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MECHANISMS FOR REACHING THE DIFFERENTIATED STATE: INSIGHTS FROM NEURAL CREST DERIVED MELANOCYTES

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Abstract

Black pigment cells, or melanocytes, are the major contributing cells to pigmentation in vertebrate organisms. Although the function of these cells is distinct depending on the organism, the events involved in their development are remarkably similar. Here, we review the mechanisms involved in the early development of melanocytes from neural crest, many of which are conserved in organisms as diverse as zebrafish, birds and humans. We also discuss recent studies that provide further insight into how melanocyte differentiation is achieved and maintained.

Keywords

melanocytes; differentiation; neural crest; specification

1. Introduction

The process of cellular differentiation during development involves the acquisition of the specific characteristics necessary for proper function in the organism. This process involves the correct temporal regulation of genetic hierarchies and extracellular signals to integrate cells into tissues and organs. Cell fate determination has been intensively studied in the development of the neural crest, a multipotent population of cells unique to vertebrates that give rise to derivatives with remarkably distinct properties and functions, including neurons and glia of the peripheral nervous system and craniofacial bone and cartilage in addition to pigment cells [1]. Likely the most well characterized of neural crest cell fate programs is that of the melanocyte lineage, the cells that produce black pigment throughout the body. Examination of neural crest in many organisms, including rodents, birds, amphibians and fish, are providing insight into the conserved mechanisms regulating its development. It is becoming clear that several signals involved in neural crest induction are also critical to regulating derivative fate choice [2]. Although the reiterated use of these signals indicates some similar mechanisms of development among neural crest-derived cell types, some specificity must be generated to turn on distinct differentiation programs. Here, we will examine signaling pathways and transcription factors known to be critical for the differentiation of melanin-containing pigment cells, known as melanocytes in mammals and birds, or melanophores in fish and amphibians.

Melanocytes have distinct functions in different organisms. In humans, they function in eye, hair and skin pigmentation, and are critical for the tanning response to protect skin cells from ultraviolet induced DNA damage (reviewed in [3,4]). The biology of pigmentation has fascinated biologists for years, and thus much is known regarding the genes important for the

development of pigment patterns in several organisms (reviewed in [5]) and the biological significance of these patterns. In animals with fur or coats, including rodents and dogs, melanocytes are responsible for the characteristic patterns and colors of hair that are observed in these organisms. Melanocytes transfer melanin pigment to surrounding cells to color hair and skin (reviewed in [6]). Vertebrates without fur, such as amphibians and fish, melanophores, generate diverse pigment patterns along with other neural crest-derived pigment cell types, including yellow xanthophores and iridescent iridophores (reviewed in [7,8]). Melanophores and other chromatophores can rapidly change pigment distribution resulting in color adaptation, warning signals, and mating signs important for camouflage, predator evasion and sexual selection (reviewed in [9]).

2. Specification of melanocytes from neural crest

A compelling model for cell fate specification involves integration of signals on a “nodal point”, with activation of a transcription factor acting as a master regulator to activate downstream differentiation genes [10]. Microphthalmia Transcription Factor (Mitf) has many properties of a master regulator for the melanocyte lineage. Mitf is a basic-helix-loop-helix (BHLH) leucine zipper protein that is the earliest marker indicating establishment of this lineage [11,12]). Transgenic mice driving Cre recombinase using a Mitf bacterial artificial chromosome confirms that expression is specific to melanocyte lineages [13]. The importance of Mitf gene regulation in melanocyte development has been demonstrated by loss-of-function studies: mutations in the mouse Mitf gene lead to a range of pigmentation defects, depending on the mutation and the amount of functional protein present (reviewed in [14]). Overexpression of Mitf confers melanocyte characteristics [15,16]). Together these results suggest that Mitf is necessary and sufficient to specify cells as melanocytes.

