Evolution of pigment cell regression in the cavefish Astyanax: a late step in melanogenesis

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SUMMARY Pigmentation and eyes are often lost in cave-adapted animals. Although the mechanisms of eye degeneration are beginning to be understood, little is known about the evolutionary and developmental processes involved in pigment cell regression. In teleost embryos, a population of neural crest cells migrates into the body wall and differentiates into melanophores, xanthophores, and iridophores. All three pigment cell types are present in the eyed surface-dwelling form (surface fish) of the teleost Astyanax mexicanus. However, melanophores are absent or substantially reduced in number in various derived populations of the conspecific blind cave-dwelling form (cavefish). We show here that tyrosinase-positive melanoblasts are present in cavefish. DiI labeling revealed a population of trunk neural crest cells in cavefish embryos that migrate to locations normally occupied by differentiated melanophores. We also discovered a cell population in cavefish embryos and adults resembling melanoblasts in several features, including the ability to synthesize melanin when supplied with the tyrosinase substrate l-dopa. Dil-tyrosinase double-labeling and neural keel explant experiments showed that the tyrosinase-positive cells are derived from the neural crest. The number of melanoblasts varies in different adult cavefish populations relative to the extent of melanophore reduction. Although cavefish melanoblasts can synthesize melanin from exogenous l-dopa, they are unable to convert exogenous l-tyrosine to l-dopa and melanin. We conclude that pigment cell regression in cavefish is mediated by an evolutionary change late in melanogenesis that may involve an impediment in the ability to convert l-tyrosine to l-dopa and melanin.

INTRODUCTION

A hallmark of cave-adapted animals, apart from their dramatic loss of eyes, is the absence or reduction in melanin body pigmentation (Culver 1982). Despite our increasing understanding of the molecular basis of pigment cell development, particularly in teleosts (Lister 2002), little is known about how pigmentation is changed during evolution. Pigment patterns are important visual cues in species recognition, predator avoidance, and mate choice (Parichy 2001) and thus are expected to have a role in major evolutionary events such as speciation. The evolution of pigmentation has sparked considerable interest (Mason and Mason 2000) because of the identification of genes and cell surface molecules that play a key role in pigment cell development (Kelsch et al. 1996; Parichy et al. 1999; Dorsky et al. 2000).

Vertebrate body pigment cells are derived from the neural crest (DuShane 1935), a diverse population of pluripotent embryonic stem cells originating at the junction between the neural plate and the nonneural ectoderm (Hall 1999; LeDouarin and Kalcheim 1999). Neural crest cells undergo an epithelial to mesenchymal transformation during neurulation and migrate from the dorsal midline along stereotypic pathways. The migrating cells give rise to numerous derivatives throughout the embryo and are thought to be important in the evolution of vertebrates (Gans and Northcutt 1983). Cranial neural crest cells migrate beneath the ectoderm to form elements of the craniofacial skeleton, cartilage, parts of the cranial sensory ganglia, and pigment cells. Trunk neural crest cells migrate along one of two different pathways. The neural crest cells that enter the ventrolateral pathway, which migrate between the neural tube and the somites or within the sclerotomal portion of a somite, differentiate into dorsal root ganglia, sympathetic chain ganglia, and glia. In contrast, the neural crest cells that migrate through the dorsolateral pathway, between the ectoderm and the somitic mesoderm, become body pigment cells. All types of body pigment cells, including melanophores, xanthophores, and iridophores, are derived from the neural crest (Erickson 1993).

