotomes or first travel medially and only secondarily migrate to the epidermis (Andres, 1963; Collazo *et al.*, 1993; Krotoski *et al.*, 1988; MacMillan, 1976; Tucker, 1986). Medial migration of melanophores or their precursors in other amphibians is suggested by the observation that many amphibians exhibit chromatophores lining internal structures (DuShane, 1943; Tucker and Erickson, 1986a).

The factors controlling amphibian neural crest and chromatophore morphogenesis are not well understood, but results from many studies suggest roles for the extracellular matrix (ECM). Work in *Ambystoma mexicanum* suggests that changes in the composition of the ECM may influence the

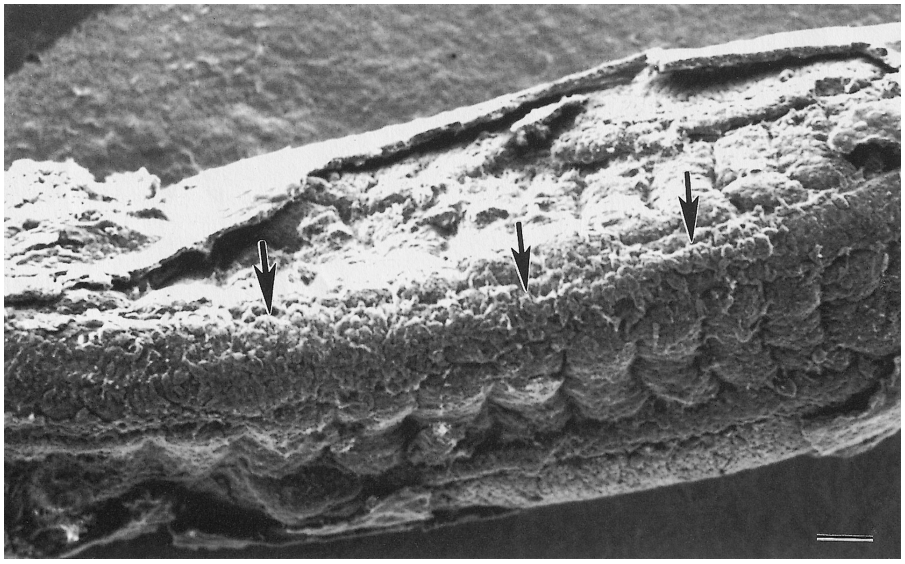

Fig. 5.3. Neural crest cells (arrows) emerging from the neural tube of a salamander embryo. Bar = 200μm.

initial dispersal and migration of neural crest cells (Löfberg and Ahlfors, 1978; Löfberg *et al.*, 1980, 1985). A variety of ECM components have been identified in the vicinity of early or later migrating amphibian neural crest cells and chromatophores, including fibronectin, laminin, collagens type I and III, chondroitin and keratan sulfate proteoglycans, tenascin, hyaluronan, and molecules that bind the lectin peanut agglutinin (PNA) (Epperlein and Löfberg, 1990; Epperlein *et al.*, 1988; Olsson *et al.*, 1996; Parichy, 1996a, 2001a; Perris *et al.*, 1990; Tucker, 1986; Tucker and Erickson, 1986a, b). Several studies have examined the behavior of salamander neural crest cells on ECM substrata *in vitro* (Epperlein *et al.*, 1988; Perris and Johansson, 1987, 1990; Tucker and Erickson, 1986a, b). These investigations have shown that salamander neural crest cells can disperse and migrate on a variety of ECM components, including fibronectin, vitronectin, laminin, and collagens type I, IV, and VI. In contrast, purified tenascin inhibits the adhesion of neural crest cells, and motility of these cells can be modulated in a concentration-dependent manner by addition of chondroitin sulfate proteoglycans and hyaluronan.

In the early larvae of several salamander taxa, melanophores first are uniformly distributed over the flank but subsequently concentrate near the dorsal apex of the myotomes and along the dorsal margin of the yolk mass. Because xanthophores typically are present over the lateral face of the myotomes, the resulting pattern consists of dark, horizontal dorsal and lateral stripes bordering a pale "melanophore-free region" along the middle of the flank (Parichy, 1996b, 2001a, b). In one of these taxa, *Taricha torosa*, fibronectin is abundant at sites of melanophore localization, whereas hyaluronan is abundant within the dorsal fin matrix where melanophores are not found (Tucker and Erickson, 1986a, b). In *Triturus alpestris*, the local appearance of tenascin and chondroitin sulfate proteoglycans over the lateral face of the myotomes correlates with the evacuation of melanophores from this region (Epperlein and Löfberg, 1990). Moreover, interspecific differences in tenascin and laminin correlate with species-specific melanophore distributions (Parichy, 2001a). In several other taxa within the families Ambystomatidae and Salamandridae, the lateral line sensory system (Northcutt, 1992; Northcutt *et al.*, 1994) is directly responsible for excluding melanophores from the middle of the flank (Parichy, 1996a, c). Lateral line-dependent perturbations of the subepidermal basement membrane and increased deposition of tenascin correlate with the evacuation of melanophores from this region (Parichy 1996a, 2001a).

Changes in the ECM also may influence chromatophores and chromatoblasts during later development. In *A. t. tigrinum* for example, staining for laminin, tenascin, and PNA-binding activity increase dramatically during terminal formation of the early larval pigment pattern, probably reflecting the elaboration of a largely acellular dermis that is hypothesized to stabilize melanophore positions (Parichy, 1996c; Stearner, 1946). Likewise, breaks in the collagenous basement membrane and the appearance of fibronectin "tracts" correlate with changes in melanophore positions at metamorphosis in the frog *Rana esculenta* (Denèfle and Lechaire, 1990, 1991, 1992; Denèfle *et al.*, 1987).

Another class of factors that probably influences the morphogenetic behavior of chromatophores is interactions among the cells themselves, as in teleosts. Based on a series of studies *in vitro*, Twitty (1944) and Twitty and Niu (1948) suggested that the directed dispersal of neural crest cells and melanophores results from the production of diffusible substances that repel neighboring cells. Although such negative chemotaxis has not been ruled out in urodeles, attempts to verify these conclusions using quail neural crest cells have not been successful (Erickson and Olivier, 1983). More recently, melanophore and neural crest cell dispersal has been suggested to result from contact inhibition of movement (Abercrombie, 1970) coupled with high cell densities in dorsal regions of the

flank [Tucker and Erickson (1986a); but see Keller and Spieth (1984)], concordant with similar proposals in avian embryos (Rovasio *et al.*, 1983; Erickson, 1985). In contrast to repellent interactions that appear to mediate pigment cell dispersion, adhesive interactions among melanophores have been suggested to contribute to the condensation of these cells during stripe formation in *T. torosa* (Parichy, 1996a; Twitty, 1945).

Cell–cell interactions also may be involved in the development of vertical barring patterns in several salamanders within the family Ambystomatidae (Epperlein and Löfberg, 1990; Lehman, 1954, 1957; Olsson, 1994; Olsson and Löfberg, 1992; Parichy, 1996a, b, 2001b); similar patterns also are present in some frog tadpoles (Altig and Johnston, 1989; Gosner and Black, 1957). In these taxa, melanophores scatter widely over the somites during early stages while xanthophores are retained in aggregates at variable intervals dorsal to the neural tube. Xanthophores subsequently disperse from these aggregates, and melanophores on the flank recede short distances from the advancing xanthophores. This results in alternating vertical bars of melanophores and xanthophores in young larvae. Epperlein and Löfberg (1990) hypothesize that aggregates arise at the level of the premigratory neural crest by xanthophores "sorting out" from surrounding melanophores via differential adhesion (Steinberg, 1970). Observations from experimentally manipulated and mutant embryos are consistent with this idea. Nevertheless, the events of aggregate formation have yet to be described *in vivo*. During later stages, repulsive interactions between melanophores and xanthophores appear to drive morphogenesis of the definitive vertical barring pattern (Epperlein and Löfberg, 1990) though the underlying mechanisms have yet to be established.

In addition to interactions among chromatophores and between chromatophores and the ECM, gross physical features of the extracellular environment probably influence the migration and localization of amphibian chromatophores. For example, a physical barrier near the dorsal margin of the yolk mass may contribute to the failure of *T. torosa* melanophores to migrate ventrally over the lateral plate mesoderm (Twitty, 1936). Melanophores of this species also localize along the lateral line nerve perhaps due to contact guidance (Dunn, 1982; Tucker and Erickson, 1986a). Finally, the lateral line nerve in *A. tigrinum* is hypothesized to act as a physical barrier that prevents dorsal melanophores from migrating further ventrally (Parichy, 1996c).

