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ARTICLE

Pigment Cell Organization in the Hypodermis of Zebrafish

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Zebrafish have a characteristic horizontal-stripe pigment pattern made by a specific distribution of three types of pigment cells: melanophores, xanthophores, and iridophores. This pattern is a valuable model to investigate how the spatial patterns form during animal development. Although recent findings suggest that the interactions among the pigment cells play a key role, the particular details of these interactions have not yet been clarified. In this report, we performed transmission electron microscopic study to show the distribution, conformation, and how the cells contact with each other in the hypodermis. We found that the pigment cells form complex but ordered, layered structures in both stripe and interstripe regions. The order of the layered structures is kept strictly all through the hypodermal regions. Our study will provide basic information to investigate the mechanism of pigment pattern formation in zebrafish. *Developmental Dynamics 227:497–503, 2003.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

In vertebrates, pigment cells are derived from neural crest cells, a transient embryonic cell population that emerges from the dorsal neural tube, migrates by means of specific pathways, and differentiates into a variety of cell types, including pigment cells (Hall, 1999; Le Douarin and Kalcheim, 1999, and references therein). Many animals have characteristic color patterns, which are composed of pigment cells (Nordlund et al., 1998). Mechanisms of how vertebrate pigment patterns are formed have been discussed for centuries (Goodrich and Nichols, 1931; Twitty, 1936; Meinhardt, 1982; Jackson, 1994). In recent years, the zebrafish (*Danio rerio*), a teleost, has become a valuable model animal in many research fields (Jesuthasan, 2002), especially in developmental biology. As the name of the organism suggests, its pigment pattern consists of an orderly arrangement of horizontal stripes. The existence of patterning mutants isolated from a large-scale screen (Haffter et al., 1996a,b; Kelsh et al., 1996), and recently developed molecular genetic techniques make the zebrafish an ideal experimental system to investigate the mechanism of pigment pattern formation.

Recently, several genes that cause the altered pigment pattern have been isolated. From the phenotypic analysis, each of them is supposed to be involved in ontogeny of pigment cells, migration, survival, specification, or differentiation (Johnson et al., 1995; Lister et al., 1999; Parichy et al., 1999, 2000a,b; Rawls et al., 2001; Quigley and Parichy, 2002; Parichy and Turner, 2003). For example, *panther* mutant that shows spot-like pattern of melanophores has defect in *c-fms*, which encodes a type III receptor tyrosine kinase (Parichy et al., 2000b). With a temperature-sensitive allele of *panther* mutant, Parichy and Turner (2003) showed that the *c-fms* is required for the development of xanthophores and the maintenance of melanophores. Another example is *c-kit*. In the embryo of *c-kit* mutant, melanophores largely fail to migrate and subsequently undergo programmed cell death (Parichy et al., 1999). Eventually, the stripe formed contains a fewer number of melanophores and the border is relatively ambiguous (Johnson et al., 1995). In the regenerating fin, *kit* is essential

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for the survival of newly differentiated melanophores (Rawls and Johnson, 2001). These studies suggest that interaction among different types of pigment cells is important in the formation of pigment patterns. Detailed study of the distribution and conformation of each class of pigment cell in respect to this striped pattern will aid in understanding how these cells interact with each other.

To date, the organization of pigment cells into the stripe pattern is understood as follows. Dark stripes consist of melanophores (black cells containing melanin granules) and iridophores (silvery and/or white cells containing ultrafine reflecting platelets). Light stripes, also referred to as interstripe; consist of xanthophores (yellow to orange cells containing pteridine and carotenoid granules) and iridophores (Kirschbaum, 1975; Johnson et al., 1995; Elliott, 2000; Parichy et al., 1999, 2000b).