We used the teleost Astyanax mexicanus to understand the evolutionary mechanisms underlying modifications in pigment cell development. A useful feature of A. mexicanus is the existence of two developmental forms: a surface-dwelling form (surface fish) with eyes and pigmentation and a cave-dwelling form (cavefish) without eyes and pigmentation. Variations in pigmentation and eyes are common in cave-adapted animals. Although the mechanisms of eye degeneration are beginning to be understood, little is known about the evolutionary and developmental processes involved in pigment cell regression. In teleost embryos, a population of neural crest cells migrates into the body wall and differentiates into melanophores, xanthophores, and iridophores. All three pigment cell types are present in the eyed surface-dwelling form (surface fish) of the teleost Astyanax mexicanus. However, melanophores are absent or substantially reduced in number in various derived populations of the conspecific blind cave-dwelling form (cavefish). We show here that tyrosinase-positive melanoblasts are present in cavefish. DiI labeling revealed a population of trunk neural crest cells in cavefish embryos that migrate to locations normally occupied by differentiated melanophores. We also discovered a cell population in cavefish embryos and adults resembling melanoblasts in several features, including the ability to synthesize melanin when supplied with the tyrosinase substrate l-dopa. Dil-tyrosinase double-labeling and neural keel explant experiments showed that the tyrosinase-positive cells are derived from the neural crest. The number of melanoblasts varies in different adult cavefish populations relative to the extent of melanophore reduction. Although cavefish melanoblasts can synthesize melanin from exogenous l-dopa, they are unable to convert exogenous l-tyrosine to l-dopa and melanin. We conclude that pigment cell regression in cavefish is mediated by an evolutionary change late in melanogenesis that may involve an impediment in the ability to convert l-tyrosine to l-dopa and melanin.
form (cavefish), which has reduced or lost these features (Jeffery 2001). Remarkably, genetic studies have shown that albinism is controlled by a single Mendelian gene in *Astyanax* cavefish (Sadoglu 1957). At least 30 different *Astyanax* cavefish populations have been described (Mitchell et al. 1977; Espinasa et al. 2001) that exhibit varying degrees of albinism and may have evolved from a surface fish ancestor independently (Dowling et al. 2002; Strecker et al. 2003). Some cavefish populations, such as those from the Pachón Cave (Pachón cavefish), have lost all body melanophores, whereas others, such as those from the Chica, Los Sabinos, Tinaja, and Curva Caves (Chica, Los Sabinos, Tinaja, and Curva cavefish, respectively), have reduced numbers of melanophores. Whereas the developmental mechanisms involved in cavefish eye degeneration are being elucidated (Jeffery and Martasian 1998; Jeffery et al. 2000; Yamamoto and Jeffery 2000; Strickler et al. 2001, 2002), those involved in the loss of pigment have not been investigated.

The regression of melanophores could be caused by changes at one or more levels in the developing cavefish embryo. First, the subset of neural crest cells responsible for generating melanophores may not be specified during embryogenesis. Second, pigment cell progenitors may be specified properly but could fail to undergo migration along their stereotypic pathways in the embryo. Third, neural crest cells may be specified and migrate properly, but their fate might be diverted to a different pigment cell type or to a nonpigment cell derivative. Finally, the melanophore progenitors (melanoblasts) may be determined properly, but they could arrest before differentiation or die, possibly as a consequence of changes in the local extracellular environment in cavefish.

Here we show that *Astyanax* cavefish embryos have a population of migratory cells that originate in the neural keel (teleost neural tube), migrate through the dorsolateral pathway, and exhibit melanoblast-specific tyrosinase activity but fail to undergo terminal differentiation into melanophores. A similar population of tyrosinase-positive melanoblasts was discovered in varying amounts in different populations of adult cavefish. Interestingly, cavefish melanoblasts are able to convert exogenous L-dopa but not L-tyrosine into melanin. The results suggest that pigment cell regression is controlled at a late step in melanogenesis, which may involve a restriction in tyrosine accessibility.

**MATERIALS AND METHODS**

**Biological materials**

Laboratory stocks of *A. mexicanus* originated from surface fish collected at Balmorhea State Park, Texas, USA and cavefish collected at La Cueva de El Pachón in Tamaulipas, Mexico and Cueva de la Curva, Cueva de los Sabinos, La Cueva Chica, and Sótano de la Tinaja in San Luis Potosí, Mexico. Stocks were maintained at 25°C on a 12:12-h light/dark cycle. Embryos were collected after natural or induced spawning and raised to adults as described previously (Jeffery and Martasian 1998; Jeffery et al. 2000). Under these conditions, hatching occurred at about 18 h postfertilization (hpf) and melanophores (in surface fish) appeared at about 24 hpf. Some control experiments were done with tail fin explants (see below) from the tropical albino catfish Corydora aeneus, which was purchased in a pet store. All methods were approved by the University of Maryland animal care and use committee and conformed to National Institutes of Health guidelines.

**Dil labeling**

Embryos were placed in calcium-free zebrafish Ringer’s-1 solution (0.65% NaCl in double-distilled water; Westerfield 2000) and dechorionated with sharpened watchmaker forceps. CM-Dil (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate, Cell Tracker, Molecular Probes, Eugene, OR, USA) was dissolved in ethanol (2μg/μl) and then diluted to 0.5μg/μl in 0.3 M sucrose. Glass electrodes were back-filled with the Dil solution. A bolus of Dil was pressure injected into the dorsal region of the neural keel above somite 10 in 24-hpf embryos. DiI-labeled embryos were reared for 24–72 h in filtered embryo medium (Westerfield 2000) at room temperature (RT), fixed in 4% paraformaldehyde, and viewed with fluorescence optics.