A variety of hormones and growth factors also could influence the behavior of embryonic amphibian neural crest and pigment cells. Candidate molecules have been identified in teleost and amniote embryos, but the distributions and functions of these molecules and their receptors are only beginning to be investigated in amphibians at stages relevant to early pigment pattern formation (e.g., Baker *et al.*, 1995).

Finally, externally visible pigment patterns of most amphibians change markedly during later larval stages and especially during and after metamorphosis (Bagnara, 1982; DuShane, 1943; Fernandez and Collins, 1988; Lehman, 1953; Lehman and Youngs, 1959; Niu and Twitty, 1950; Parichy 1998, 2001b; Smith-Gill, 1974; Stearner, 1946; Twitty, 1936). These changes presumably reflect persistent or renewed migration of existing pigment cells as well as neural crest cells or chromatoblasts that have not yet overtly differentiated. Nevertheless, it seems likely that these chromatophore movements are influenced by endocrine-derived hormones (reviewed in Frost-Mason *et al.*, 1995). Hormones and localized growth factors probably also influence the massive restructuring of skin that occurs during metamorphosis (Heatwole and Barthalmus, 1995), which may indirectly impact the establishment of a new pigment pattern. For example, thyroid hormones trigger a cascade of events during metamorphosis, including extensive remodeling of the ECM, cell death, proliferation, and differentiation (e.g., Patterton *et al.*, 1995). Intriguingly, thyroxine stimulates the migration of melanophores from the dermis to the epidermis in cultured frog integument, possibly reflecting a direct effect of the hormone on melanophores (Yasutomi, 1987).

Differentiation of Amphibian Chromatophores

At least some amphibian neural crest cells begin to differentiate into melanophores and xanthophores shortly after dispersing, or even while in the premigratory position dorsal to the neural tube (Epperlein and Löfberg, 1984, 1990; Olsson and Löfberg, 1992; Tucker and Erickson, 1986a). In contrast, iridophores typically are detectable only after the appearance of melanophores and xanthophores. It seems likely that additional populations of chromatoblasts remain covert within the integument and differentiate at later larval stages, during metamorphosis, or during adult life (Bagnara *et al.*, 1979; Brown, 1997; DuShane, 1943; Fernandez and Collins, 1988; Frost *et al.*, 1984a; Ohsugi and Ide, 1983; Parichy, 1998, 2001b; Stearner, 1946).

In *A. mexicanum*, differentiation into melanophores and xanthophores is stimulated by serum (Dean and Frost-Mason, 1991), or alternatively if the cells are co-cultured with neural tube, epidermis, or ECM isolated from the dorsolateral (but not medial) pathway (Perris and Löfberg, 1986; Perris *et al.*, 1988). Similar requirements for serum are observed in cultures of neural crest cells isolated from *T. torosa* (Tucker and Erickson, 1986b; Twitty, 1944, 1945) and *X. laevis* (Fukuzawa and Ide, 1988).

One molecule known to stimulate melanogenesis specifically is α-melanocyte stimulating hormone (α-MSH), which is produced in the pituitary gland of adult animals. α-MSH originally was identified as a circulating hormone that causes the dispersion of melanin-containing granules (melanosomes) within melanocytes (e.g., Bagnara and Hadley, 1969; Fernandez and Bagnara, 1991; Sawyer *et al.*, 1983). In addition to these short-term physiological responses, α-MSH probably also mediates long-term morphologic changes in pigmentation (reviewed in Bagnara and Hadley, 1969). *In vitro*, α-MSH stimulates both proliferation and melanization of anuran neural crest cells (Fukuzawa and Bagnara, 1989;

Wilson and Milos, 1987) and melanophores (Fukuzawa and Bagnara, 1989). A possible role for α-MSH or related peptides during early development also has been suggested in a preliminary report demonstrating α-MSH immunoreactivity in the epidermis of *A. mexicanum* embryos and larvae (Frost-Mason *et al.*, 1992). It will be interesting to see if α-MSH activity contributes to the early differentiation of neural crest cells into chromatophores, particularly in light of taxon- and stage-specific differences in the ability of epidermis to promote melanophore differentiation (DeLanney, 1941; Thibaudeau and Frost-Mason, 1992; Twitty, 1936).

In addition to α-MSH, presently unidentified factors in the dorsal integument and serum of *Rana pipiens* and *X. laevis* stimulate both melanization and outgrowth in cultured *X. laevis* neural crest cells (Fukuzawa and Bagnara, 1989; Mangano *et al.*, 1992). Conversely, a partially purified factor from ventral integument of *Rana* and *X. laevis* inhibits outgrowth and melanization of these cells (Fukuzawa and Bagnara, 1989; Fukuzawa and Ide, 1988), whereas medium containing factors derived from ventral integument stimulates adhesion and proliferation of iridophores *in vitro* (Bagnara and Fukuzawa, 1990). An antibody raised against a putative "melanization-inhibiting factor" has been shown to block inhibitory effects *in vitro*, and this antibody stains the ventral but not dorsal integument of *R. pipiens* (Fukuzawa *et al.*, 1995). Further characterization of this factor could provide important insights into the control of regional differences in pigmentation (e.g., the dark dorsal and white bellies seen in many amphibians and other vertebrates).

Mutants

Numerous pigment mutants have been found across several amphibian taxa, including *R. pipiens* (Davison, 1964; Smith-Gill, 1973, 1974, 1975; Volpe and Dasgupta, 1962), *Bombina orientalis* (Ellinger, 1980; Frost *et al.*, 1982), *X. laevis* (Droin, 1992), and *P. waltl* (Collenot *et al.*, 1989). Perhaps the most studied are those of *A. mexicanum*: melanoid, albino, axanthic, and white (reviewed in Frost and Malacinski, 1980; Frost *et al.*, 1984b). Early larval "wild-type" *A. mexicanum* display abundant melanophores and xanthophores, whereas older larvae and paedomorphic adults exhibit melanophores, xanthophores, and iridophores in an irregular mottled green and black pattern that covers the integument.

melanoid (m)

In contrast to wild-type *A. mexicanum*, *m/m* animals exhibit few xanthophores as larvae or adults and completely lack iridophores at all stages of development (Frost *et al.*, 1984c). Thus, melanoid adults are a velvety black. Genetic mapping places the melanoid locus on linkage group 14 (Smith *et al.*, 2005). The melanoid defect is believed to act autonomously within the neural crest lineage (Sawada and Dalton, 1979). Total pigment cell numbers do not differ between melanoid and wild-type animals (Hoerter, 1977).

Biochemically, the deficit of xanthophores and iridophores correlates with a decrease in overall pteridine levels during development (Frost *et al.*, 1984c) as well as lower levels of pteridines compared to wild-type animals (Benjamin, 1970; Frost *et al.*, 1984b; Thorteinsdottir and Frost, 1986). Treatment of wild-type *A. mexicanum* with an inhibitor of the pteridine synthesis enzyme xanthine dehydrogenase results in a phenocopy of the melanoid defect (Frost *et al.*, 1989; Thorteinsdottir and Frost, 1986).

albino (a)

The *a* gene was originally introduced into *A. mexicanum* from the closely related *A. tigrinum* [an undertaking that required truly heroic efforts; see Humphrey (1967)]. Homozygotes for the albino gene completely lack melanin but do have unmelanized "melanophores" containing organelles typical of early stages in melanogenesis (Frost *et al.*, 1986a). Because *a/a* individuals possess both xanthophores and iridophores, their overall coloration is a golden yellow. Albino *A. mexicanum* also exhibit differences in pteridine profiles within the integument compared to wild-type *A. mexicanum* (Frost *et al.*, 1986a). The biochemical defect underlying the albino phenotype remains unknown.

axanthic (ax)

Little is known about the axanthic defect. The *ax/ax A. mexicanum* completely lacks iridophores whereas "xanthophores" are identifiable but remain unpigmented; melanophores are apparently unaffected (Frost *et al.*, 1984a, 1986b).

white (d)

Homozygous *d/d* individuals lack all three types of chromatophores in the integument during most or all of their lives (Frost *et al.*, 1984a), resulting in a white phenotype. Notably, eye pigmentation is unaffected, suggesting that the defect is specific for neural crest-derived pigment cells. Deficient cutaneous pigmentation is thought to result from a failure of neural crest cells and chromatophores to disperse into the dorsolateral migratory pathway (Dalton, 1950; Keller *et al.*, 1982), though lineage tracers have yet to be employed to test explicitly the relative roles of inhibited dispersal versus inhibited differentiation or survival [see also DuShane (1939)]. Indeed, some chromatophore precursors must disperse, because some *d/d* animals repigment spontaneously in haphazard patches after reaching sexual maturity (Frost and Malacinski, 1980; Frost *et al.*, 1984b).

