RESEARCH ARTICLE

Pigment Cell Distributions in Different Tissues of the Zebrafish, With Special Reference to the Striped Pigment Pattern

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The orderly pigment pattern of zebrafish (*Danio rerio*) is a good model system for studying how spatial patterns form in animals. Recent molecular genetic studies have shown that interactions between the pigment cells play major roles in pattern formation. In the present study, we performed comparative transmission electron microscopy of pigment cells, in order to clarify the structural interactions of pigment cells in tissues with and without a striped pattern. In patterned tissues, pigment cells were distributed as a one-cell-thick sheet. The layer order of the sheets is always kept strictly. In tissues without a striped pattern, the layer order was often disturbed or the cells were distributed in a scattered, double-sheeted, or an accumulated pile. Our observations suggest that the underlying mechanism that controls the vertical order of the pigment cells is related to that controlling the stripe pattern. *Developmental Dynamics 234: 293–300, 2005.* © 2005 Wiley-Liss, Inc.

Key words: zebrafish; pigment cells; pigment pattern; neural crest; transmission electron microscopy

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INTRODUCTION

Recently, the zebrafish (*Danio rerio*), a teleost, has become increasingly important as a representative vertebrate model in many fields of research (Jesuthasan, 2002). This is especially true of developmental biology, in which large-scale screening approaches can be used (Haffter et al., 1996a,b; Kelsh et al., 1996), and of methodologically advanced developmental genetics. The pigment patterns of vertebrates are formed by pigment cells that are derived from neural crest cells. The neural crest is a transient, embryonic tissue unique to vertebrates. Neural crest cells emerge from the dorsal region of the neural tube and migrate widely throughout the developing embryo. These cells play essential roles in the formation of a variety of tissues in vertebrates including the pigment cells (reviewed in detail by Nordlund et al., 1998; Hall, 1999; Le Douarin and Kalcheim, 1999).

The characteristic external feature of zebrafish is the horizontal stripe pigment pattern (Fig. 1A), which is known to consist of three classes of neural-crest-derived pigment cells: melanophores, xanthophores, and iridophores (Raible et al., 1992; Raible and Eisen, 1994; Schilling and Kimmel, 1994; Dutton et al., 2001). Melanophores contain black pigment granules (melanosomes), xanthophores contain yellow to orange pigment granules (pterinosomes), and silver iridophores contain ultra-fine reflecting platelets (Kirschbaum, 1975; Johnson et al., 1995; Parichy et al., 1999, 2000; Elliott, 2000; Ziegler et al., 2000; Hirata et al., 2003). The pigment pattern of zebrafish can be considered to be a construct of these three classes of pigment cells.

Recent molecular genetic studies

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have uncovered the mechanism by which pigment cells form the striped pattern (reviewed in detail by Rawls et al., 2001 and Kelsh, 2004). Several mutant genes that cause pigment pattern abnormalities have been isolated (Johnson et al., 1995; Parichy et al., 1999, 2000; Rawls and Johnson, 2000; Rawls et al., 2001; Kelsh, 2004). Most of them code for molecules required for development of melanophores and xanthophores (Parichy et al., 1999, 2000; see also Kelsh, 2004). In vivo studies with zebrafish chimeras suggest that interactions between the different types of pigment cell play a key role in formation of the striped pattern (Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003).

To understand how pigment cells interact with each other, it is necessary to have a detailed understanding of the morphology of pigment cells in addition to the identification of physiologically relevant genes and proteins. In a previous study utilizing transmission electron microscopy (TEM), we gave a detailed account of the morphology of the striped pigment pattern region of the trunk. We found that the pigment cells in the hypodermis of the trunk region with a pigmented pattern kept a specific layer order (Hirata et al., 2003). In the present study, we have carried out another comparative TEM analysis of zebrafish pigment cells, focusing on different tissues with and without a striped pigment pattern (Fig. 1A). Our purpose in this study is to identify conformational order commonly shared among pigment cells located within organized pigment patterns.