Some features that are easily observed by light microscopy are not well understood on a cellular level. For example, the interstripe region can be divided into two subregions with different appearances when illuminated from a specific angle (Fig. 1C,D). The stripe region is usually recognized as composed of only melanophores. However, the stripe region contains scattered bronze-colored spots (Fig. 1B–D), which turn out to be xanthophores on melanophores (see Results and Discussions section). Xanthophores in the stripe region are more obvious in *albino* mutant (Fig. 1A). (It can also be conceivable that the xanthophores in the stripe region occur by the *albino* mutation. However, the number of xanthophores in the fins is not changed in *albino* mutant.) These findings suggest that the striped pigment pattern of zebrafish is more complex than the current understanding. Clearly, more detailed analysis is needed to elucidate the organization of pigment cells with respect to the stripe pattern. To provide basic information to investigate the mechanism of pigment cell patterning, we have performed a detailed study of the morphology and conformation of various pigment cell components in zebrafish skin using transmission electron microscopy.

RESULTS AND DISCUSSIONS

Binocular Dissecting Microscopic Observation

We began our analysis of the pigment cells in zebrafish stripes with gross observation by using a binocular dissecting microscope. All classes of pigment cells were distributed also in the epidermis over the scales. The pigment cells within the overlapping epidermal tissues, however, were not organized in the stripe pattern (Kirschbaum, 1975); therefore, we focused our observation on pigment cells around the dermal region. In stripe regions, we can observe black dendritic cells (melanophores), reflecting silver plates (iridophores), and some sparsely distributed blue- or bronze-colored spots.

In interstripe regions, yellow- to orange-colored spots created by xanthophores are distributed ubiquitously, but two qualitatively different subregions were found that differ in their transparency. The middle portions of the interstripe region exhibit a relatively dull iridescence (Fig. 1C,D). On the other hand, the edges of this region appear shiny or transparent, depending on the angle of illumination (Fig. 1C,D). It was impossible to identify the cellular structure underlying these apparent differences in coloration.

Light Microscopic Observation Around Zebrafish Dermal Region

Patterns of scalation are diverse among fish (Elliott, 2000). In zebrafish, the epidermis covers the imbricate scales, whose tips lie in small pockets of dermal tissue (Fig. 2A). Transverse histologic sections revealed the distribution of melanophores in the hypodermal region, just beneath the dermis (Fig. 2A,C).

Melanophores did show clear localization in the stripe region of hypodermis (Fig. 2A). However, we could not detect xanthophores or iridophores in this region by light microscopy, although the shining light spots and background reflection are observed by dissecting microscopy. In the hypodermis of interstripe regions, whitish-colored cells, which may consist of iridophores, were observed (Fig. 2A,B). This layer is thicker in the middle portions of interstripe regions and thinner at their edges (Fig. 2C). Again, we could not identify xanthophores by histologic observation using their characteristic orange color as a criterion, even in unstained sections (data not shown).

Identification of Each Pigment Cell Type by Transmission Electron Microscopy

Although no detailed ultrastructure-based analyses on pigment cells have yet been reported in zebrafish, the ultrastructures of the various classes of pigment cell have been described in other teleost species (Roberts et al., 1971; Hawkes, 1974; Fujii et al., 1989; Goda et al., 1994; Goda and Fujii, 2001). We have used these descriptions to help in the identification of these pigment cell types around the dermal region of the zebrafish.

Melanophores.

The most common class of fish pigment cell is the melanophore, which possess round or slightly ellipsoid electron-dense granules known as melanosomes, which contain deposited melanoprotein (Fujii, 1993; Elliott, 2000). Transmission electron microscopy (TEM) revealed a clear distribution of melanophores in the stripe region of the zebrafish hypodermis (Fig. 3C). Size of the melanosomes in zebrafish looks almost uniform ($\sim$0.5 μm in diameter). They are always packed densely in the cytoplasm of melanophores.

Iridophores.