The defect associated with the *d* gene traditionally has been attributed to a change within the epidermis, since wild-type epidermis grafted to white embryos permits melanophore dispersal whereas the converse does not (Keller *et al.*, 1982). Löfberg *et al.* (1989) obtained analogous results when microcarriers coated with only white or wild-type subepidermal ECM were grafted between embryos. In contrast, microcarriers coated with subepidermal ECM from *older* white embryos permitted neural crest dispersal in both white and wild-type hosts. This led to the hypothesis that changes in the subepidermal ECM normally required for neural crest cell migration are transiently retarded in white embryos. Unfortunately, com-

parisons of white and wild-type ECM have yielded conflicting results (Epperlein and Löfberg, 1993; Olsson *et al.*, 1996; Perris *et al.*, 1990) and do not adequately explain what the white defect might be. Genetic mapping places *d* on linkage group 1, allowing the exclusion of several candidate genes, including *steel*, encoding the kit ligand, SLF (Parichy *et al.*, 1999; Smith *et al.* 2005; Voss *et al.*, 2001).

Reptiles

Little is known concerning neural crest and pigment cell development in reptilian embryos. Hou and Takeuchi (1994) used the HNK-1 antibody to show that neural crest cells of the softshell turtle *Trionyx sinensis* migrate along both ventromedial and dorsolateral pathways, as in other vertebrates. Melanophores in this species are found cutaneously and internally (Hou and Takeuchi, 1991), suggesting that both ventromedially and dorsolaterally migrating neural crest cells can differentiate as melanophores. Intriguingly, early migrating neural crest cells also enter the myotome (Hou and Takeuchi, 1994). This could reflect enhanced invasiveness of a melanogenic subpopulation of neural crest cells traveling along the medial pathway, as has been suggested in the Silkie chicken (Faraco *et al.*, 2001; Reedy *et al.*, 1998b).

Birds

Avian embryos are amenable to experimental manipulation at virtually all stages of embryogenesis and thus have been widely exploited as a model system for studying neural crest and pigment cell development. By far the two most commonly used avian species for these studies are the domestic chicken, *Gallus gallus*, and the Japanese quail, *Coturnix japonica*. Many studies have taken advantage of the fact that quail neural crest cells, when grafted into a chick embryo host, can be distinguished by their characteristic nuclear staining pattern with Feulgen dye (Le Douarin, 1982) or the quail-specific antibody QCPN. In addition, avian neural crest cells can be identified on the basis of their immunoreactivity with the HNK-1 antibody (Tucker *et al.*, 1988) and by their expression of specific cadherins (Nakagawa and Takeichi, 1995) or transcription factors, such as Sox 10 (Cheng *et al.*, 2000).

The neural crest origin of avian melanocytes, the sole skin pigment cell type found in birds, was demonstrated over 50 years ago using experimental embryological techniques (Dorris, 1936, 1938, 1939; Ris, 1941; Watterson, 1942; Willier and Rawles, 1940). In adult birds, melanocytes are distributed in both the dermis and the epidermis (Hulley *et al.*, 1991). In addition, melanocytes are found internally in some birds, for example the Silkie chicken (e.g., Faraco *et al.*, 2001; Makita and Mochizuki, 1984; Makita and Tsuzuki, 1986).

Morphogenesis of Avian Melanocytes

The dynamics of neural crest migration in the avian trunk have been well documented. In chickens, neural crest cells first detach from the neuroepithelium in the trunk at stage 11 (Tosney, 1978) and invade the ventromedial pathway shortly thereafter (Bronner-Fraser, 1986; Loring and Erickson, 1987). These neural crest cells differentiate into the neurons and glial cells of the peripheral nervous system. The entry of avian neural crest cells into the dorsolateral path is delayed by about 24 hours with respect to ventromedial migration and begins at stage 20 at the level of the forelimb (Erickson *et al.*, 1992; Serbedzija *et al.*, 1989). Neural crest cells destined for the dorsolateral path initially pause between the lateral neural tube and the tip of the dermamyotome (Fig. 5.4). This region has been termed the migration "staging area" (Weston, 1991). Cells that enter this pathway will differentiate into pigment

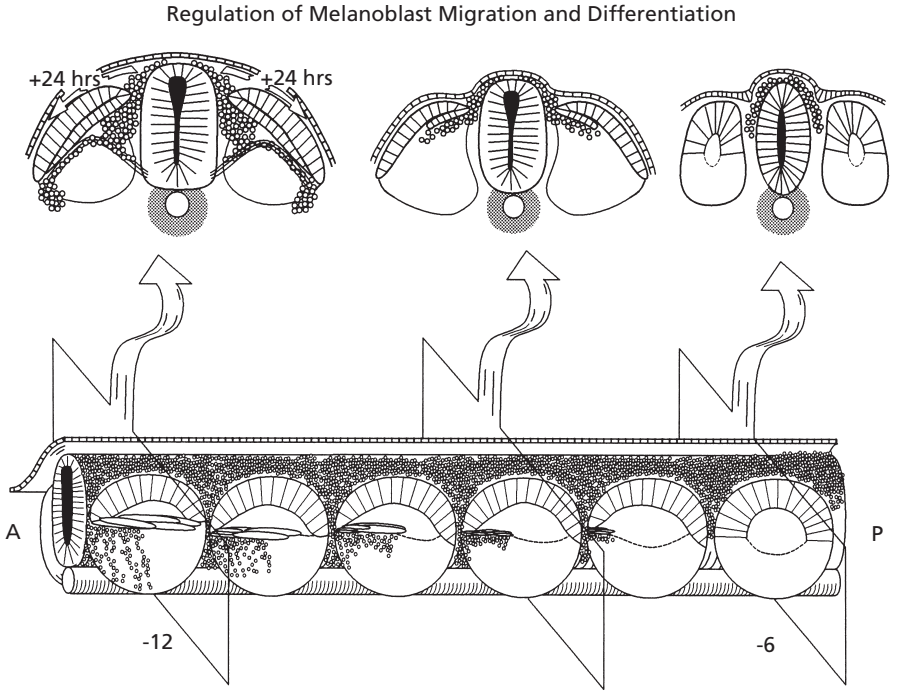

Fig. 5.4. Schematic diagram of the major migratory pathways followed by neural crest cells in the trunk of amniote embryos. Anterior is to the left. (Bottom) Sagittal section through somites -6 to -12 indicating the simultaneous development of the myotome and neural crest invasion into the somite from the ventromedial path. (Top) Cross-sections at different axial levels show the ventromedial and dorsolateral pathways taken by neural crest cells. Diagram by Martha Spence and used with permission from Academic Press.

cells of the skin. Melanoblasts begin entering the ectoderm approximately two stages after entering the dorsolateral path (Erickson *et al.*, 1992). Eventually, they invade the feather follicle and complete their differentiation into melanocytes (Hulley *et al.*, 1991).

A major question about neural crest morphogenesis is what determines the positioning of neural and glial cells in the ventral pathway and pigment cells in the dorsolateral path. The generally accepted model for patterning neural crest derivatives proposes that neural crest cells are a pluripotent population of cells that migrate haphazardly into the ventral or dorsolateral paths, and differentiate according to the cues encountered in these paths (Le Douarin, 1982, 1986; Le Douarin *et al.*, 1993; Selleck *et al.*, 1993; Stemple and Anderson, 1993). This model is supported by experimental evidence from heterotopic grafting experiments (Le Douarin and Teillet, 1974; Le Douarin *et al.*, 1975), as well as back-transplantation of crest-derived structures into the early migratory crest pathways (Ayer-Le Lievre and Le Douarin, 1982; Dupin, 1984; Le Douarin *et al.*, 1978; Le Lievre *et al.*, 1980), which show that, *as a population*, neural crest cells migrate and differentiate according to their new environment and not their origin.

However, an alternative mechanism directs the migration of chicken melanoblasts into the dorsolateral path at the trunk level. Specifically, melanoblasts are developmentally biased at the time that they depart from the neural tube: only neural crest cells that are specified as melanocytes enter the dorsolateral path (Reedy *et al.*, 1998a), and melanoblasts are the only neural crest cells that can exploit the dorsolateral path under experimental conditions (Erickson and Goins, 1995). Thus, the final distribution of pigment cells is directed by cell-autonomous migratory properties unique to that subpopulation. The evidence that melanoblasts are specified prior to entering the dorsolateral pathway will be discussed in the following section. Here we will first consider what controls the choice of pathway.