**Neural keel explant cultures**

Embryos were raised to approximately the 10 somite stage and then placed in Ringer’s-2 solution (0.75% NaCl, 0.02% KCl, 0.02% CaCl₂, 5 mM Hapes, pH 7.3) in a 60 × 15-mm plastic Petri dish and dechorionated. Dissections were performed under a dissecting microscope with two sharpened tungsten needles: one straight and one bent at a 90-degree angle. First, the cranium and yolk mass were removed from the embryos. Then, using the straight needle as an anchor and the bent needle to push, the inner portion of the embryo was rolled out of the epidermis and transported to a separate dish in Ringer’s-2. Embryos were dissociated into parts by a brief (90–120 sec) incubation in 0.25% pancreatin (Sigma Chemical Co., St. Louis, MO, USA) while being drawn through a large-orifice plastic pipette tip (200 μl, Fisher Scientific, Newark, DE, USA) to agitate the solution. When the neural keel with adhering somites began to dissociate from the rest of the embryo, the explants were removed and washed four times in Ringer’s-2 to stop enzyme activity. Most of the somites separated from the neural keel with further media agitation, and the remainder was removed with tungsten needles.

Neural keel explants were cultured in Lab-Tek 4 chamber slides coated in 10 μg/ml fibronectin (Roche, Indianapolis, IN, USA) in phosphate-buffered saline (PBS). The media consisted of L-15 (Cellgro, Mediatech, Inc., Herndon, VA, USA) with 20% fetal bovine serum (Sigma), 1 × penicillin-streptomycin (Roche; 1 × is 100 IU penicillin, 0.1 mg streptomycin-HCl), 1 × gentamicin (Roche; 50 μg/ml), and 0.2 μM α-MSH (Sigma). Cultures were maintained at RT for 3 days.

For tyrosinase assay (see below), the culture medium was removed, the explant cultures were dipped briefly in PBS to wash away extra medium, and the explants were fixed by immersion in 5% formalin (pH 7.5) for 30 min at RT. The explants were then
was conducted using 0.1% L-tyrosine (pH 7.4, Sigma) instead of L-dopa or L-tyrosine (pH 7.4), and incubated at 37°C for about 5 h.

Preparation of scale and tail fin explants
Adult surface fish, cavefish, or albino catfish were immobilized by a brief immersion in iced aquarium water. A few scales were removed from their flanks with a spatula, and clips of tail fin were excised with a sharp scissors. The fish were returned to the aquarium where tail fin lesions regenerated within a month. The scales and fin explants were immersed in Ringer’s-1 at RT, fixed, and processed for tyrosinase assay.

Tyrosinase assay
Tyrosinase assays were carried out according to Humason (1972) or Laidlaw (1932) with similar results. Embryos, hatched larvae, scales, fin explants, or neural keel explants were fixed overnight at 4°C in 5% formalin or 4% paraformaldehyde, dissolved in PBS, and washed in several changes of PBS. The PBS was replaced with 0.1% 3,4-dihydroxyphenylalanine (L-dopa, pH 7.4, Sigma), and the specimens were incubated at 37°C. The L-dopa solution was replaced at 30-min intervals. After completion of the assay, some of the embryos were embedded in polyester wax and sectioned at 10 μm. The sections were mounted on slides and viewed unstained under a compound microscope. In some experiments, the assay was conducted using 0.1% L-tyrosine (pH 7.4, Sigma) instead of L-dopa. After 2 h, embryos were washed through several PBS changes and mounted on glass microscope slides for viewing. Controls were preincubated in 1 mM 1-phenyl-2-thiourea, a potent inhibitor of tyrosinase, and then subjected to tyrosinase assay in media supplemented with the inhibitor.

Dil-tyrosinase double-labeling
Embryos were injected in the trunk neural keel region with Dil as described above and raised until labeled migratory cells were present in the flank, about 60 hpf. The labeled embryos were then fixed in 5% formalin and assayed for tyrosinase as described above. The embryos were viewed with fluorescence and bright-field optics in which double-labeled cells appeared red (Dil) and black (melanin).