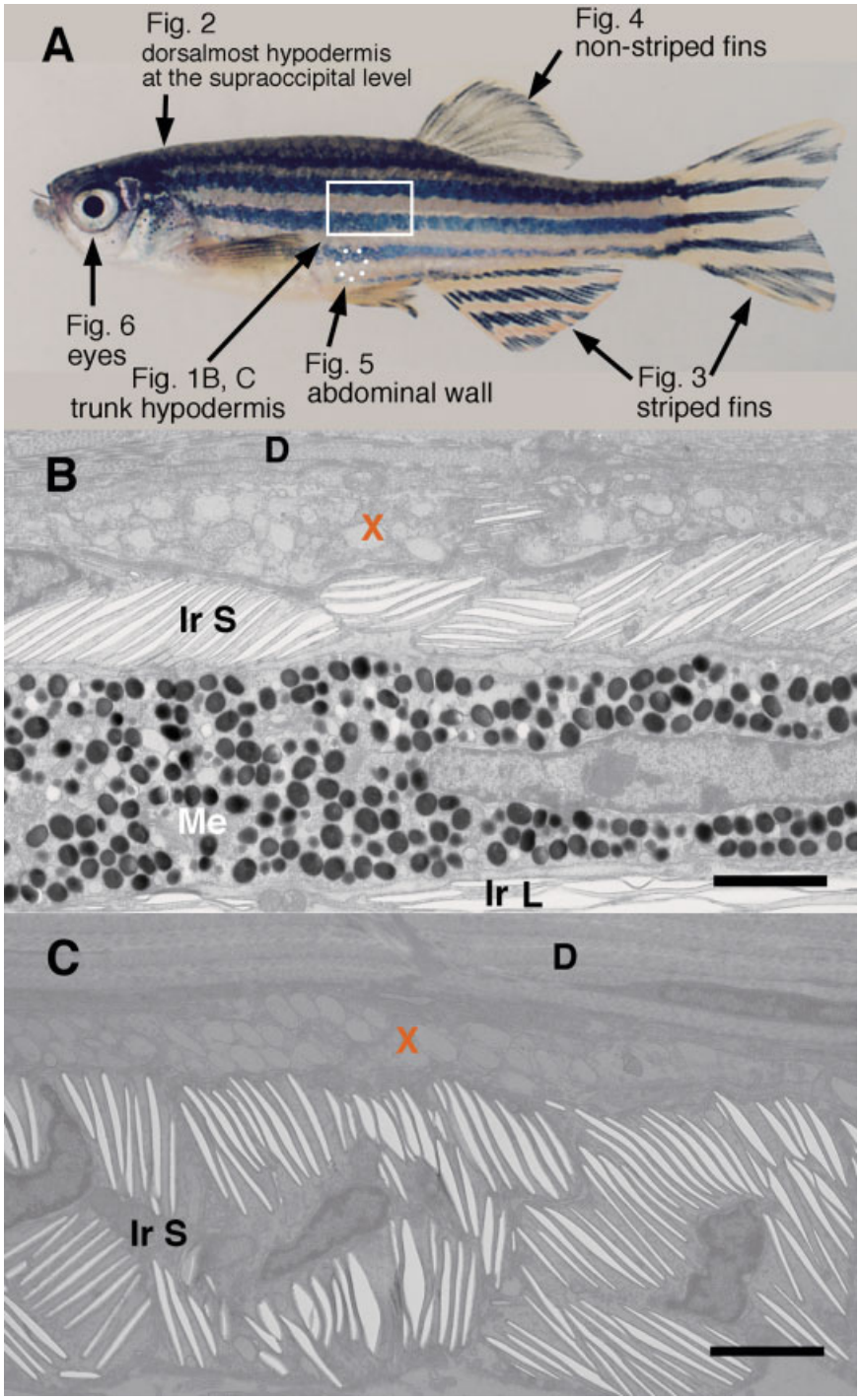

Fig. 1. **A:** Pigment pattern in zebrafish. Wild type zebrafish have a characteristic striped pigment pattern in the trunk as well as caudal and anal fins. Arrows indicate the tissues in which we carried out TEM analyses of pigment-cell conformations in this study. **B,C:** Transmission electron micrographs showing the pigment-cell conformation in the hypodermis of the trunk pigment pattern region. Surface is to the top. Vertical order (i.e., from surface to inside) of the pigment-cell sheets was xanthophore/type S iridophore/melanophore/type L iridophore in dark stripe regions (B), and xanthophore/type S iridophore in light stripe regions (C). See also Hirata et al. (2003). D, dermis; Ir L, type L iridophores; Ir S, type S iridophores; M, muscle layer; Me, melanophores; X, xanthophores. Scale bars = 2 μm.