Iridophores contribute to white- or silver-colored regions. They contain uniformly spaced, flat-appearing reflective crystalline platelets. Each platelet is composed primarily of guanine but may also contain other purines such as hypoxanthine, adenine, and uric acid (Fujii, 1993; Le Douarin and Kalcheim, 1999). They usually appear as stacks of empty space in the cytoplasm due to loss of their contents during tissue prep-

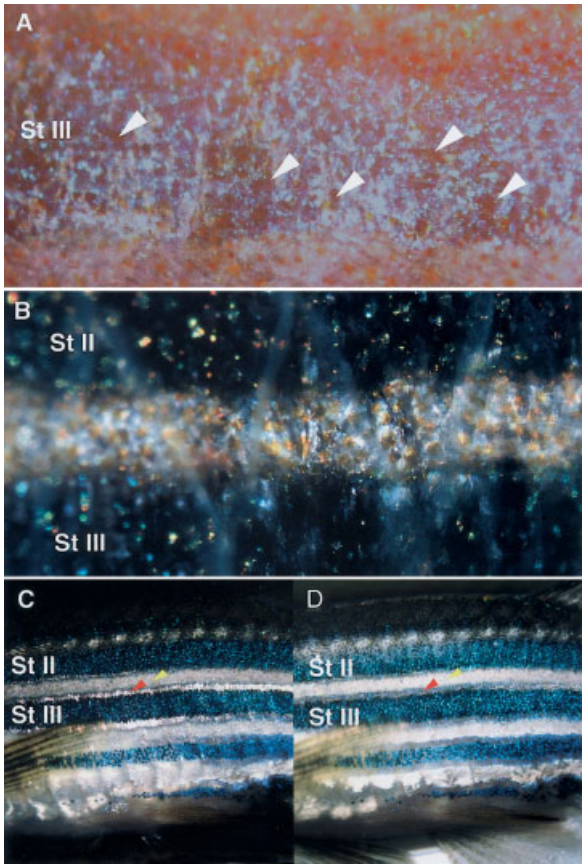

Fig. 1.

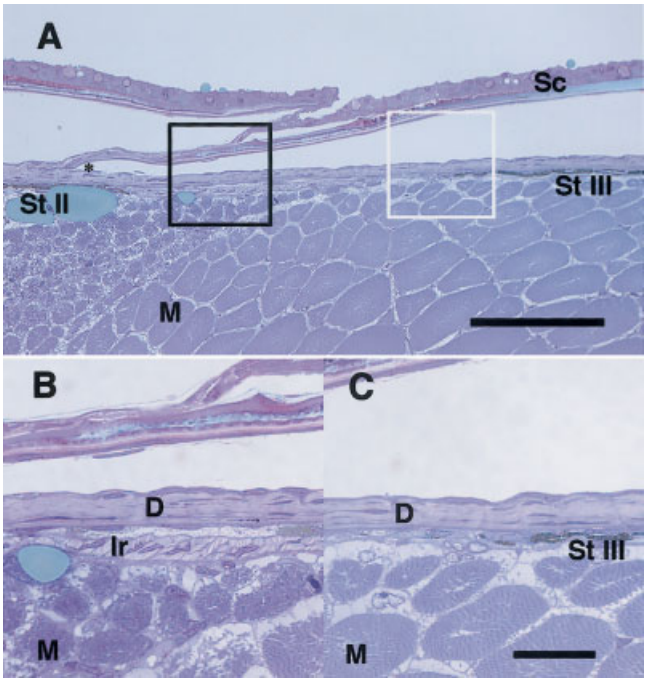

Fig. 2.

Fig. 1. Pigment cells in the zebrafish trunk. **A:** *Albino* mutant. Xanthophore populations (arrowheads) distributed in the third stripe (St III). **B:** Wild-type. Pigment cells in the second (St II) and third (St III) stripes. **C,D:** Two subregions corresponding to the middle and edges (yellow and red arrowheads, respectively) of the interstripe region between the second (St II) and third (St III) stripes. Note that the iridescence depends on the angle of illumination. Anterior is to the left.

Fig. 2. Pigment cells in the hypodermis of the zebrafish lateral trunk. **A:** Transverse section through the second (St II) to the third (St III) stripes. Black and white boxes indicate the areas of higher magnification shown in B and C. The asterisk indicates small numbers of xanthophores (X) observed in a small pocket of dermal tissue, where the tip of the scale lies. **B:** Higher magnification image of black-boxed area in A, corresponding to the middle of the interstripe region between the second and third stripes. **C:** Higher magnification image of white-boxed area in A, corresponding to the dorsal part of the third stripe and the interstripe edge. D, dermis; Ir, iridophores; Sc, scale; M, muscle. Scale bars = 10 μm in A, 2 μm in C (applies to B,C).