Neutral red detection of macrophages and tyrosinase staining
The neutral red vital staining method (Herbomel et al. 2001) was used to determine the relationship between macrophages and tyrosinase-positive cells. Tail fin explants prepared from adult surface fish and cavefish were stained with 2.5 μg/ml neutral red in the dark for 20 h (RT). After photography, the neutral red-stained explants were fixed, processed for tyrosinase assay, and the tyrosinase-stained explants were photographed again. Although subsequent processing for tyrosinase assay removed neutral red staining, the distribution of neutral red-stained macrophages and tyrosinase-positive cells could be determined by comparing photographs and merging images of the same neutral red- and tyrosinase-stained specimens in Adobe Photoshop.

RESULTS

Migration of Dil-labeled neural crest cells in cavefish embryos
A region of the trunk neural keel was labeled with Dil and the migration of labeled cells followed to determine whether neural crest cells delaminate and migrate in cavefish embryos (Fig. 1). Pachón cavefish were used in this experiment because they are usually devoid of all body pigmentation (Fig. 2D). (However, Pachón cavefish frequently exhibit melanophores around the eye.) Placement of a Dil bolus in the neural keel at 24 hpf resulted in cell migration from the initial site of Dil introduction during the next day (Fig. 1, B–D). The labeled cells migrated in rostral, ventral, and caudal directions beneath the trunk body wall (Fig. 1, B and C) and yolk sac (Fig. 1D), another region containing melanophores in teleost embryos (Lister 2002). The results show that trunk neural crest cells are present in Pachón cavefish embryos and migrate into the positions normally occupied by melanophores.

Tyrosinase-positive cells in cavefish embryos
Tyrosinase assays using L-dopa as a substrate were conducted to determine whether cells capable of melanin synthesis are present in Pachón cavefish embryos. Figure 2, A and B, shows the results of assays initiated at 36 hpf, about the time melanogenesis began in the trunk region of surface fish embryos (Fig. 2C). In cavefish embryos, melanophores were absent from the trunk, yolk sac, and usually the eye (Fig. 2D). After L-dopa was provided, dendritic tyrosinase-positive cells were detected surrounding the eye, in the trunk, and in the yolk sac in both surface fish and cavefish embryos (Fig. 2, A, B, and J). Pretreatment of embryos with the tyrosinase inhibitor 1-phenyl-2-thiourea prevented the appearance of melanized cells (data not shown; see Fig. 4J). Tyrosinase-positive cells were first detected in the ventral trunk posterior to the yolk sac in 24-hpf cavefish embryos (Fig. 2E), the stage and location in which body melanophores first appear in surface fish embryos (Fig. 2C). Sections showed that these cells were located beneath the epidermis or in the developing dorsal fin (Fig. 2I), positions normally occupied by melanophores in surface fish embryos. Tyrosinase-positive cells were seen throughout cavefish development (Fig. 2, F–H). Therefore, despite the absence of body pigmentation, cavefish embryos retain the ability to synthesize melanin in cells that resemble melanoblasts in their morphology, timing of appearance, and location in the embryo.

Tyrosinase-positive cells are melanoblasts derived from the neural keel
Tyrosinase assays are often used to specifically detect melanoblasts, although the assay is not absolutely specific for melanoblasts: other cell types, particularly leukocytes, also
Two different approaches were used to determine whether the tyrosinase-positive cells were neural crest derivatives. First, we injected DiI in the dorsal neural keel region of 7–10 somite cavefish embryos and the labeled embryos were allowed to develop to 60 hpf, when tyrosinase-positive cells are abundant in the trunk. The DiI-labeled embryos were then fixed and assayed for tyrosinase. As shown in Figure 3, A–D, some trunk and yolk sac cells showed both DiI and tyrosinase staining, indicating that they had migrated from the neural keel region. Second, we asked whether neural keel explants are able to produce tyrosinase-positive cells in culture. As reported previously in teleost embryos (Sadaghiani and Vielkind 1990), outgrowth of cells from *Astyanax* neural keels were obtained in culture (Fig. 3, E–I). In surface fish, but not cavefish, some of the outgrowing cells differentiated into melanophores (Fig. 3, E, F, and H). The same explants were then assayed for tyrosinase activity. In cavefish (as well as surface fish), outgrowing cells with dendritic morphology showed tyrosinase activity (Fig. 3, G and I). The results suggest that cells produced by the cavefish neural keel can synthesize melanin in vitro when supplied with L-dopa, although they do not differentiate into melanophores. The double-labeling and explant results demonstrate that tyrosinase-positive cells are melanoblasts that arise from the neural keel region and are neural crest derivatives.