RESULTS

Trunk Hypodermis

Our previous study on the wild-type trunk hypodermis, where the pigment cells form a clear, striped pattern, revealed all three classes (four types) of pigment cell forming strictly organized, sheeted structures in the hypodermis of both dark stripe and light stripe regions (Fig. 1B,C; Hirata et al., 2003). Briefly, at the most superficial level of the hypodermis in the dark stripe region, immediately below the dermal tissue, xanthophores were observed. However, pterinosomes found in the xanthophores in dark stripe are smaller in number and less uniform in their shape and size (Fig. 1B; Hirata et al., 2003). Below this layer, a sheet of iridophores, which contains reflecting platelets of relatively small di-

mension, was always located (type S iridophores). Those two classes of pigment cell were always concomitant with each other. Below these, a sheet of melanophores was observed. In the deepest hypodermis, a sheet of another type of iridophore (type L iridophores) was found.

In the hypodermis of the trunk light stripe region, sheets of xanthophores and iridophores were observed. They were always coupled to one another (Fig. 1C; Hirata et al., 2003). The sheet of iridophores was located just beneath the xanthophore sheet. Iridophores in this region have small-sized, reflecting platelets similar to type S iridophores in the dark stripe region.

The three classes of pigment cells conformed strictly to this order in the sheeted structures in the hypodermis of both dark stripe and light stripe regions. Each sheet was composed of a one cell thick layer of a class of pigment cells. Vertical order of the sheets (i.e., from surface to inside) was unchanging: xanthophore/type S iridophore/melanophore/type L iridophore in dark stripe regions; and xanthophore/type S iridophore in light stripe regions, respectively (Fig. 1B,C; Hirata et al., 2003).

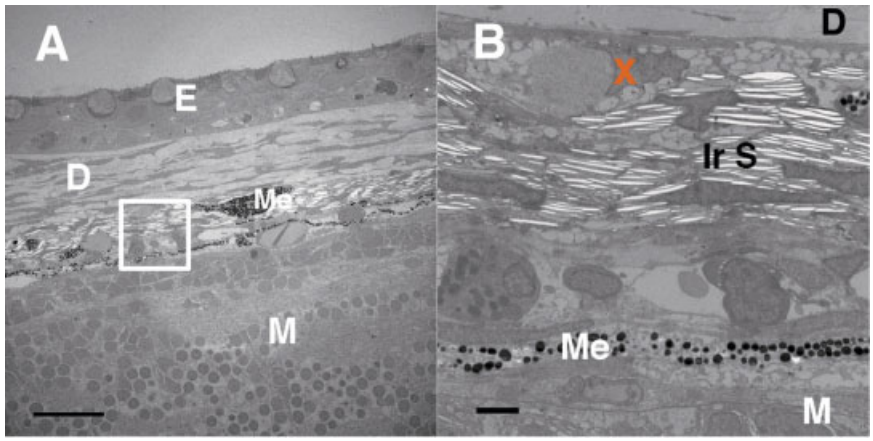

Fig. 2. Transmission electron micrographs showing pigment-cell conformation in the dorsal-most hypodermis at the supraoccipital level. Dorsal is to the top. **A:** Lower magnification image. Accumulating type S iridophores contain thinly scattered melanophores. White box indicates the area of higher magnification image shown in B. **B:** Higher magnification image of boxed region in A. Though this portion is also hypodermis, distribution of pigment cells is disordered in comparison to the hypodermis of the striped pigment pattern region of the trunk. D, dermis; E, epidermis; Ir S, type S iridophores; M, muscle layer; Me, melanophores; X, xanthophores. Scale bars = 10 μm in A, 2 μm in B.

Hypodermis of Non-Striped Pigment Pattern Region in the Body Trunk

In the body trunk of the zebrafish, some peripheral regions do not have a striped pattern although all types of pigment cell are present. To determine whether there was any conformational difference to that of the striped pigment pattern regions, we examined pigment cells in the dorsal-most hypodermis at the supraoccipital level. As shown in the TEM pictures in Figure 2A and B, the conformation and the layer order of the pigment cells are different from the trunk hypodermis of either dark or light stripe regions. The hypodermis of this dorsal region consisted of roughly three layers of pigment cells. From the surface, we found a single sheet of xanthophores, groups of type S iridophores, and a single sheet of melanophores. Type L iridophores, which always lie just beneath the melanophore-layer in the dark stripe region, were not observed. Melanophores were found thinly scattered within groups of type S iridophores. Capillaries were inserted between the groups of type S iridophores and the sheet of melanophores, whereas these pigment cells are always in direct contact in dark stripe regions.