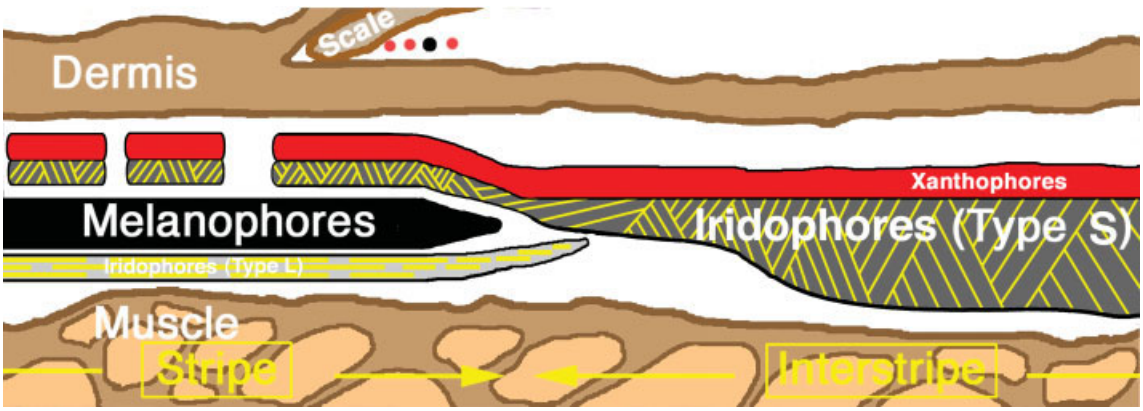

Fig. 4. Summary of pigment cell distribution, in relation to the stripe pattern.

aration, sectioning (Taylor, 1969; Bagnara et al., 1979), and the damage by electron during TEM observation (Matsumoto, 2001). In our study, iridophore platelets mainly were found as stacks of empty space, although we did sometimes observe iridophores containing platelets surrounded by unit membrane, which may represent more intact condition (Fig. 3E). We classified these iridophores into two types that differ in the size of the platelets contained therein. Type L iridophores contain a quite large ($\sim$10 μm) but smaller number ($<$20) of platelets lying parallel to the dermis (Fig. 3C, Ir L), which make the mirror-like reflection observed by binocular microscopy. Type S iridophores contain uniform-sized (2 μm) and large number ($>$50) of platelets (Fig. 3A–C, Ir S), which cause variety of appearances depending on the angles relative to skin surface.

Xanthophores.

We observed cells in the uppermost layer of the hypodermis that contain low electron-density chromatosomes surrounded by unit membrane, which is similar to those in cells previously reported as xanthophores (Hawkes, 1974, Kirschbaum, 1975; Fig. 3A–D). We thereby designated these cells as xanthophores. The maximum size of the chromatosome in a xanthophore (xanthosome) is as large as in a melanosome. However, the shape and the size of xanthosomes are less uniform compared with melanosomes. Occasionally, we found xanthophores that contain few xanthosome sparsely packed in the cytoplasm. We were unable to identify these cells by histologic observation by light microscopy, perhaps because xanthophore pigments, which mainly consist of carotenoids and pteridines, which are, respectively, fat- and water-soluble (Le Douarin and Kalcheim, 1999), may be lost during sample preparation. Indeed pigments themselves have been lost in a greater or less degree, but characteristic fine structures of pigment granules have been kept. Therefore, we could identify xanthophores as cells with characteristic granules by TEM observation (Fig. 3A–D). The layered structure of pigment cells, which corresponds to the zebrafish pigment pattern, is summarized in Figure 4.