**Tyrosinase-positive cells in adult cavefish**

Tyrosinase assays were performed on body scales and tail fin explants and the results compared with surface fish to determine whether adult cavefish also exhibit tyrosinase-positive melanoblasts (Fig. 4). Large melanophores are present at the periphery of surface fish scales but are absent from Pachón cavefish scales (Fig. 4, A–D). In contrast, iridophores (Fig. 4, A and B) and xanthophores (data not shown but see Fig. 4, G–J) were detected in both surface and cavefish scales. After tyrosinase assay, tyrosinase-positive cells with dendritic morphology were visible in surface fish and cavefish scales. After tyrosinase assay, tyrosinase-positive cells with dendritic morphology were visible in surface fish and cavefish scales. The results show that surface fish and Pachón cavefish scales have a population of tyrosinase-positive melanoblasts.

Similar experiments were conducted with tail fin explants (Fig. 4, G–N). Surface fish contain abundant melanophores located on the surface and lateral sides of the fin rays, but the interray regions are almost devoid of these cells (Fig. 4G). Some of the fin melanophores showed elaborate cell processes, whereas others lacked melanosome-containing processes, and pigment deposition appeared to be attenuated to their central cores. In contrast, Pachón cavefish fins lacked melanophores (Fig. 4H), although both cave and surface forms had xanthophores (Fig. 4, G, I, and J). Tyrosinase assays of cavefish fin explants with L-dopa substrate resulted in the appearance of tyrosinase-positive cells: some cells were

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**Fig. 1.** DiI labeling showing cell migration from the neural keel region into the trunk and yolk sac of Pachón cavefish embryos. (A) Bright-field image of a 48-hpf embryo 1 day after DiI injection. (B, C) Fluorescence images of the same DiI-injected embryo as in A. (B) Low-magnification image showing the DiI bolus (DiI) and some of the migratory cells extending in rostral, caudal, and ventral directions from the injection site (C, inset in B). Higher magnification image showing DiI-labeled migratory cells (arrows) in the trunk. (D) Higher magnification image of a 48-hpf embryo showing DiI-labeled migratory cells (arrows) in the yolk sac. Scale bars: 20 μm in A; magnification is the same in B and approximately two times in C and D.

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**can be stained** (Laidlaw 1932; Tucker and Erickson 1986). Therefore, it was essential to determine whether the cavefish tyrosinase-positive cells were derived from the neural crest and thus of the melanophore lineage.
present in and along the fin rays but most were localized in the interray regions (Fig. 4I). Although many of these cells were dendritic (Fig. 4I, inset), they did not show the stellate mor-

Fig. 2. Melanophores and tyrosinase-positive cells in surface fish and cavefish embryos. (A, B) Tyrosinase-positive cells are present around the eye and in the yolk sac (arrows) in 36-hpf surface fish (A) and cavefish (B) embryos. (C, D) Control surface fish (C) and cavefish (D) embryos at the same stage of development as A and B showing lack of melanophore development around the eye and yolk sac. Note that naturally melanized cells (arrowhead) have started to appear in the trunk of the surface fish (C) but not the cavefish (D) embryo. (E–H) Distribution of tyrosinase-positive cells during Pachón cavefish development. (E) 24 hpf. (F) 32 hpf. (G) 60 hpf. (H) 84 hpf. Scale bar: A, 40 μm; magnification is the same in A–H. (I) A section through the trunk region of a 36-hpf cavefish embryo showing tyrosinase-positive cells lying under the epidermis in the normal position of dermal melanophores. Scale bar: I, 80 μm. (J) Higher magnification image showing dendritic morphology of tyrosinase-positive cells in a 36-hpf Pachón cavefish embryo. Scale bar: J, 25 μm.