Pigment Cell Conformation in the Fins

Localization of pigment cells in the fin.

Fin tissues are roughly divided into three major cellular compartments (Huang et al., 2003): pairs of bony rays (fin rays; see Fig. 3E inset); "epidermal" epithelia; and the central mesenchymal layer, in which pigment cells are located. In the TEM analysis, we found a thin layer with low electron density between the "epidermal epithelia" and the "mesenchymal layer" (see white arrowheads in Figs. 3A–D,F, 4). Within the thin layers, we observed a collagen-fiber-like morphology (data not shown) similar to that reported in larval zebrafish by Le Guellec et al. (2004). Therefore, these thin layers are likely to be homologous to the dermal tissue found in the body trunk. These thin layers, which have a thickness of no more than 1 μm, were observed in every fin of the zebrafish (see white arrowheads in Figs. 3A–D,F, 4). Therefore, almost every pigment cell in zebrafish fins was observed in a position between the pair of dermal layers that is homologous to the hypodermal region in the trunk. On rare occasions, we observed round-shaped melanosome-containing cells within the epidermal tissue (see supplemental data A).

Fins with striped pigment pattern.

Zebrafish have a horizontal striped pigment pattern in both caudal and anal fins, whereas there is no clear pigment pattern in other fins (Fig. 1A).

In the dark stripe regions of striped fins, we observed two classes of pigment cell forming a three-layered structure in the hypodermis (i.e., the region sandwiched by a pair of dermal layers). A sheet of xanthophores was inserted between two sheets of melanophores, which were positioned adjacent to the dermal layers (Fig. 3A,C). In the light stripe regions, only a sheet of xanthophores was observed (Fig. 3B,D). In both regions, gaps in the cell sheets were rare. One of the conformational rules kept in the hypodermis of trunk pigment pattern region, that each pigment cell type forms a gapless sheet of a single cell thickness, is also kept in the striped fin. The order of the layers was different; in the fin,

iridophores did not form layers and the xanthophores were located between two separate sheets of melanophores. However, the fin-specific layer order (melanophore/xanthophore/melanophore) was strictly maintained. Around the most ventral level of the anal fin, the density of pigment cells decreased, and the striped pigment pattern became less clear (Fig. 3F, inset). In these regions, pigment cells did not form the sheet structure observed in the clear pigment pattern region, but the conformation was similar to that of non-striped fins (see below).

Iridophores are thinly distributed along fin rays and in the hypodermis of light stripe regions. Iridophores found in striped fins are typeS iridophores. All of them are in association with xanthophores, without forming the sheet conformation (Fig. 3E). We found that some of the xanthophores located close to the fin rays contained melanosome-like granules of higher electron density (see Supplementary Material, which is available at www.interscience.wiley.com/jpages/1058-8388/suppmat). Although we do not have any information about the identity of such cells, it is conceivable that they might be in a transient state before becoming fully developed melanophores or xanthophores.

Fins without striped pigment pattern.

Dorsal, ventral, and pectoral fins in zebrafish do not have a striped pigment pattern (Fig. 1A). In these fins, both xanthophores and melanophores were distributed in a scattered manner. They were never present as a sheet or layered structure (Fig. 4A,C). Melanophores in these fins usually showed a round shape (Fig. 4A,B). In regions where neither pigment cell type is present, the bilateral dermal layers are often fused (Fig. 4B,D).

We found few iridophore-like cells in non-striped fins. When present, such cells showed almost the same morphology as type S iridophores in striped fins (data not shown).