Why are neural and glial precursors unable to invade the dorsolateral path, whereas the later-migrating melanoblasts can exploit this pathway? The dorsolateral path at the trunk level is initially refractory to neural crest cell migration (Erickson and Goins, 1995; Erickson *et al.* 1992; Serbedzija *et al.*, 1989). By stages 19–20, beginning at the wing-bud level, dorsolateral migration is initiated as ventral migration gradually ceases (Kitamura *et al.*, 1992; Oakley *et al.*, 1994; Serbedzija *et al.*, 1989). The molecular basis for this switch in pathways, initially suggested by Oakley *et al.* (1994), was that inhibitory molecules are gradually lost, so that the environment in the dorsolateral path is now permissive for migration. However, experimental studies do not support this proposal. They instead suggest that there are changes in those neural crest cells specified as pigment cells that allow them to overcome the inhibitory cues. When nonmelanogenic neural crest cells are grafted into stage-19 embryos at the wing-bud level, which is the time when the host neural crest cells would be embarking on the dorsolateral path, the grafted cells only migrate ventrally (Erickson and Goins, 1995). However, when melanoblasts are grafted into the early neural crest migratory pathway (stage-15 host), the grafted cells invade the dorsolateral space immediately, well in advance of the time when dorsolateral migration usually takes place (Erickson *et al.*, 1992). These experimental studies indicate that molecular changes occurring during melanoblast specification, rather than changes in the environment, permit dorsolateral migration.

One possible molecular change in melanoblasts is that they develop unique sensitivity to positive guidance cues from the dermamyotome, allowing them to migrate dorsolaterally. In the mouse, melanoblasts depend upon SLF produced by the dermamyotome for their initial dispersal onto the dorsolateral path (Wehrle-Haller and Weston, 1995, 1997; Wehrle-Haller *et al.*, 1996; and see Mammals below), and they express the receptor for SLF, kit, prior to embarking on the dorsolateral path. However in the avian embryo, kit is first expressed by melanoblasts long after they have migrated dorsolaterally (stage 25), and SLF is not produced by the dermamyotome, but rather by the ectoderm and only after stage 25 (Lecoin *et al.*, 1995; Reedy *et al.*, 2003). Furthermore, morpholino knockdown of kit does not affect either melanoblast migration or specification (Harris and Erickson, unpublished data). Thus, acquiring responsiveness to SLF owing to kit upregulation is unlikely to control the timing of dorsolateral migration in the chicken.

In the past few years a number of guidance molecules have been identified that cause the collapse or repulsion of growth cones, filopodia or lamellipodia (Tessier-Lavigne and Goodman, 1996). These include the netrins, the collapsin/semaphorins, Slits and ephrins (Gale and Yancopoulos, 1997). Three laboratories have shown that ventrally migrating neural crest cells are constrained to migrate in the anterior half of somites because inhibitory ephrins are expressed in the posterior somite (Flenniken *et al.*, 1996; Krull *et al.*, 1997; Wang and Anderson, 1997). Ephrins are also distributed in the dorsolateral space at the time of early crest migration and they have also been shown to be responsible for preventing neural and glial precursors from entering this space (Santiago and Erickson, 2002). In contrast, ephrins stimulate melanoblast migration, and this positive guidance cue permits melanoblasts to invade the dorsolateral space. This is not unlike the netrins and semaphorins, which can be inhibitory under some circumstances and stimulatory under others. A major task will be to understand how Eph receptors, the receptors for ephrins, can differentially signal in melanoblasts and nonmelanoblasts. Further, we do not yet understand how ephrin signaling results in enhanced melanoblast migration, although integrin activation is likely to be a factor (Beauvais *et al.*, 1995; Santiago and Erickson, 2002).

Ephrins are likely not the only signaling molecules that control the directional migration of melanoblasts into the dorsolateral space. Recently, Jia *et al.* (2005) have demonstrated that Slits, which are secreted chemorepellants, expressed in the dermamyotome also prevent the migration of early migratory neural crest cells into the dorsolateral space. When the Slit

receptor, Robo 1, is inactivated by expressing a dominant negative form in the premigratory neural crest, ventrally migrating crest cells now precociously invade the dorsolateral pathway. Still unanswered is how melanoblasts can overcome the chemorepellant action of Slit. Potentially the Robo receptors could be downregulated on neural crest cells during melanoblast specification, or melanoblasts may be attracted to Slits rather than being repelled.

Yet another signaling molecule/receptor pair that potentially controls directed migration into the dorsolateral space is endothelins/endothelin receptors (reviewed by Pla and Larue, 2003). Endothelins are 21-amino acid peptides originally identified for roles in vasoconstriction (Yanigasawa *et al.*, 1988) and their receptors are G-protein-coupled, heptahelical, cell-surface receptors. In the chick, ventrally migrating neural crest cells express Ednrb, whereas Ednrb2 is upregulated just prior to neural crest cells migrating in the dorsolateral pathway. Endothelin-3, the presumptive ligand for avian Ednrb and Ednrb2, is expressed by the ectoderm (Nataf *et al.*, 1998). When mouse embryonic stem (ES) cells are transfected with Ednrb2 and grafted into an early chick embryo, they take the dorsolateral pathway precociously, unlike the wild-type ES cells or ES cells expressing Ednrb, which migrate ventrally (Pla *et al.*, 2005). This study suggests that endothelin signaling may positively attract melanoblasts into the dorsolateral space. It would be interesting to manipulate the composition of endothelin receptors on neural crest cells *in situ* to test this possibility directly. Beyond effects on migration, several studies of cultured quail neural crest cells show that endothelin-3 clearly affect proliferation of melanoblasts (Lahav *et al.*, 1996). Additionally clonal analysis reveals that endothelin signaling enhances the survival of clonogenic cells that give rise to melanocytes only, glial cells only, and the melanocyte/glial bipotent precursors (Lahav *et al.*, 1998).

During later morphogenesis, melanoblasts enter the ectoderm, which is separated from the dermis by a basal lamina. Although previous studies have shown that basal laminae are impenetrable by early-migrating neural crest cells, melanoblasts clearly have enhanced invasive properties (Cuddenec, 1977; Erickson, 1987; Erickson and Goins, 1995; Sears and Ciment, 1988; Teillet, 1971). Localized discontinuities in the basal lamina adjacent to neural crest cells in the dorsolateral pathway suggest that melanoblasts selectively degrade the basal lamina prior to invading the ectoderm (Erickson *et al.*, 1992). The mechanisms of melanoblast invasiveness are not clear, but one possibility is that they produce matrix-degrading proteases that allow them access to sites where other crest cells normally cannot enter (Duong and Erickson, 2004; Erickson and Isseroff, 1986; Valinsky and Le Douarin, 1985).

Differentiation of Avian Melanocytes

The melanoblast lineage was believed to be specified only after neural crest cells had migrated from the neural tube, because a number of cloning or culture studies using avian neural crest showed that pigment cells could arise from a pluripotent precursor in 24-hour outgrowths (Baroffio *et al.*, 1991; Dupin and Le Douarin, 2003; Le Douarin *et al.*, 1993; Sieber-Blum *et al.*, 1993; Stocker *et al.*, 1991; Weston, 1991). However, when quail trunk-level neural tubes are cultured and the fate of those neural crest cells that emigrated during the first six hours is assessed, no melanocytes differentiate, even after six days in culture under conditions that are permissive for melanogenesis (Reedy *et al.*, 1998a). Thus there is heterogeneity in the melanogenic potential of different temporal subpopulations of neural crest cells from the same axial level. Corroborating studies by Henion and Weston (1997) showed that many of the neural crest cells are fate-restricted (that is, the progeny from a single neural crest cell all have the same phenotype) at the time that they detach from the neural tube, and that neurogenic and some gliogenic precursors migrate from the neural tube before any melanogenic precursors. They did note that there are some clones that are bipotent and could give rise to glial cells and pigment cells, which are likely to be the intermediate precursor population identified in previous cultures studies (Sherman *et al.*, 1993; Sieber-Blum and Cohen, 1980). Together these two studies show that many neural crest cells are developmentally restricted when they leave the neural tube, and that neurogenic, gliogenic, and melanogenic neural crest cells are specified at different times prior to their migration. Similar observations have been made in the zebrafish (Raible and Eisen, 1994; Dorsky *et al.*, 1998, 2000a) and in axolotls (Epperlein and Löfberg, 1984). See also reviews by Anderson (2000), Dorsky *et al.* (2000b), and Erickson (2003).