Fig. 3. Double-labeling and neural keel explant experiments showing that tyrosinase-positive cells are derived from the neural crest. (A–D) Embryonic cells labeled for DiI and tyrosinase. Fluorescence (A, C) and bright-field (B, D) images showing cells labeled with DiI (red) and melanin (black) in the trunk (A, B) and yolk sac (C, D) of 36-hpf Pachón cavefish embryos. Scale bars: A, 100 μm; magnification is the same in A–D. (E–G) Cultured explants of neural keels from surface fish (E) and cavefish (G–I) embryos showing outgrowing cells. Note that isolated neural keels curl into spheres in culture. Outgrowing cells differentiate into melanophores in surface fish (E, arrows) but not Pachón cavefish (F, H, arrows) explants. (G, I) Outgrowing cells in the same Pachón cavefish explant as F can produce melanin after tyrosinase assay (arrows). H and I represent higher magnifications of the insets shown in F and G, respectively. Scale bar: E, 100 μm; magnification is the same in E–G.
tyrosinase inhibitor 1-phenyl-2-thiourea before supplying L-dopa substrate prevented the appearance of melanized cells (Fig. 4J). Thus, cavefish fins also contain tyrosinase-positive cells.

Macrophages in the interray regions of surface fish and cavefish fin explants could also be detected by the tyrosinase assay (Tucker and Erickson 1986). This possibility was tested in two ways. First, we conducted tyrosinase assays on fin explants from an albino catfish, which did not reveal any tyrosinase-positive cells, suggesting that the tyrosinase-positive cells detected in cavefish are not macrophages. Second, we determined the distribution of macrophages in fin explants directly by neutral red staining (Fig. 4M), and the results were compared with the distribution of tyrosinase-positive cells in the same explant (Fig. 4N). No overlap was observed between melanin-containing cells and the neutral red-staining cells, excluding the possibility that the tyrosinase-positive cells are macrophages.

Tyrosinase assays of Pachón cavefish fin explants were also conducted using l-tyrosine, the metabolic precursor of L-dopa, as a substrate. The results showed that fin melanoblasts could not synthesize melanin using exogenous l-tyrosine (Fig. 4K). l-Tyrosine did not serve as a competitive

Fig. 4. Tyrosinase-positive cells in adult scales and tail fin explants. (A–F) Scales. (A, B) Surface fish (A) and Pachón cavefish (B) scales showing melanophores (upward pointing arrows) and iridophores (downward pointing arrows). Melanophores are absent in cavefish scales. Scale bar, 100 µm; magnification is the same in A and B. (C, D) Higher magnification images (approximately four times of A and B) of control surface fish (C) and cavefish (D) scales. Arrowhead tips indicate the scale edges, where large melanophores are located in surface fish but absent in cavefish. (E, F) Higher magnification images (approximately four times of A and B) of surface fish (E) and cavefish (F) scales after tyrosinase assay showing the presence of melanized cells (arrows) of dendritic morphology (inset in F). Arrowhead: edges of scales and melanophore (in E). (G–N) Tail fin explants. (G) Surface fish tail fin explant showing melanophores (arrowheads) distributed on the surface and along the bases of the fin rays but not in the interray regions. (H) Pachón cavefish tail fin explant showing the absence of melanophores. (I) Pachón cavefish tail fin explant assayed with L-dopa substrate showing tyrosinase-positive cells (arrows) distributed on the surface and along the bases of the fin rays but primarily in the interray regions. Inset: higher magnification image (approximately 10 times of I) showing the dendritic morphology of tyrosinase-positive cells. (J) Pachón cavefish tail fin explant assayed as in I but preincubated with the tyrosinase inhibitor 1-phenyl-2-thiourea before L-dopa and showing no tyrosinase-positive cells. (K) Pachón cavefish tail fin explant assayed as in I except with l-tyrosine instead of L-dopa substrate and showing no tyrosinase-positive cells. (L) Tail fin explant from the albino catfish C. aeneus assayed as in I showing absence of tyrosinase-positive cells. X, orange xanthophores are present in both surface fish and cavefish fins. Scale bar: G, 100 µm; magnification is the same in G–L. (M, N) Controls comparing the distribution of macrophages and tyrosinase-positive cells in Pachón cavefish tail fin explants. (M) Neutral red-stained macrophages in an interray of a tail fin explant. (N) The same tail fin explant as in M after tyrosinase assay showing nonoverlapping distribution of macrophages (red) and tyrosinase-positive cells (black, arrows), which are slightly out of focus because they reside in a tissue layer internal to the macrophages. Scale bar: M, 75 µm; magnification is the same in M and N.
inhibitor of tyrosinase activity because supplying of this substrate along with low concentrations of L-dopa (0.01%) to the same fin explants resulted in the appearance of melanin-containing cells (data not shown). These experiments were repeated with cavefish embryonic neural keel explants with the same results: the outgrowing cells were able to use l-dopa but not l-tyrosine as a substrate to synthesize melanin (data not shown). It is concluded that melanoblasts with potential to synthesize melanin when supplied with the exogenous substrate L-dopa, but not its precursor L-tyrosine, are present in adult Pachón cavefish.