Pigment Cell Organization in the Interstripe Region

Iridophores and xanthophores were observed ubiquitously and always in coupling with one another, in the hypodermis of the interstripe region (Fig. 3A,B). Xanthophores were found exclusively between the stratum compactum of the dermis and the iridophore layer, which lies just above the muscular layer. Although all the iridophores locate in the interstripe region have small reflecting platelets (type S), we observed regional differences with respect to the orientation of the platelets they contain. In the middle interstripe regions, the layer of iridophores is thick and contains multilayered platelets that face in a variety of directions (Fig. 3A). Iridophores at the interstripe edges are thin and contain platelets lying subparallel to the dermis (Fig. 3B). Such conformational differences in the reflecting platelets of iridophores explain the differences in appearance of these subregions observed by binocular microscopy (Fig. 1C,D). Type L iridophores protrude from stripe region into interstripe edge region (Fig. 4), which cause the mirror-like reflection. On the type L iridophores, type S iridophores locate. The platelets of the type S iridophores in this region are mutually parallel and the angle against the skin plane is small (Fig. 3B) in comparison to those in middle interstripe region (Fig. 3A). (In this region, 74% of type S iridophore platelet arranged at the angle less than 30 degree to the dermis; 32% in the stripe region.) Such arrangement may enhance the mirror-like reflection. When the illumination is from other angles, iridophores in this region appear transparent and only the xanthophores were visible. On the other hand, platelets in iridophores in the middle interstripe region are piled up in a variety of angles, so that the gross appearance of this region does not change with the angle of illumination.

Pigment Cell Organization in the Stripe Region

We have observed, all three classes of pigment cells forming an unexpectedly complex layered structure in the hypodermis (Fig. 3C). In the most superficial hypodermal layer, immediately below the dermis, xanthophores were observed. Just below this layer of xanthophores, type S iridophores are always located (Fig. 3A,B). We have never observed each of the cells solely or stacking in the opposite order. We found the stacking of xanthophore and iridophore (type S) almost ubiquitously in this region, although the density of xanthophores in the stripe region is apparently lower than that in the interstripe region when observed by dissecting microscope. This apparent discrepancy could be explained by the amount of yellow pigment possessed in the xanthophores in different regions. However, we do not have quantitative data to confirm this hypothesis.

Just beneath the layers of iridophores and xanthophores, a layer of melanophores was observed (Fig. 3C). They showed a highly ubiquitous distribution throughout the stripe regions.

At the deepest layer of the hypodermis, just above the skeletal muscle, another type of iridophore (type L) was observed (Fig. 3C). This type of iridophore has characteristic morphologic features distinguishing them from iridophores in other regions (Fig. 3A–C). Type L iridophores, at a thickness of only several sheets of platelets, contain quite large platelets lying parallel to the dermis, making the mirror-like reflection observed by binocular microscopy. No type L iridophores have been observed in contact with xanthophores.

Blue (Fig. 1B–D) and bronze (Fig. 1B) iridescent spots observed in the stripe region can be explained as follows. Goda et al. (1994) described the morphologic features of blue-colored regions in surgeonfish (*Paracanthurus hepatus*) and have shown that clusters of iridophores together with underlying melanophores generate both light and dark blue colorations. When the overlying iridophore contains orderly stacked