The function of Mitf is conserved across species. Mutations in Mitf cause pigmentation defects in rat [17], hamster [18,19], quail [20] and dog [21]. Moreover, mutations in Mitf underlie Waardenburg Syndrome 2A in humans [22], a disorder characterized by hearing loss and defects in hair, eye and skin pigmentation. Along with hearing loss, zebrafish also rely on Mitf (*mitfa*) expression and activity throughout embryonic and metamorphic development for pigmentation. Zebrafish *nacre* mutants, which do not express functional *mitfa* protein, never develop differentiating melanophore precursors, and thus completely lack pigmentation throughout their lives. Unlike in mammals, all other systems, including the retinal pigment epithelium (RPE), develop and function normally in zebrafish [23]. A second *mitf* gene, *mitfb*, appears to compensate for pigmentation of the RPE [24], a characteristic shared with other fishes [25]. Overexpression of *mitfa* in fishes results in ectopic pigmentation [23,26], suggesting that as in mammals it is sufficient to confer melanophore-specific traits.

At its simplest, a model for specification of melanocyte fates from the neural crest would be achieved by activating Mitf transcription. Expression of Mitf is regulated both by extrinsic signaling pathways and intrinsic factors. Wnt signaling is known to be necessary and sufficient for promoting melanocyte fate from neural crest via regulation of β -catenin activity [27–30]. The Mitf promoter/enhancer responds directly to β -catenin through Lef/TCF binding sites [31–33], and Wnt signaling cooperates with Mitf to transactivate its own promoter [34]. Additional regulators of Mitf expression include neural crest transcription factors: the Sry-related factor Sox10 and the paired homeodomain factor Pax3. Loss-of-function mutations in both of these genes results in pigmentation defects in mice [35–37] and Waardenburg syndrome in humans [22,38]. Expression of Sox10 or Pax3 is sufficient to transactivate the Mitf promoter *in vitro*. Moreover, when both proteins are present on the Mitf promoter, synergism is observed [39–44].

Aspects of the Wnt, Sox, Pax and Mitf regulatory relationship are conserved in species other than mammals. Loss of Sox10 function in zebrafish results in loss of melanophores and additionally loss of other pigment cell types including yellow xanthophores and shiny iridophores [45]. Sox10 is necessary for *mitfa* expression in zebrafish and directly transactivates the *mitfa* promoter [44,45]. Sox10 is also necessary for pigment cell development in *Xenopus* [46]. However, there are also differences, particularly with respect to Pax genes. In addition to Pax3, the closely related Pax7 is expressed in avian melanocytes [47]. The roles of Pax3 and Pax7 in neural crest development appear to be variable among species; for example, Pax7 is required for formation of avian neural crest [48] but dispensable for mammalian neural crest [49]. In zebrafish, loss of Pax3 and Pax7 results in loss of the yellow pigmented xanthophores with only subtle effects on melanophores [50]. Whether another Pax gene plays a role in zebrafish melanophore development as in other species remains to be determined.

3. The differentiated state of melanocytes

The specification of melanocytes from neural crest relies heavily on the spatial and temporal control of gene expression and interactions. While some regulation of gene expression continues during differentiation, it is becoming clear that additional, post-translational regulatory mechanisms function to modulate and maintain the differentiated state (Figure 1). Several recent studies provide interesting insights into how this is achieved in melanocytes.

3.1 The differentiated state defined

Whereas the functions of melanocytes are diverse among different organisms, the differentiated state is relatively similar. Differentiated melanocytes are characterized by three properties: 1) black pigment or melanin accumulation 2) melanin trafficking within organelles called melanosomes and 3) dendritic morphology. First, the production and accumulation of melanin involves several enzymes from the tyrosinase related protein (Trp) family, including Trp1, dopachrome tautomerase (Dct; originally known as Trp2) and the rate limiting enzyme, tyrosinase (Tyr) (reviewed in [51]). These enzymes catalyze reactions critical for production of melanin and also interact with one another to regulate each other's activities [52].

A second characteristic of differentiated melanocytes involves cytoskeleton dependent trafficking of their unique organelles, the melanin containing melanosomes (reviewed in [53]). In mammals, the transport of melanosomes to the periphery of the cell (dispersion) via kinesin motors is important for transfer of melanosomes to surrounding keratinocytes. Positioning of these melanosomes at the periphery depends on complex interactions between Rab27a, melanophilin and myosin Va proteins that link melanosomes to the actin cytoskeleton. Disruption of any of these components results in lighter coat color as demonstrated by *dilute*, *ashen* and *leaden* mutant mice (reviewed in [53]). Zebrafish melanophores provide additional insight into the mechanisms regulating melanosome trafficking. As these cells aggregate and disperse their melanosomes in response to hormones (cAMP dependent), melanosome movements can be carefully examined following addition of cAMP stimulants or inhibitors to the fish water. Recent studies examining zebrafish melanophores provide evidence that *melanophilin* is an important mediator of cAMP-dependent regulation of melanosome trafficking. Zebrafish *melanophilin* mutants demonstrate defective melanosome dispersion in their melanophores, showing continued aggregation, even in response to dispersion inducing stimulants [54]. Thus, zebrafish *melanophilin* is also important for the correct positioning of the melanosomes within the cell, although the melanosomes are not transferred to surrounding cells as done in mammals.