Melanoblasts and melanophores in different cavefish populations

Chica, Los Sabinos, Tinaja, and Curva cavefish show differing degrees of melanophore differentiation (Wilkens 1988). To determine whether melanoblasts also accumulate in these cavefish, tyrosinase assays were done on tail fin explants and compared with those of Pachón cavefish. Tyrosinase-positive cells were observed in the tail fins of each cavefish population (Fig. 5). We next investigated the ratio of melanophores and melanoblasts in these cavefish. The number of melanophores was counted in a specific area within or bordering a fin ray in each explant before tyrosinase assay. Then, the number of tyrosinase-positive cells was counted in a comparable area of the same fin explant after the tyrosinase assay was completed. To distinguish between natural and l-dopa-induced melanized cells, tyrosinase-positive cells were counted only in the interray areas, which lack melanophores (Fig. 4G). Although this underestimates the total number of tyrosinase-positive cells, it allows unambiguous determination of the relationship between the two cell types.

The results showed that the number of tyrosinase-positive melanoblasts is increased in every cavefish population relative to surface fish (Fig. 6). In addition, an inverse relationship was observed between the number of melanophores (in the cavefish populations that have them) and melanoblasts (Fig. 6). Surface fish have a melanoblast to melanophore ratio of about 1:2. The ratio is reversed in Chica, Tinaja, and Los Sabinos cavefish, which have about half the number of the melanophores as surface fish. In contrast, Curva cavefish, which show the greatest reduction in melanophores among the cavefish populations with pigmentation, have a melanoblast to melanophore ratio of almost 8:1 (Fig. 6). Finally, Pachón cavefish, which have no melanophores, exhibited fewer melanoblasts than any other cavefish population, although there were more of these cells than in surface fish (Fig. 6). These results suggest that melanoblasts accumulate in numbers proportional to differentiated melanophores in Chica, Tinaja, Los Sabinos, and Curva cavefish. In Pachón cavefish, the number of melanoblasts is reduced in the absence of melanophore differentiation.

DISCUSSION

We studied the developmental mechanisms responsible for pigment cell regression in cavefish. Our results indicate that
Pigment cell development is blocked at a late step in melanogenesis after neural crest cells delaminate from the neural keel, migrate into the body wall, and are specified as tyrosinase-expressing melanoblasts. Because of the expression of functional tyrosinase enzyme, cavefish melanoblasts have the ability to convert exogenous L-dopa to melanin. However, they cannot convert L-tyrosine to L-dopa, although tyrosinase also catalyzes this reaction, suggesting a role for tyrosine accessibility in arrest of melanophore differentiation. This study provides a foundation for understanding the regressive evolution of melanogenesis through examination of developmental changes within surface-dwelling and cave-adapted forms of a single species exhibiting multiple pigment phenotypes.

Several lines of evidence indicate that cavefish exhibit an abundant population of melanoblasts derived from the neural keel, migrate into the body wall, and are specified as tyrosinase-expressing melanoblasts. Because of the expression of functional tyrosinase enzyme, cavefish melanoblasts have the ability to convert exogenous L-dopa to melanin. However, they cannot convert L-tyrosine to L-dopa, although tyrosinase also catalyzes this reaction, suggesting a role for tyrosine accessibility in arrest of melanophore differentiation. This study provides a foundation for understanding the regressive evolution of melanogenesis through examination of developmental changes within surface-dwelling and cave-adapted forms of a single species exhibiting multiple pigment phenotypes.