We know a great deal about melanogenesis, including the biochemical pathways that generate pigment, the regulation of expression of the enzymes in that pathway, and the role of numerous growth factors that play a role in melanoblast maintenance, proliferation, and terminal differentiation (discussed in detail in many chapters in this textbook). Nevertheless, very little is known about the molecular events that specify the lineage as distinct from the other neural crest derivatives. The studies cited above suggest that there is a lineage switch from the nonmelanogenic lineages to the melanogenic lineage; that is, the premigratory neural crest cells may be specified first as neuro/gliogenic cells [or bipotent neural/glial and glial/melanogenic precursors (Le Douarin *et al.*, 2004)] and subsequently some switch to the melanogenic fate. Further, this lineage switch occurs while the neural crest precursors are still resident in the neural tube or shortly after they detach from it.

Studies in the chick (Jin *et al.*, 2001) and in zebrafish (Dorsky *et al.*, 1998, and see Fishes, above) show that this lineage switch is controlled, at a minimum, by two competing classes of signaling molecules: the Wnts and the BMPs. Specifically, Wnt 1/3a signaling specifies melanocytes whereas BMP 2/4 signaling specifies neural and glial cells. Despite the evidence that Wnt and BMP signaling is involved in fate specification, we do not know the cellular mechanisms by which this specification is achieved, or how Wnt- and BMP-dependent signals compete with each other in this context.

There are undoubtedly many genes involved in the specification of the melanogenic lineage downstream of Wnt

signaling, including transcription factor cascades (Anderson, 1994; Groves and Anderson, 1996; Groves *et al.*, 1995). Only a few transcription factors have been identified to date that regulate the melanogenic fate. Microphthalmia (Mitf), a basic-helix-loop-helix-zipper transcription factor, has been shown in human, mouse, zebrafish (*nacre*, *mitfa*), and chickens to bind to the so-called M-box, an 11-bp sequence containing the core element CATGTG (Hodgkinson *et al.*, 1993), in the upstream regulatory region of the tyrosinase and tyrosine-related protein 1 (TRP-1) genes and activate their transcription (review by Goding, 2000; see Mammals, below, for more detail). Downregulation of *Mitf* in chick neural crest cells using morpholinos eliminates melanoblasts and misexpression of Mitf by electroporation gives rise to excess pigments cells (Thomas and Erickson, unpublished). Mitf appears to be the primary transcription factor that drives melanoblast specification (Goding, 2000).

Specification of different neural crest subpopulations at the trunk level occurs in sequential temporal waves, and yet many transcription factors that control neural, glial, and melanogenic lineages are expressed at the same time in the dorsal neural tube. For example, Sox10 and Pax3 are both required for the development of all three lineages in mouse, chick, and probably zebrafish (Britsch *et al.*, 1998; Cheung *et al.*, 2005; Dutton *et al.*, 2001; McKeown *et al.*, 2005; Lacosta *et al.*, 2005; Potterf *et al.*, 2001), so what prevents neuroblasts, glioblasts, and melanoblasts from developing simultaneously? One possibility is that a transcriptional repressor for melanogenesis is expressed early, which would prevent the early migratory crest from having melanogenic potential, whereas Wnt signaling may cause the downregulation of this putative repressor, which would then permit melanogenesis. We predict that the lineage switch from neural/glial to melanoblast occurs as a result of the downregulation of this repressor.

A good candidate for such a transcriptional repressor is FoxD3. FoxD3 is a member of the winged-helix family of transcription factors (Kaestner *et al.*, 2000). FoxD3 represses melanogenesis but is required for gliogenesis in the chick (Kos *et al.*, 2001). The zebrafish homolog (formerly, *fkh-6*) similarly stimulates glial cell differentiation (Kelsh *et al.*, 2000) and *foxd3* mutants exhibit defects in neurons, glia, and ectomesenchymal lineages, though not chromatophore lineages (Stewart *et al.*, 2005). In the neural epithelium, FoxD3 controls another lineage switch from neural crest to interneuron (Dottori *et al.*, 2001), and in the early mouse embryo it is critical for maintaining embryonic stem cells (Hanna *et al.*, 2002). Since FoxD3 in most experimental contexts acts as a transcriptional repressor (Freyaldenhoven *et al.*, 1997b; Hromas *et al.*, 1999), the most parsimonious hypothesis that accounts for the current data is that FoxD3 represses *Mitf* expression directly by binding to the *Mitf* promoter. Using a luciferase assay, chick FoxD3 expressed in B16 melanoma cells will reduce luciferase activity that is being driven by a chicken *Mitf* enhancer (Thomas and Erickson, unpublished results). This model further predicts that when FoxD3 is downregulated under the influence of Wnt signaling, *Mitf* is no longer repressed by FoxD3, and is therefore transcribed by the transcriptional activators Tcf/Lef-1, Sox10, and Pax3 (Goding, 2000).

Mutants

There is considerable variation in plumage and skin pigmentation among domestic fowl (reviewed in Smyth, 1990; Staples, 2001) and a variety of mutants with melanocyte defects have been isolated (e.g., Niwa *et al.*, 2002) including a mutation in Mitf (Mochii *et al.*, 1998). Congenital hypopigmentation resulting from inefficient melanoblast migration, differentiation, or both also has been shown in several instances (Jimbow *et al.*, 1974). Some chicken strains have been used as model systems for studying pigment defects such as vitiligo (Boissy and Lamoreux, 1988). Most of these defects are characterized by the spontaneous depigmentation of normally pigmented birds and probably represent mutants in melanocyte physiology rather than true developmental mutants. One exception is the Silkie strain of chickens, which is characterized by extensive dermal and internal pigmentation caused by a prevalence of melanocytes in ectopic locations (Faraco *et al.*, 2001; Hallet and Ferrand, 1984; Makita and Mochizuki, 1984; Makita and Tsuzuki, 1986).

Unlike White Leghorn and quail melanoblasts, Silkie melanoblasts migrate ventrally, as well as dorsolaterally, during neural crest morphogenesis (Faraco *et al.*, 2001; Reedy *et al.*, 1998b). The abnormal migration of melanocytes in Silkie chickens does not reflect a defect in the neural crest, because Silkie neural tubes grafted into White Leghorn hosts never exhibit internal pigmentation, whereas quail neural tubes placed in Silkie embryos do generate internal melanocytes (Hallet and Ferrand, 1984). A molecular marker for barrier molecules, PNA (Oakley *et al.*, 1991), indicates that inhibitory molecules are normally distributed beneath the dermamyotome in regions through which melanoblasts do not migrate. These PNA barriers are absent in the Silkie embryo (Faraco *et al.*, 2001; de Freitas *et al.*, 2003), suggesting that barriers develop during the late migration of melanoblasts that keep them in the dorsolateral path and prevent them from dispersing ventrally. Thus this mutant has identified a process late in melanoblast migration that constrains melanoblasts to the skin. Identification of the factors expressed in the Silkie environment responsible for melanocyte morphogenesis will be of considerable interest.

Mammals

As in birds, the only pigment cell found in mammalian skin is the melanocyte. The neural crest origin of mammalian epidermal melanocytes was demonstrated by Rawles (1947, 1948) in an elegant study in which she grafted isolated mouse neural tube, somite, or skin (ectoderm plus dermis) of varying ages into the chick coelom and then scored for the presence of pigmented cells. Studies of mammalian development are hampered by several factors, including the inaccessibility of the embryo to experimental manipulation and higher maintenance costs compared with other vertebrate systems. Nevertheless, the mouse has proved to be a popular system for

studying pigment cell development because of the ability to make and analyze transgenic embryos and the availability of many coat color mutants. In this section we discuss the development of pigmentation in mammals, focusing primarily on mouse and to a lesser extent on humans.

Morphogenesis of Murine Melanocytes

Several studies have followed the early stages of trunk neural crest migration in mouse embryos (e.g., Erickson and Weston, 1983; Serbedzija *et al.*, 1990). At the level of the forelimb, neural crest cells first emerge from the neural tube at day E8.5 (i.e., 8.5 days postcoitus). As in avian embryos, mammalian trunk neural crest cells take one of two migratory pathways: ventrally between the somite and neural tube, or dorsolaterally in the space between the dermamyotome and overlying somite (Rawles, 1947; Serbedzija *et al.*, 1990). Unlike the situation in avians, however, the entry of mouse neural crest cells into the dorsolateral path does not appear to be delayed significantly with respect to ventral migration (Rawles, 1947; Serbedzija *et al.*, 1990, 1994). By E10.5, the emigration of neural crest cells from the dorsal neural tube has ceased (Serbedzija *et al.*, 1990, 1994).