The last property not unique to melanocytes, but important to their function, is their dendritic shape or morphology. Human melanocytes, critical for donation of DNA-protecting melanosomes to surrounding epidermal skin cells or keratinocytes (tanning response), take on

a highly dendritic appearance, with cell projections interacting with neighboring keratinocytes at a ratio of one melanocyte to approximately 40 keratinocytes (reviewed in [4]). Once this cell interaction is accomplished, the melanocyte has achieved balance, or homeostasis, with the surrounding keratinocytes. The importance of melanocyte adhesion and homeostasis promoting proteins, such as CCN3 (Cyr61, CTGF, Nov), is indicated by the correlation of their decrease in function with melanoma progression [55]. Although aquatic vertebrates do not have a tanning response similar to that observed in humans, their melanocyte morphology is similar to that of mammalian cells depending on their location. Unlike mammalian melanocytes, which are restricted to the epidermis of the skin, frog and fish melanocytes are found in both epidermal and dermal tissue. The dermal cells are dendritic while epidermal melanocytes are round and smaller [56,57].

3.2 Promoting the differentiated state

Melanocyte differentiation involves changes in gene expression to establish the differentiated state, including production of pigment, melanosome trafficking and cell morphology. In the simplest model, expression of *Mitf* would be sufficient to promote the differentiated state in the melanocyte lineage. Indeed, *Mitf* has been shown to regulate expression of several key genes involved in melanin production (reviewed in [58]). However, several studies suggest that the nodal point in melanocyte differentiation is more complex than simply the activation and subsequent functioning of *Mitf*. Intriguingly, it is interactions amongst *Mitf* and its activators Wnt, Sox and Pax that together regulate melanocyte differentiation. *Mitf* cooperates with Wnt signaling components to regulate downstream genes involved in pigment synthesis through direct interactions with Lef and β -catenin [59,60]. Sox10 also acts synergistically with *Mitf* to regulate melanocyte-specific genes [61–65]. Pax3 and *Mitf* both positively regulate the *Trp1* gene [66,67], although direct synergy has not been tested. A more elegant and complete model for the nodal point regulating melanocyte differentiation involves the combination of Pax3, Wnt signaling and *Mitf* [68]. In this model, Pax3 both activates *Mitf* expression and competes with *Mitf* for space on the *Dct* promoter. Activation of Wnt signaling allows displacement of Pax3 by *Mitf* and subsequent *Dct* transcription.

3.3 Modulating the differentiated state

Melanocytes change their properties in response to environmental stimuli. Melanin plays an important role in protecting against damage from ultraviolet light, and melanocytes increase pigmentation in response to UV exposure and other types of stress (reviewed in [69]). Melanogenesis is also under hormonal regulation (reviewed in [70,71]). A key signaling pathway involved in these melanocyte responses to environmental signals is the cAMP pathway, and a major target is *Mitf* (reviewed in [72]). Transcription of *Mitf* in response to increased cAMP levels is modulated by Sox10 [73]. Additionally, pigmentation is modulated by Dickkopf, an inhibitor of the Wnt signaling pathway [74]. Thus, many of the same pathways involved in modulating levels of pigmentation are those involved in the initial differentiation of melanocytes.