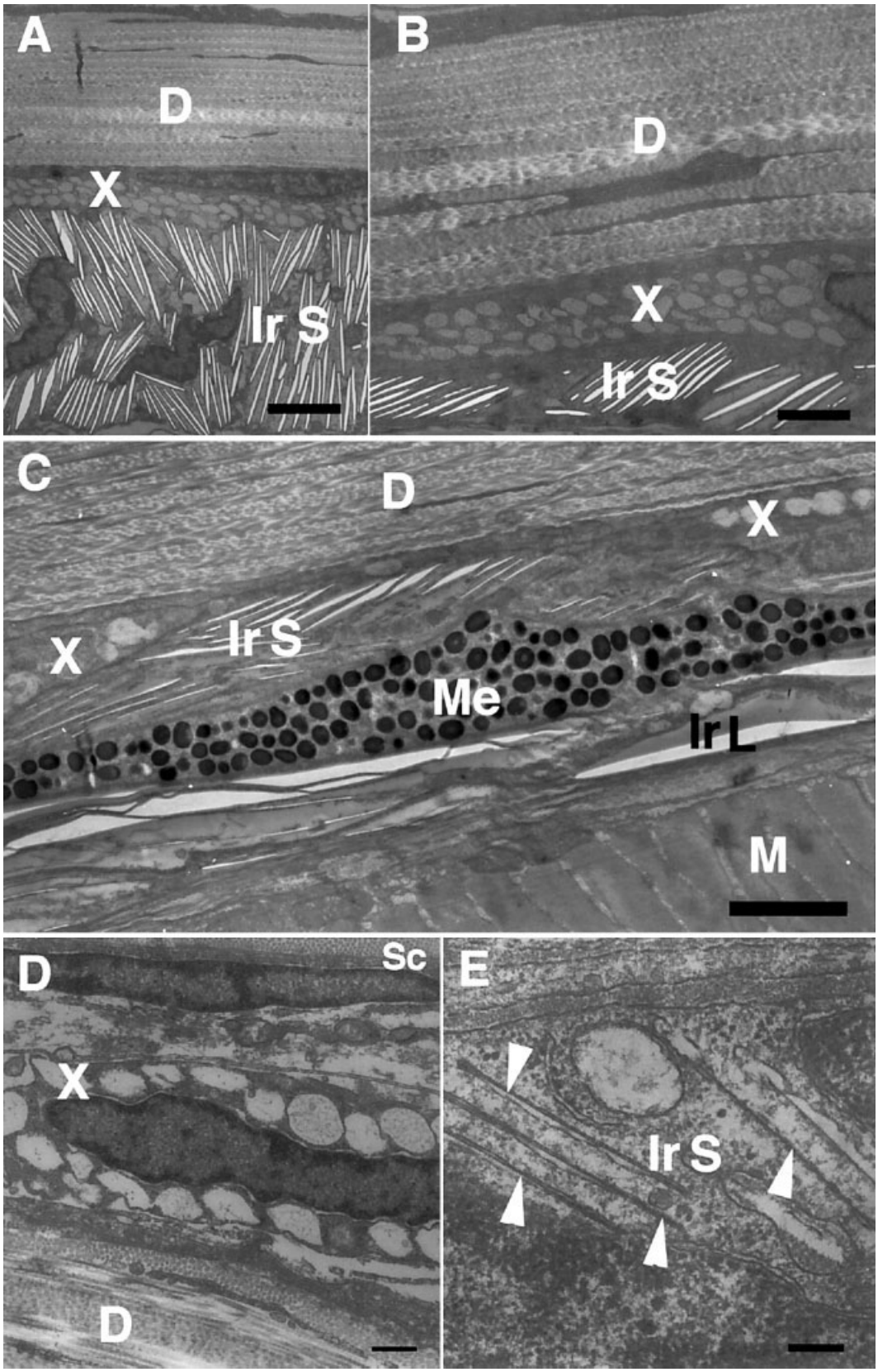

Fig. 3. Transmission electron photomicrographs of pigment cells around the dermis. **A:** Hypodermal pigment cells in the middle interstripe region between the second and third stripes. Type S iridophores (Ir S) and xanthophores (X) are observed ubiquitously. **B:** Hypodermal pigment cells in the interstripe edge. Iridophores (type S, Ir S) and xanthophores (X) are concomitant with each other as observed in A. Note that small reflecting platelets are observed in the horizontal axis (i.e., subparallel to the dermis). **C:** Hypodermal pigment cells at the level of second stripe. Xanthophores (X), type S iridophores (Ir S), melanophores (Me), and type L iridophores (Ir L) are seen. **D:** Small numbers of xanthophores (X) observed in a small pocket of dermal tissue, where the tip of the scale (Sc) lies (correspond to the position of asterisk in Fig. 2A). **E:** Reflecting platelets (arrowheads) observed in type S iridophores of the second stripe. Scale bars = 2 μm in A,B, 1 μm in C,D, 500 nm in D, 200 nm in E.

platelets, it appears light blue. When the iridophore contains fewer, randomly oriented platelets, however, it appears dark blue (Goda et al., 1994). In the stripe regions of the zebrafish trunk, type S iridophores were found to overlie the layer of melanophores; this combination may generate the blue-colored spots. TEM revealed that xanthophores always overlie type S iridophores. The color of the iridescent spots may be dependent on the amount of yellow to orange pigment (carotenoids and pteridines) contained by the xanthophore. When the xanthophore contains fewer yellow and orange pigments, the spot looks blue; when much is contained, the spot appears bronze.