Mayer (1973) recombined flank dermis and ectoderm from albino or normally pigmented mouse embryos to demonstrate that melanoblasts begin entering the epidermis at E12. This was subsequently confirmed using probes against the melanogenic enzyme dct to identify melanoblasts (Cable *et al.*, 1995; Pavan and Tilghman, 1994; Steel *et al.*, 1992). Interestingly, the first melanoblasts to enter the ectoderm are located lateral, as opposed to dorsal, to the neural tube (Pavan and Tilghman, 1994). This corresponds to the spatial pattern of the onset of dct immunoreactivity and suggests that entry into the epidermis depends on the cells reaching a particular point in melanogenesis. Once in the epidermis, melanoblasts migrate into the developing hair follicles, and by E16 premelanosomes are detectable by the dopa reaction (reviewed in Hirobe, 1995).

More recently, several studies have used mice that express the *lacZ* reporter under the control of the *Dct* promoter/enhancer elements to track murine melanoblasts more carefully. For example, Wilkie and coworkers (2002) used *Dct-lacZ* transgenic mice to study the spatial and temporal patterns of melanoblast appearance and migration. They noted that melanoblasts do not arise uniformly along the length of the neural tube. Rather, a large pool of melanoblasts is present in the cervical region by E11.5, and significantly fewer melanoblasts are found in the trunk. The cervical pool appears to be the source of craniofacial melanocytes, as no melanoblasts were found to originate in rostral regions. Also using *Dct-lacZ* reporter mice, Jordan and Jackson (2000b) found evidence that there is a *kit*-independent population of melanoblasts present in the trunk between E15.5 and E16.5, and that these cells are capable of colonizing areas of the flank that are otherwise devoid of melanoblasts in the *Ph* mutants. The ontogeny of these cells is as yet unclear, but if confirmed this is a very interesting observation because of the obvious parallels reported in zebrafish (discussed in Fish, above).

Distribution and patterning of pigment cells is controlled not only by guidance molecules but also by signaling systems that control cell proliferation and cell survival. In the mouse there is, as yet, little information about the molecular nature of directed migration of melanoblasts. However, two general classes of mouse mutants have identified critical regulators of melanoblast proliferation and survival that in turn affect patterning: *piebald/lethal spotting* and *Steel/Dominant spotting*.

piebald mutants display a range of white spotting depending on the allele. Only neural crest-derived melanocytes are affected, however, as retinal pigmentation is normal (Silvers, 1979). In addition to white spotting, *piebald* mutants exhibit dramatically reduced numbers of two other neural crest derivatives: the cochlear melanocytes of the inner ear, and the enteric ganglia of the myenteric plexus (Deol, 1967, 1970; Lane, 1966; Silvers, 1979; Webster, 1973). In homozygotes of the most severe allele, *piebald-lethal* (s^l), this results in deformities of the cochlear duct and in megacolon, the latter of which causes neonatal death.

The product of the *s* locus was identified as the endothelin-B receptor (Ednrb) (Greenstein *et al.*, 1994; Hosoda *et al.*, 1994). Analysis of the Ednrb gene in *piebald* mutants provides an explanation for the variation in allelic severity; whereas the entire *Ednrb* gene is deleted in the s^l allele, the less severe *s* allele is structurally normal but expressed at much lower levels compared to wild type (Hosoda *et al.*, 1994). Interestingly, early tissue recombination experiments (Mayer 1965, 1967a, b, 1977) suggested that although the *s* phenotype is largely cell autonomous, there were non-cell autonomous effects as well. More recent tissue recombination experiments by Lou and coworkers (2004) support this conclusion.

Pavan and Tilghman (1994) demonstrated that the white spotting phenotype in s^l mutants is manifested very early in development by an almost complete lack of melanoblasts in the trunk. Experiments by Shin and coworkers (1999) using a conditional allele confirmed that melanocytes are dependent upon Ednrb very early in their development, between E10 and E12, consistent with *Ednrb* expression in the head fold and future dorsal neuroepithelium prior to neural crest dispersal (Tilghman *et al.*, 1995).

What happens to melanocyte precursors in the absence of *Ednrb* function? Recent studies by Lee *et al.* (2003) have shown that melanoblasts form and even begin to disperse from the neural tube in *Ednrb*-null embryos. However, *Ednrb*-null melanoblasts do not migrate very far along the dorsolateral pathway and eventually disappear. Whether these cells ultimately die or simply fail to complete melanogenesis remains unclear. It should also be noted that in the chicken embryo (see above), melanoblasts chemotact toward endothelin-3. Whether endothelins provide directional cues to murine melanoblasts has not been explored.

The identification of *Ednrb* as the gene at the piebald locus suggested that one or more of the endothelins might play a role

in melanocyte development. The phenotype of mutants at the *lethal spotting (ls)* locus is virtually identical to that of *piebald* mutants, including white spotting and megacolon (Silvers, 1979). Baynash *et al.* (1994) demonstrated that the product of the *ls* locus is EDN-3. In addition, they showed that *ls* mutants carry a point mutation that prevents normal proteolytic processing of the inactive polypeptide precursor of EDN-3.

The other mouse mutants that have provided important insights into morphogenesis are *Dominant spotting* (*W*) and *Steel* (*Sl*). *W* and *Sl* mutants are phenocopies of one another and are characterized by being sterile, anemic, lacking in mature mast cells, and devoid of epidermal pigmentation (Silvers, 1979). These deficiencies are caused by the abnormal development of three stem cell populations: the germ cells, hematopoietic precursor cells, and the neural crest [Silvers (1979) and references therein; reviewed in Fleischman (1993), Morrison-Graham and Takahashi (1993)]. Early work demonstrated that the *Sl* mutation, but not the *W* mutation, could be rescued by a "normal" environment, implying that the *W* mutation was cell autonomous whereas the *Sl* mutation was not (Mayer, 1970; Mayer and Green, 1968). These results, together with the complementary mutant phenotypes, suggested that the *W* and *Sl* gene products act in a common pathway, possibly as a receptor–ligand complex. The cloning and characterization of these genes proved that this is, in fact, the case, with *W* encoding Kit and *Sl* encoding the Kit ligand, SLF (Anderson *et al.*, 1990; Flanagan and Leder, 1990; Geissler *et al.*, 1988; Huang *et al.*, 1990; Martin *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990a, b).

Several studies have attempted to define exactly when melanoblasts require Kit and SLF activity (see Wehrle-Haller, 2003 for a comprehensive review). The patterns of expression of both Kit and SLF mRNAs suggest an early role in melanocyte development, with Kit^+ melanoblasts detectable in the trunk as early as E10.5 (Keshet *et al.*, 1991; Manova and Bacharova, 1991; Wehrle-Haller and Weston, 1995). Likewise, *in utero* injection of ACK2, a Kit function-blocking antibody, revealed that Kit is required at this time (Nishikawa *et al.*, 1991; Yoshida *et al.*, 1993). These results are consistent with several studies that examined the dependence of melanogenesis on SLF *in vitro* (Morrison-Graham and Weston, 1993; Murphy *et al.*, 1992; Reid *et al.*, 1995).

I*n vitro* and *in vivo* studies support the idea that mammalian Kit signaling is required for the survival of melanoblasts and their proper migration (Cable *et al.*, 1995; Ito *et al.*, 1999; Morrison-Graham and Weston, 1993; Murphy *et al.*, 1992; Reid *et al.*, 1995; Steel *et al.*, 1992; Wehrle-Haller and Weston, 1995; Wehrle-Haller *et al.*, 2001; reviewed in Yoshida *et al.*, 2001) as in fish (Parichy *et al.*, 1999; Rawls and Johnson, 2003). Later in development, Kit/SLF signaling is also required for the entry of melanoblasts into the developing hair follicle, having largely chemokinetic effects on melanoblasts (Jordan and Jackson, 2000a; Peters *et al.*, 2002); the precise factors that guide these precursors to hair follicles remain unknown.