While in some cases differentiation factors are reutilized, additional factors with similar properties and functions are also used in response to extrinsic stimuli. For example, Usf-1, a bHLH-leucine zipper transcription factor related to *Mitf*, promotes tyrosinase activity in response to UV light [75,76]. *Mitf* has not been shown to be UV responsive. Similarly, Passeron *et al.* recently reported a melanocyte-specific function for the Sry transcription factor Sox9 in response to ultraviolet B (UVB) stimulus. Normal human epidermal melanocytes upregulate their Sox9 expression levels in response to UVB stimulus, which works in a cAMP/Protein Kinase A-dependent manner [77]. Using Chromatin Immunoprecipitation assays, the authors show that this cAMP dependent upregulation of Sox9 expression correlates with increased Sox9 protein binding to the *Mitf* promoter. Expression of *Dct* and *Tyr* are upregulated in

response to UVB, most likely due to Sox9 mediated activation of Mitf expression. Sox9 apparently is functioning in a fashion analogous to Sox10, to which it is closely related. Surprisingly, Sox10 expression remains unaltered [77]. This difference in SoxE (Sox8/9/10) protein-mediated regulation of Dct expression in rodent and human melanocytes, may suggest a mechanism that is unique to melanocytes capable of participating in the tanning response.

3.4 Maintaining the differentiated state

Following differentiation of neural crest cells, the differentiated state of the lineage must be maintained. This process likely involves cell response to signals in the environment. Real, Glavieux-Pardanaud and colleagues report the ability of differentiated quail melanocytes to revert to a dedifferentiated state in culture. Following isolation of melanocytes from quail epidermal tissue, melanocytes were propagated in culture in the presence or absence of endothelin 3, a ligand that activates the endothelin receptor B2 resulting in signals shown to be important for melanocyte proliferation [78]. Melanocytes isolated in this fashion showed a decrease in melanin content and an increase in early neural crest marker expression, such as Sox10 and FoxD3 [79]. These results indicate differentiated melanocytes may maintain some progenitor cell like properties allowing them to revert to other cell types when the appropriate inhibitory signals (from surrounding tissue *in vivo*) are absent.

Recent evidence suggests that nearby epidermal cells provide signals necessary to maintain the differentiated state and behavior of melanocytes [80]. Weiner, Han and colleagues found that in mice pigment pattern generation, pigment recipient cells (skin cells) are just as critical as pigment donor cells (melanocytes). Furthermore, later in development it appears recipient cells can affect the localization and pigmentation of melanocytes. When the forkhead transcription factor, FoxN1, is ectopically expressed, melanocytes move away from FoxN1 negative areas, re-establishing themselves in areas containing FoxN1 positive cells. This phenotype is partially rescued when Fgf2 activity is inhibited [80].

Kit signaling also plays a role in maintaining melanocyte differentiation. Activation of the Kit tyrosine kinase receptor via its ligand, Stem Cell Factor, is necessary to maintain proper levels of pigmentation. Both mice [81] and zebrafish [82] harboring null mutations in Kit show defects in pigmentation due to reduced survival of melanocytes. Activation of Kit signaling leads to Mitf phosphorylation via the MAPK pathway [83] and a subsequent increase in Bcl-2 gene expression, an antiapoptotic gene shown to be important for melanocyte survival in mice [84]. It will be interesting to examine how Kit signaling interacts with other extrinsic signals and intrinsic factors necessary to maintain the differentiated state.

Last, differentiated cells must occasionally be replaced to maintain proper melanocyte function. Much has been learned regarding the mechanisms involved in melanocyte stem cell maintenance using the hair follicle as a model [84,85]; reviewed in [86,87]. The hair follicle contains areas termed the bulb and the bulge. The bulge contains slowly proliferating cells that can renew or leave the niche (bulge) to differentiate into mature melanocytes in the bulb region. As these cells have the ability to become mature melanocytes, they have been called melanocyte stem cells (MSCs). The interactions occurring between Pax3, Sox10 and Mitf to regulate the Dct promoter are especially critical in the bulge where decisions to self-renew or to differentiate are being made [68]. In addition, the function of the antiapoptosis transcription factor, Bcl-2, is also involved in MSC maintenance. Following the examination of mouse models of hair graying, Nishimura *et al.* found that failure of MSC maintenance in the bulge leads to loss of hair pigmentation [85,88]. This loss of MSC maintenance has also been confirmed in human hair graying (reviewed in [87]). Not only do melanocytes need to promote their differentiation, several signaling pathways and intrinsic signals are at work to maintain differentiation in a manner that is appropriate to the function of melanocytes in that specific organism.