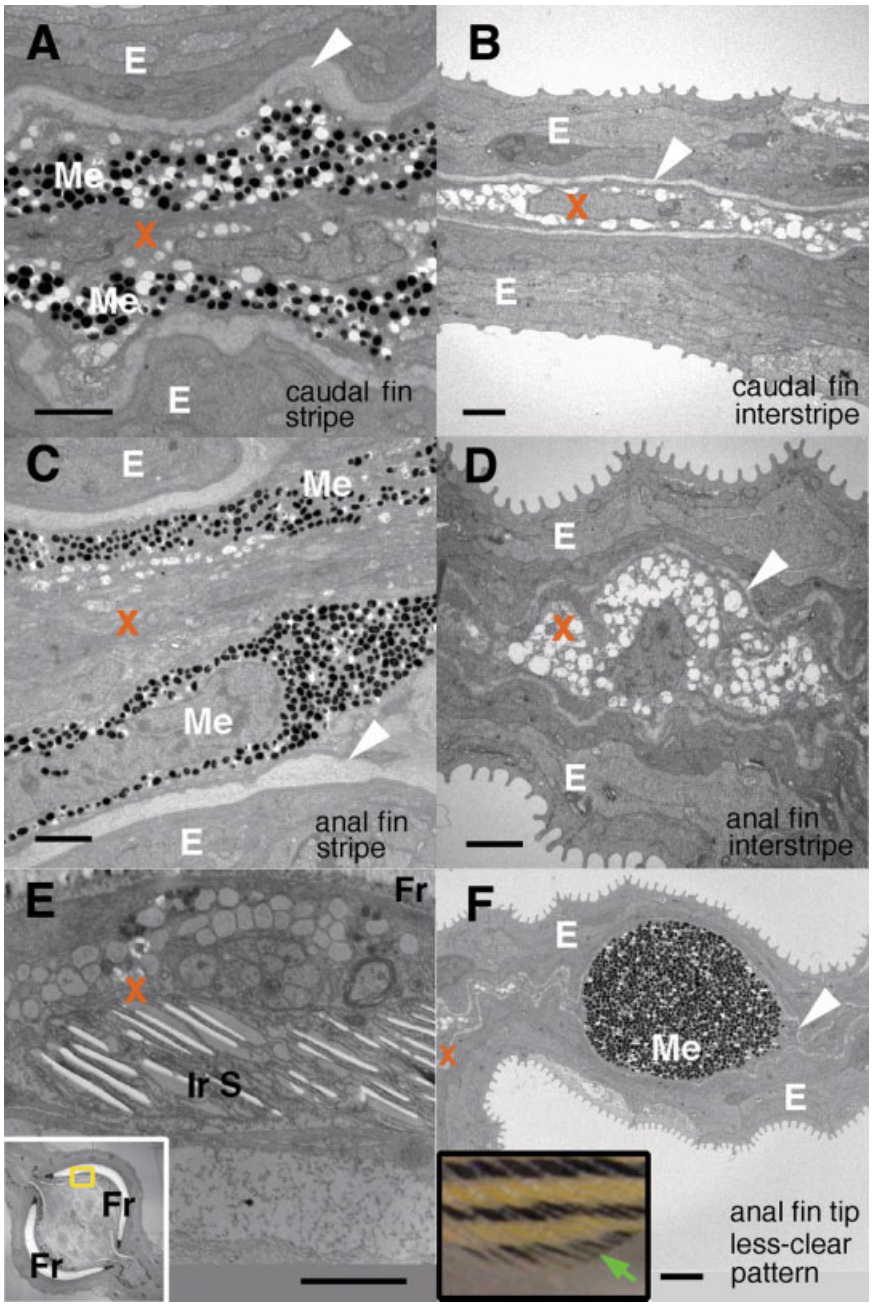

Fig. 3. Transmission electron micrographs showing pigment cells in the striped fin. Lateral is to the top. **A,B:** Hypodermal pigment cells in the caudal fin. In the hypodermis of dark stripe region (A), a sheet of xanthophores was inserted between two independent sheets of melanophores. Only one sheet of xanthophores was observed in light stripe region (B). **C,D:** Hypodermal pigment cells in the anal fin. Both in the dark stripe (C) and light stripe regions (D), conformations of the pigment cells were basically same as that in the caudal fin (A, B). **E:** Thin-scattering type S iridophores in the caudal fin. Type S iridophores were seen, associated with fin rays in both striped fins. Type S iridophores and xanthophores are always concomitant with each other as observed in the hypodermis trunk pigment pattern region. Yellow box in inset indicates the position of this image. **F:** Pigment cells at the ventro-most level of the anal fin, where the striped pigment pattern becomes less clear (inset). Only thinly scattered xanthophores and round-shaped melanophores were observed. White arrowheads (A–D, F) indicate dermal tissue. E, epidermal tissue; Fr, fin ray; Ir S, type S iridophores; Me, melanophores; X, xanthophores. Scale bars = 2 μm.