Remaining unresolved is the importance of two forms of SLF. Alternative splicing of the primary *Sl* transcript results in two isoforms, soluble and membrane bound, both of which are equally capable of binding to and activating Kit (Flanagan *et al.*, 1991; Majumdar *et al.*, 1994). In *Sl dickey* (Sl^d) mutants, the entire transmembrane and cytoplasmic domains of the gene are deleted. As a result, Sl^d mutants produce functional, soluble SLF but no transmembrane SLF (Brannan *et al.*, 1991; Flanagan *et al.*, 1991). Sl^d/Sl^d homozygotes are phenotypically identical to homozygotes for *Sl* null alleles (Silvers, 1979), indicating that soluble SLF alone is not sufficient for normal melanogenesis. Wehrle-Haller and Weston (1995) demonstrated that soluble SLF is, however, sufficient for the initial dispersal of melanoblasts along the dorsolateral pathway, while membrane-bound SLF is required for later survival, differentiation, or both. One explanation for the importance of membrane-bound SLF is that it would be expected to result in a higher local concentration of SLF because it cannot diffuse away from the cells producing it. An additional possibility, however, is that the membrane-bound form of SLF functions as a cell adhesion partner for Kit expressing melanoblasts. The ability of SLF/Kit binding to promote cell adhesion directly has been demonstrated in cell lines (Flanagan *et al.*, 1991), primordial germ cells (Marziali *et al.*, 1993), mast cells (Adachi *et al.*, 1992; Kaneko *et al.*, 1991), and megakaryocytes (Avraham *et al.*, 1992).

Morphogenesis of Human Melanocytes

Despite the evidence for a neural crest origin for pigment cells in other vertebrate species, many early studies of human pigmentation were prefaced on the theory that human melanocytes are derived from the basal cells of the epidermis, rather than the neural crest [Bloch (1917, 1921), as reported in Zimmerman and Becker (1959a)].

Information on human melanocyte development is reviewed in Holbrook *et al.* (1988) and Boissy and Nordlund (1995) and is summarized briefly here. The migration of neural crest through the lateral pathway brings melanoblasts first to the dermis. From the dermis, melanoblasts migrate into the epidermis to their eventual location on the basal lamina of the basal epithelial layer of the epidermis. Early work on the timing of appearance of fetal melanoblasts and melanocytes was limited to indirect approaches that utilized the reactivity of premelanosomes with reduced silver and subsequent identification of reactive cells by electron microscopy. Mature melanocytes have also been identified using the dopa reaction and morphologic criteria. More recently, the discovery of a cytoplasmic epitope shared by melanoma cells and fetal melanocytes, the HMB-45 monoclonal antibody (Gown *et al.*, 1986), has allowed a reanalysis of data on the timing of appearance of human melanocytes (Holbrook *et al.*, 1988).

Early studies indicated that in human embryos neural crest migration is complete by seven weeks and that melanoblasts appear in the dermis by 11 weeks (Zimmerman and Becker, 1959a, b). The timing of appearance of melanoblasts in the epidermis has since been refined to earlier than seven weeks using HMB-45 staining (Holbrook *et al.*, 1988). The density

of this initial population of melanocytes observed by seven weeks is about 50% of the density observed at birth. By 10 weeks, melanocytes in the epidermis are observed to have premelanosomes based on both the reaction with reduced silver and direct electron microscopic observation (Fujita *et al.*, 1970; Zimmerman and Becker, 1959b).

It was thought that a large influx of melanoblasts occurred at the 12–14-week stage of development based on counts derived by reduced silver staining, but this has been reinterpreted by the HMB-45 staining data, which seem to imply that the levels of melanocytes thought to appear at the twelfth week are already in place by the eighth week and thus there is no new influx. There is a rough doubling of the number of melanocytes between weeks 10 and 14, with the numbers observed by HMB-45 staining much greater than the numbers observed by reduced silver staining. This may be due to mitosis of melanocytes that are already in the epidermis.

The final number and distribution of melanocytes in the adult is relatively constant, although it can be affected by such stimuli as ultraviolet (UV) light. The distribution of melanocytes appears to be nonrandom (Holbrook *et al.*, 1988), and each melanocyte is found with 36 keratinocytes in an epidermal melanin unit. Melanocytes that are associated with hair follicles arrive at their final location by following the downgrowth of epidermal cells in the developing hair germ (Mishima and Widlan, 1966). The production of melanin pigment developmentally begins at around 12 weeks based on both electron microscopic studies and dopa staining (Zimmerman and Becker, 1959a; Zimmerman and Cornbleet, 1948). The transfer of pigment to keratinocytes occurs late in the second trimester of development (Zimmerman and Cornbleet, 1948).

The human piebald trait, characterized by a white forelock and patchy pigmentation, is likely due to a defect in melanocyte morphogenesis resulting in reduction or absence of these cells in discrete areas of the skin. Like white spotting in mice, piebaldism is congenital (Halaban and Moellmann, 1993) and many piebald patients have been shown to have mutations in the human homolog of *Kit* (e.g., Fleischman, 1992, 1993; Fleischman *et al.*, 1991; Giebel and Spritz, 1991; Spritz *et al.*, 1992, 1993; reviewed in Baxter *et al.*, 2004). Defects in melanocyte and melanoblast morphogenesis resulting from disruptions in endothelin-3 and Ednrl signaling are likewise implicated in Hirschsprung disease in humans, which is also characterized by aganglionic megacolon (McCallion and Chakravarti, 2001).

Differentiation of Mammalian Melanocytes

As discussed previously, it is well documented in both teleost and avian embryos that melanocyte lineage segregation begins very early in neural crest development, and that melanoblasts are specified prior to or at least coincident with their emigration from the neural tube. A recent study by Wilson *et al.* (2004) demonstrates that the same is true in murine embryos. Using mice that express lacZ under the control of the kit regulatory elements (W^{lacz}), they identified distinct melanogenic (kit^+) and neurogenic ($p75^+$) subpopulations of premigratory neural crest cells in the midline of the dorsal neural tube prior to neural crest emigration.

What signaling pathways are responsible for the initial segregation of the melanocyte lineage? There is strong evidence to suggest that Wnt/β-catenin signaling plays a critical role in this process. Wnt signaling promotes melanocyte formation in both birds and fish (see previous sections), and targeted disruption of the β-catenin gene in the dorsal neural tube of mouse embryos results in specific loss of the melanocyte and sensory ganglia neural crest subpopulations (Hari *et al.*, 2002). The most likely activators of β-catenin *in vivo* are Wnt1 and Wnt3a, both of which are expressed in the dorsal neural tube and required for melanocyte formation (Ikeya *et al.*, 1997). Dunn *et al.* (2005) have recently shown that forced overexpression of β-catenin, Wnt1, or Wnt3a *in vitro* results in an endothelin-dependent increase in melanocyte differentiation as well.

Several transcription factors have been identified that are required for neural crest development overall (e.g., slug/snail, foxd3, and Pax 3) and are therefore important for melanocyte formation as well. The first transcription factor that is relatively specific for melanocyte differentiation was identified in the *microphthalmia* (*mi*) mutant. Both neural crest-derived and non-neural crest-derived melanocytes are affected in *mi* mutants. As a result, homozygotes of many alleles display white spotting, small, unpigmented eyes, and a lack of melanocytes in the inner ear (Silvers, 1979). Mice with certain *mi* alleles are also deficient in osteoclasts and mast cells (Silvers, 1979).

mi encodes the transcription factor Mitf (Hodgkinson *et al.*, 1993). Members of this family bind DNA as homo- or heterodimers (Hemesath *et al.*, 1994), which explains why the original *mi* allele is semidominant and may also explain the high degree of intrallelic complementation and interallelic interactions that characterize *mi* mutants (Hodgkinson *et al.*, 1993; Silvers, 1979; Steingrimsson *et al.*, 1994). As previously discussed, Mitf regulates the expression of several melanocyte-specific genes, including *dopachrome tautomerase*, *tyrosinase*, *tyrosinase-related protein 1*, *Pmel17*, and *Melan-a* (Baxter and Pavan, 2003; Bentley *et al.*, 1994; Du *et al.*, 2003; Yasumoto *et al.*, 1995; Yokoyama *et al.*, 1994).

In transgenic mice carrying mutations in *mi*, melanoblasts form initially but fail to survive and reach the migration staging area (Hornyak *et al.*, 2001). It is unlikely that this phenotype is related to Mitf's role regulating expression of the melanin biosynthesis genes mentioned above, as mutations at the *brown* (TRP-1), *slaty* (dct), and *albino* (tyrosinase) loci do not result in melanocyte death but rather prevent the formation of normal melanin (see Silvers, 1979; Bennett, 1993; and references therein). However, McGill *et al.* (2002) have shown that Mitf is a critical transcriptional regulator of the anti-apoptotic gene *Bcl2* in melanocytes, suggesting that *Mi*-deficient melanoblasts die as a result of Bcl2 downregulation.