Recently, an interaction between xanthophores and melanophores has been demonstrated by using the temperature-sensitive allele of the *panther* gene (Parichy and Turner, 2003). *panther* gene codes the c-fms protein, a homologue of the CSF-1 receptor in mouse. In the zebrafish skin, *panther* gene is expressed only in xanthophore and is necessary to maintain the cell type. However, when the mutant fish lose the *panther* gene activity, melanophores, which do not express *panther* gene, disappear soon after xanthophores are lost. This finding shows that melanophores need some molecular stimulation released by xanthophores to be in hypodermis steadily. From the layer order found in this TEM study, it appears that iridophores preclude direct interactions between xanthophore and melanophore, suggesting that the interaction between xanthophore and melanophore is mediated by soluble substances or by means of iridophores.

Our TEM observation revealed that the organization of the pigment cells in the zebrafish, which provides basic information for the study of pattern formation in the zebrafish. We found that the vertical (i.e., the direction perpendicular to the skin) order of pigment cells in each region is kept strictly throughout the entire hypodermis. We never observed two different types of pigment cells stacking in opposite order or a pigment cell lying upon the same class of pigment cells. To form and to maintain such a strict order in the pigment cell conformation, active interactions between pigment cells must be required. We suppose such interactions may also have roles in the horizontal pattern of the pigment cells. Therefore, studying the pigment cell organization in the mutant fish that have altered pigment pattern is of great interest.

EXPERIMENTAL PROCEDUES

Experimental Animals

Wild-type zebrafish (*Danio rerio*) were obtained from the zebrafish stock center of the Max Planck Institute (Tübingen). They were kept and bred in the laboratory aquarium as described in "The Zebrafish Book" (Westerfield, 1993). Just before experiments, fish were anesthetized with a 1/10,000 dilution of FA 100 (Tanabe Seiyaku, Japan).

Observation by Binocular Dissecting and Light Microscope

Binocular dissecting microscope observations were carried out by using a Nikon SMZ 1500 microscope, and photographs were taken by using a Nikon DXM 1200 digital still camera. Histologic sections were made in two ways. In the first, Alcian blue (pH 2.5) and hematoxylin and eosin were used to stain traditional histologic sections based on the procedures in Hirata et al. (1997, 1998) with one modification; Alcian blue staining was performed for 30 min in this study. In the second, toluidine blue-stained semithin sections were made as described in the next section. The two procedures produced indistinguishable results with respect to the morphology of pigment cells (data not shown). More than 40 fish were examined by light microscopy.

Transmission Electron Microscopy

TEM analysis was based on Toma et al. (1999). Experimental fish were immersed in a mixture of 3% paraformaldehyde and 3% glutaraldehyde in a 0.1 M cacodylate buffer. For some specimens, 1% acrolein (Tokyo Kasei, Japan) was added to this fixative for better tissue preservation (Kalt and Tandler, 1971). Specimens were cut into small pieces, immersed in the same fixative for 2 hr at room temperature, and post-fixed in 2% osmium tetroxide for 2 hr at 0°C. They were stained en bloc with 2% uranyl acetate in 50% methanol for 2 hr and then embedded in Epon 812 resin (TAAB, USA) after dehydration. Ultra-thin and semi-thin sections were cut by using an Ultracut N microtome (Reichert Nissei, Austria). Semithin sections (cut at a thickness of 500 nm) were used for histologic observation after toluidine blue staining, as mentioned above. More than 20 fish were examined in TEM analysis.

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