Pigment cells in the visceral portion (abdominal wall)

The surface of the abdomen is a silver to white color in zebrafish. Along the abdominal wall (Fig. 5A), type S iridophores were observed in accumulations several cells thick (Fig. 5A,B). Melanophores were also distributed around this region. However, they did not take a sheeted form, but were

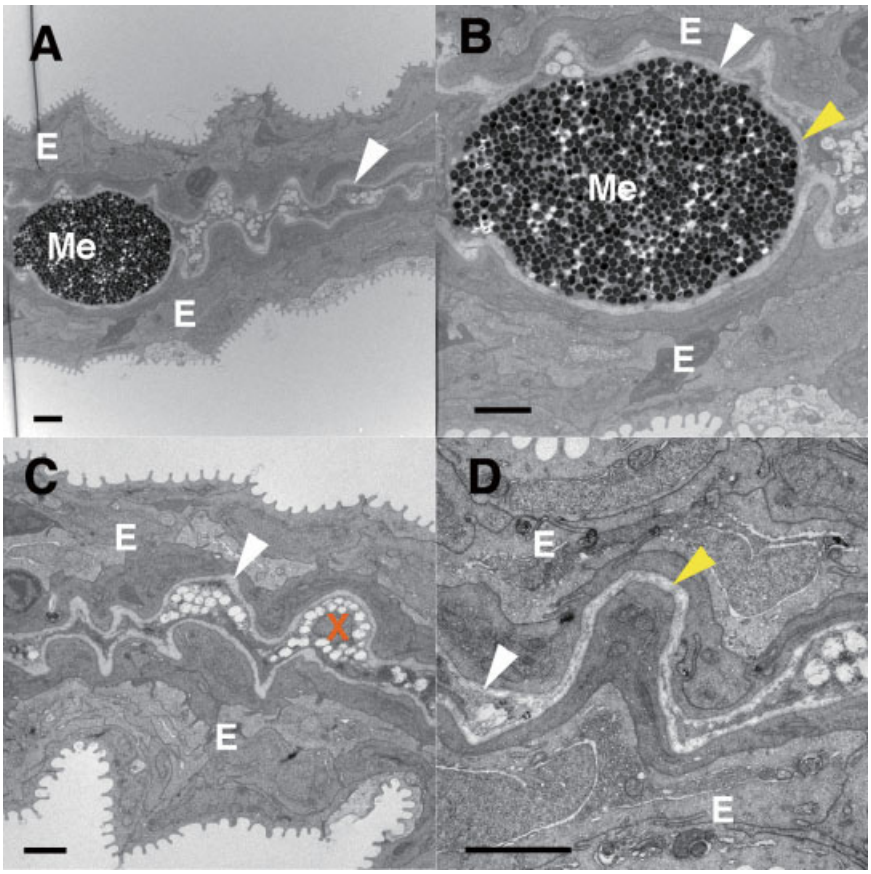

Fig. 4. Transmission electron micrographs of pigment cells in the non-striped fin. Lateral is to the top. **A–C:** Hypodermal pigment cells in the dorsal fin. Melanophores (A, B) and xanthophores (C) were observed. Neither pigment cell type formed sheeted structures. In non-striped fins, fusions of dermal tissues (B, **D**, yellow arrowheads) were frequently observed. White arrowheads indicate dermal tissue. E, epidermal tissue; Me, melanophores; X, xanthophores. Scale bars = 2 μm.