Mitf can activate transcription *in vitro* through the M box found in the promoters of several melanocyte-specific genes (Bentley *et al.*, 1994; Lowings *et al.*, 1992; Shibahara *et al.*, 1991; Yasumoto *et al.*, 1994; Yavuzer and Goding, 1994).

Moreover, misexpression of Mitf can convert nonmelanocytes into melanin-producing cells and rescue melanogenesis in mutant mice (Opdecamp *et al.*, 1997). As in other vertebrates, Mitf appears to be one of the major links between Wnt signaling and melanogenesis in two ways. First, *MITF* is a direct target of Wnt3a-mediated LEF-1 activation (Takeda *et al.*, 2000; see also Fishes). Second, the Mitf protein interacts in *trans* with LEF-1 to regulate expression of melanocyte-specific genes (Yasumoto *et al.*, 2002).

In humans, mutations in *Mi* are associated with Waardenburg's syndrome type 2A (WS2A), an autosomal dominant condition exhibiting deafness and patchy, abnormal pigmentation (reviewed in Baxter *et al.*, 2004; Hughes *et al.*, 1994; Tassabehji *et al.*, 1994b). The importance of Mitf in WS may be even more far reaching because two other genes linked to WS discussed below, *sox10* and *Pax3*, regulate Mitf expression (Lee *et al.*, 2000; Potterf *et al.*, 2000; Verastegui *et al.*, 2000). Thus, it is possible that the common etiologies between the various forms of WS ultimately result from a lack of Mitf expression.

Sox10 is another transcription factor that is critical for melanocyte development (reviewed in Mollaaghababa and Pavan, 2003). Sox10 directly regulates the expression of *MITF* (Watanabe *et al.*, 2002), and the sox10 protein also cooperates with the Mitf protein to regulate expression of *Dct* (Potterf *et al.*, 2001; Ludwig *et al.*, 2004). *Sox10* is also associated with WS2A in humans.

A third transcription factor in the melanogenesis regulatory network was identified in the *Splotch* mutant. The pigment defect in *Splotch* heterozygotes is characterized by white spotting on the feet, tail, and belly (Silvers, 1979). For most alleles, the homozygous phenotype is lethal due to defects in the neural crest and to the failure of the neural tube to close properly (Jackson, 1994; Silvers, 1979 and references therein). Other neural crest derivatives affected in homozygous *splotch* mutants include the spinal root ganglia and Schwann cells (Silvers, 1979). Interestingly, these derivatives are not noticeably affected in heterozygotes. The *splotch* locus corresponds to *Pax3*, encoding a member of the paired-box transcription factor family (reviewed in Chalepakis *et al.*, 1993) that is expressed in the dorsal neural tube and early neural crest cells (Goulding *et al.*, 1991). Melanoblasts do develop in *splotch* mice, but they are present in far lower numbers than normal (Hornyak *et al.*, 2001). This may be due at least in part to the role of Pax3 in regulating expression of Mitf. Some patients with WS1 have been shown to have mutations in the human homolog of *Pax3* (e.g., Tassabehji *et al.*, 1992, 1994a). A recent report shows that Pax3 activates Mitf, while simultaneously competing with Mitf for sites in the *dct* promoter. Wnt signaling and β-catenin activation relieve this repression, allowing melanogenesis to proceed. Thus, *Pax3* appears to prime cells for the differentiation whereas Wnt signaling allows the cells to proceed down this pathway (Lang *et al.*, 2005).

Mutants

Much of what we know about melanocyte development in mammals has come from studies of the over 70 mouse pigment mutants. A detailed discussion of the development and initial characterization of these lines can be found in Silver's classical 1979 treatise, *The Coat Colors of Mice*. In general, mouse coat color mutants can be assigned to one of three categories: those that affect the subcellular structure of melanocytes, those that perturb the biochemical pathways of melanin synthesis, and those that disrupt normal melanocyte development. Mutants in the latter category typically display various degrees of white spotting, i.e., an absence of melanocytes from distinct regions of the head, trunk, or rump.

Comprehensive discussions of these coat color mutants can be found elsewhere (e.g., Baxter *et al.*, 2004; Bennett, 1993; Jackson, 1994; Silvers, 1979). While many have been identified at the molecular level, much work remains to be done. For example, the *patchwork* mouse, which exhibits a salt-and-pepper phenotype, may be especially interesting for understanding melanoblast colonization and survival in the hair follicle (Aubin-Houzelstein and Panthier, 1999; Aubin-Houzelstein *et al.*, 1998).

A final mutant worth mentioning for historical reasons is *Patch*, a homozygous-lethal dominant mutation. Heterozygotes have a sharply demarcated white belly spot that is variable in size, sometimes encircling the entire trunk (Silvers, 1979). The *Patch* mutation was mapped to a deletion that encompasses the gene for platelet-derived growth factor receptor-α (Pdgfra) (Stephenson *et al.*, 1991).

The *Pdgfra* gene is linked to *Kit* on chromosome 5 (Nagle *et al.*, 1994). Interestingly, the *Patch* phenotype (Morrison-Graham *et al.*, 1992) is similar to that of $Kit^{W\text{–}sh}$ mutants. The $Kit^{W\text{–}sh}$ mutation is a gross rearrangement of the 5′ upstream region of the *Kit* gene that results in the ectopic misexpression of Kit in a variety of tissues, including the dermatome and developing limb (Duttlinger *et al.*, 1993). It is hypothesized that this misexpression results in a reduction of the amount of SLF available to migrating melanoblasts (Besmer *et al.*, 1993; Duttlinger *et al.*, 1993). The phenotypic similarity of $Kit^{W\text{–}sh}$ and *Patch*, together with the fact that the two genes are closely linked, led to the hypothesis that the *Patch* phenotype is due to the misexpression of *Kit*, rather than a reflection of melanoblast or neural crest dependence on Pdgfra activity *in vivo*. This hypothesis is supported by the observation that *Kit* mRNA is expressed ectopically in the dorsal neural tube and dermatome of *Patch* heterozygotes, despite the fact that *Kit* is not affected structurally by the *Patch* deletion (Duttlinger *et al.*, 1995). The absence of pigment defects in targeted *Pdgrfa* knockout mice is consistent with a primary role for *Kit* misexpression in explaining the *Patch* phenotype (Soriano, 1997).

Perspectives

The study of pigment cell development has been an area of active research in developmental biology for over a century because pigment cells are so readily identifiable and pigment patterns vary strikingly both within and among vertebrate

taxa. Until recently, embryologists studying pigment cell development were limited to careful descriptive analyses or physical perturbations of developing embryos. Now, these techniques are complemented by a growing understanding of the factors at the molecular and cellular level that regulate the morphogenetic behavior and differentiation of pigment cells.

A comparative approach to studying pigment cell development has been, and will continue to be, absolutely critical for a thorough understanding of the subject. Although each system has it own unique advantages, no single system is ideal for studying both the molecular and cellular aspects of neural crest and pigment cell development. For example, identification of the molecular basis for some of the mouse coat color mutants has provided definitive evidence for specific factors involved in melanocyte development. Nevertheless, murine embryos typically are not an experimentally tractable system, so characterizing the cellular effects of these factors is difficult. Conversely, amphibian and avian embryos are highly accessible to experimental manipulation, but are only beginning to emerge as useful systems for genetic or molecular analyses. For this reason zebrafish has become a powerful model system to study pigment patterns since they are both genetically and experimentally amenable.

Comparative studies of pigment cell development also are useful for studying questions of developmental biology in an evolutionary context. Pigment cells in all vertebrates are derived from the same progenitor cell population, the neural crest, and at least one type of pigment cell, the melanophore/cyte, utilizes a common pigment biosynthesis pathway across species. These commonalities, and considerable research over the last decade, suggest that specific molecules will play similar roles in the morphogenesis or differentiation of pigment cells in different taxa. Equally interesting, however, are those instances where this is not true. For example, the importance of Kit signaling in the early migration and survival of zebrafish melanophores and murine melanocytes is well established. However, the expression patterns of *kit* in amphibians and birds suggest this gene is not involved in the same events as in other vertebrates (Baker *et al.*, 1995; Lecoin *et al.*, 1995; Reedy *et al.*, 2003). Discrete cells (pigment cells) from a well-defined progenitor cell population (the neural crest) will thus continue to provide intriguing experimental materials for cell and developmental biologists in their quest to understand how eukaryotic cells differentiate and evolve.

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