Several lines of evidence indicate that cavefish exhibit an abundant population of melanoblasts derived from the neural crest. First, Dil-labeling experiments showed that neural crest cells delaminate and migrate along their stereotypic pathways in the trunk region of cavefish embryos. The presence of a subset of cavefish neural crest cells devoted to pigment cell development is consistent with the normal differentiation of iridophores and xanthophores in cavefish, which are also derived from the neural crest (Erickson 1993; LeDouarin and Kalcheim 1999). Second, histochemical assays demonstrated that dermal cells contain functional tyrosinase and are able to produce melanin when L-dopa is supplied as a substrate in both cavefish embryos and adults. Tyrosinase activity was also detected in dermal cells of pigmented surface fish before the differentiation of melanophores. The appearance of active tyrosinase much earlier than melanophore differentiation has also been reported in ascidian (Whittaker 1973), amphibian (Epperlein and Lofberg 1990), and zebrafish (Camp and Lardelli 2001) embryos, suggesting that this phenomenon is widespread. Third, tyrosinase-positive cells were first detected during cavefish embryogenesis in the same location as developing melanophores in surface fish embryos. Fourth, tyrosinase-positive melanoblasts arise from the cavefish neural keel (teleost neural tube), the source of all body pigment cells in vertebrate embryos. This important point was demonstrated by experiments in which Dil-labeled neural crest cells were shown to express tyrosinase after their migration into the body wall and by the outgrowth of tyrosinase-positive cells from cultured neural keel explants. Finally, in five different cavefish populations, melanoblasts accumulate to levels substantially above those of surface fish. These results suggest that a late block in the melanophore differentiation pathway has evolved repeatedly during the evolution of different cavefish populations.

We conclude that the early steps of pigment cell development, including neural crest cell specification and migration, melanophore determination, and tyrosinase expression, are functional in cavefish. Tyrosinase catalyzes three consecutive steps of the melanin biosynthetic pathway: the conversion of L-tyrosine to L-dopa, L-dopa to dopa quinone, and 5,6-dihydroxyindole (the spontaneous reaction product of dopa quinone) to indole-5,6 quinone (Korner and Pawelek 1982). Therefore, the reactions of the melanogenic pathway that operate downstream of tyrosinase, including those catalyzed by tyrosinase-related protein-1 (dopa chrome-tautomerase) and tyrosinase-related protein-2 (DHICA oxidase) (Tsukamoto et al. 1992; Kobayashi et al. 1994), must be operational in cavefish. Likewise, a lesion in the melanogenic pathway before the conversion of L-tyrosine to L-dopa is also unlikely because inability to convert the essential amino acid L-phenylalanine to L-tyrosine, the immediate precursor of L-dopa, would confer lethality. Therefore, we suggest that melanogenesis may be blocked because of sequestration of L-tyrosine from the melanin-synthesizing cascade rather than because of a missing component. This suggestion is consistent with our demonstration that cavefish can use exogenous L-dopa but not L-tyrosine to produce melanin. Many years ago, Whittaker (1973) suggested that melanin deposition in the sensory cells of ascidian embryos might be regulated by tyrosine accessibility. Likewise, a permanent block in tyrosine accessibility seems to have occurred during cavefish evolution.
Dorsky et al. (2000) showed that the local environment is an important determinant of neural crest cell differentiation in vertebrate embryos. Our ability to culture undifferentiated melanoblasts in neural keel explants may assist in determining whether a critical extracellular signal is modified or lacking in cavefish that would normally release L-tyrosine as a tyrosinase substrate and produce differentiated melanophores.

Pigment cell regression in cave animals has been proposed to be an evolutionary consequence of the accumulation of neutral mutations under conditions of relaxed selection (Culver 1982; Wilkens 1988). Accordingly, a gene controlling a late step in a relic metabolic pathway, such as the reactions that produce melanin from L-dopa, is expected to be more tolerant to genetic drift as long as it is not required for another process in development. In contrast, loss of a gene(s) that functions in earlier steps, such as in the specification or migration of neural crest cells, could not be permitted because of its critical pleiotropic role in development. However, the neutral theory would not adequately account for the conservation of tyrosinase, assuming that its function is restricted to melanogenesis or that other pigment cell types (iridophores and xanthophores) do not seem to show regression in cavefish that would normally release L-tyrosine as a tyrosinase substrate and produce differentiated melanophores.

In conclusion, we show that cavefish embryos and adults have retained a population of neural crest-derived melanoblasts, despite the loss or reduction of melanophores. Thus, the early developmental mechanisms governing melanogenesis are functional in cavefish and loss or reduction of pigment cells must be due to a late change in the melanin synthesis pathway. Our results indicate that this step may be related to the conversion of L-tyrosine to L-dopa. Astyanax has provided an excellent model to study the molecular and developmental mechanisms responsible for pigment cell regression and to compare evolutionary changes in melanogenesis.

Acknowledgments

We thank Deidre Heyser for technical assistance. This work was supported by NSF grants (DEB-9726561 and IBN-0110275) and an NIH grant (EY014619) to W. R. J. The comments of an anonymous reviewer greatly assisted us in preparing the final version of this manuscript.

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