4. Achieving specificity in reiterated pathways

It is striking that Sox8/9/10 (SoxE subgroup), Pax3/7 and Wnt genes are necessary both for melanocyte development and for the initial formation of neural crest. This regulatory network is likely of ancient origin, with aspects found in lamprey [89] and hagfish [90]. While neural crest cells have long been thought to be a vertebrate-specific characteristic [91], migrating mesenchymal cells with neural crest-like properties have been recently identified in ascidians [92,93]. These findings have led to the supposition that the primitive role of the neural crest may have been the production of pigment cells and their dispersal [94]. The reiterated use of SoxE, Pax3/7 and Wnt genes during initial neural crest induction and later melanocyte differentiation may reflect this core developmental program.

The mechanisms that allow these pathways to act in neural crest development with distinct functions in different lineages, or at different times within the same lineages, are only beginning to be understood (reviewed in [2]). Post-translational modifications of proteins may be important for promoting specificity in neural crest development. This model is supported by a recent paper examining the role of Sox10 subdomains in neural crest differentiation. Schreiner and colleagues characterize novel domains differentially involved in Sox10 function in neural crest lineages. Sox10 has several characteristic transcription factor domains, including the DNA-binding high mobility group (HMG) domain, dimerization domains and transactivation domains that are also found in the other SoxE subgroup family members. Alterations in these domains differentially affect different neural crest lineages including melanocytes [95]. Different alleles of the zebrafish Sox10 mutation *colourless* also differentially affect neural crest derivatives [96]. These data suggest distinct functions for Sox10 domains may have evolved to regulate neural crest cell fate choice. Additional modifications of Sox proteins within specific lineages, such as SUMOylation [97], may also be important for their distinct functions.

5. Future directions

Melanocytes are a remarkable model for understanding diverse biological questions. Due to their easy detection in many vertebrate organisms, they can be used to answer questions regarding the evolution, genetics, developmental biology and function of cellular development. These cells are also an attractive model for understanding general mechanisms of organelle trafficking and cell death. As the incidence of melanocyte cancer, or melanoma, increases (reviewed in [98]), gaining a more complete understanding of normal melanocyte biology could provide extensive insight to the diseased cell. What additional signals inhibit cell proliferation, maintain differentiated characteristics and promote melanocyte function? It will be interesting to examine whether acquisition of these characteristics in developing melanocytes are dependent on one another, happen in a particular sequence and are perturbed in melanoma.

It is also apparent the cellular environment or niche plays a critical role in several aspects of melanocyte biology. Melanocytes receive secreted signals from distant and local cells that guide their specification, migration, survival and function. While reception of some of these signals are regulated via mechanisms controlling melanocyte-specific receptor turnover or activity (i.e. for Kit tyrosine kinase receptor, reviewed in [99]), other functional signals are likely processed and received simultaneously. How does the melanocyte generate specific responses to these signals? Are known differentiation signals constitutively utilized and processed during maintenance of the differentiated state? What unknown factors, important for acquisition and maintenance of differentiation remain to be discovered?

Last, what can understanding melanocyte differentiation tell us about other neural crest derivatives? We know that several overlapping extrinsic signals (i.e. Wnt signaling) and

transcription factors (Sox10, Pax3, FoxD3) function in the differentiation of alternative fates. Understanding the mechanisms of how specific signals are interpreted and processed by melanocytes could provide insight into activity important to the development of these alternate neural crest lineages.

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Figure 1. Extrinsic and intrinsic factors involved in melanophore development and function in zebrafish

A) Unpigmented, specified melanoblasts expressing mRNA for the melanin producing enzyme, Dct. The blue product reveals the locations of *dct* transcript. (white arrow, e; eye) B) Melanophores in the early stages of melanin production (~30 hours post fertilization). Cell morphology is highly dendritic at this stage. C) Mature melanophore at 2 days post fertilization. Cells have accumulated high levels of melanin and have taken on a flattened morphology. D). Alteration in melanophore appearance in response to environmental cues. Melanophores are capable of aggregating (black arrow) and dispersing (black arrowhead) melanin containing organelles, melanosomes. Here, both events are observed simultaneously in this zebrafish melanophore mutant. E) Regulated survival of melanophores. Without Kit signaling, larval melanophores undergo apoptosis. Characteristic morphologies of apoptosing melanophores include cell rounding and clumping.