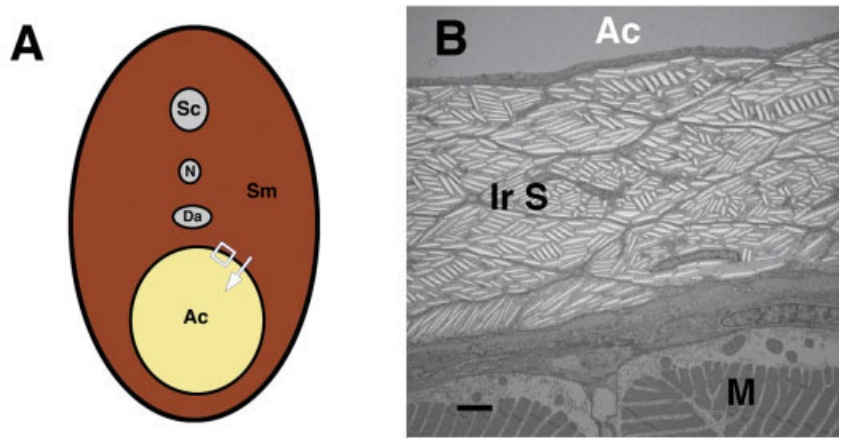

Fig. 5. Transmission electron micrographs of pigment cells along the abdominal wall. **A:** Schematic drawing of cross section of zebrafish mid-trunk. In the ventral part of the mid trunk is the abdominal cavity space containing the internal organs. Along the wall of this space, large numbers of type S iridophores were observed. Arrow and box indicate the position and direction of the TEM image shown in B. **B:** Accumulations of type S iridophores were observed along the abdominal wall. The position and direction of this picture are indicated by the box and arrow in A. Ac, abdominal cavity; Ir S, type S iridophores; Sc, spinal chord; N, notochord; Da, dorsal aorta; M, muscle layer; Sm, skeletal muscle. Scale bar = 2 μm.

thinly scattered around the type S iridophores, as well as peritonea (data not shown). Lapennas and Schmidt-Nielsen (1977) reported that the submucosa of the swim bladder contains crystals of guanine, the principal component of the reflective platelets of iridophores (Elliott, 2000), in the conger eel (*Conger oceanicus*) and pigfish (*Orthopristis crysoptera*). Such crystals are considered to cause resistance to gas permeability as well as the specific silvery coloration of the swim bladder (Lapennas and Schmidt-Nielsen, 1977). Although the swim bladder of zebrafish also exhibits a dull, silvery color among the visceral tissues, we could not find iridophore-like cells with reflective platelets (data not shown).

Pigment Cells in the Cranial Region (Sheeted Pigment Cells in the Eye)

In the iris, accumulations of type S iridophores were observed over a single sheet of melanophores (Fig. 6A,B). The thickness of the accumulation of iridophores gradually increased from central (pupil) to the peripheral portion of the eye (Fig. 6A). Cells, which contain melanosome (high electron density) or pterinosome (low electron density) -like granules, were thinly observed over the iridophore population (Fig. 6A,B).

At the boundary between the iris and the choroidea (Fig. 6C,E), two layers of melanophores are added as shown in Figure 6F. The vertical (i.e., along the axis from the orbit to the lens) layer order in the choroidea is single-sheeted melanophores/single-sheeted type S iridophores/double-sheeted melanophores. The sheet of iridophores is a single cell thickness in the choroidea. We found that this ordered structure was kept all through the choroidea (Fig. 6C,D).

DISCUSSION

In the present study, we have carried out comparative TEM analyses of zebrafish pigment cells focusing on conformational differences between tissues with and without a striped pigment pattern. The distribution of pigment cells varies among the different cell types and tissues. Each type of

pigment cell often forms a two-dimensional sheet of one cell thickness. However, in some tissues, they form accumulations or are thinly scattered without contact with any other pigment cells. The vertical layer order of the cell sheet also differs among the tissues.

Iridophores

Iridophores show the greatest variation in the nature of their distributions. In the hypodermis of regions of the trunk with a striped pigment pattern, iridophores always form a one-cell-thick sheet. However, the morphology and the shapes of the reflecting platelets of each cell differ depending on the type of stripe. In dark stripes, there are two layers of iridophores. One has large horizontal platelets (type L), the other has small platelets (type S). In light stripes, type S iridophores form a one-cell-thick sheet, but the thickness of each cell is much larger than in dark stripes (see also Hirata et al., 2003). In the iris and abdominal wall, as well as dorsalmost hypodermis at the supraoccipital level, iridophores are present as accumulations. In the fins, iridophores are not present as discrete structures but are scattered. The conformations and distribution patterns of iridophores are summarized in Table 1. How and why does this cell type take such a variety of morphologies? Possibly, they are in different states of differentiation, or alternatively, the different environments of the tissues may cause morphological changes. The answer to this problem may come from the appropriate transplantation experiments.

Although iridophores in the trunk

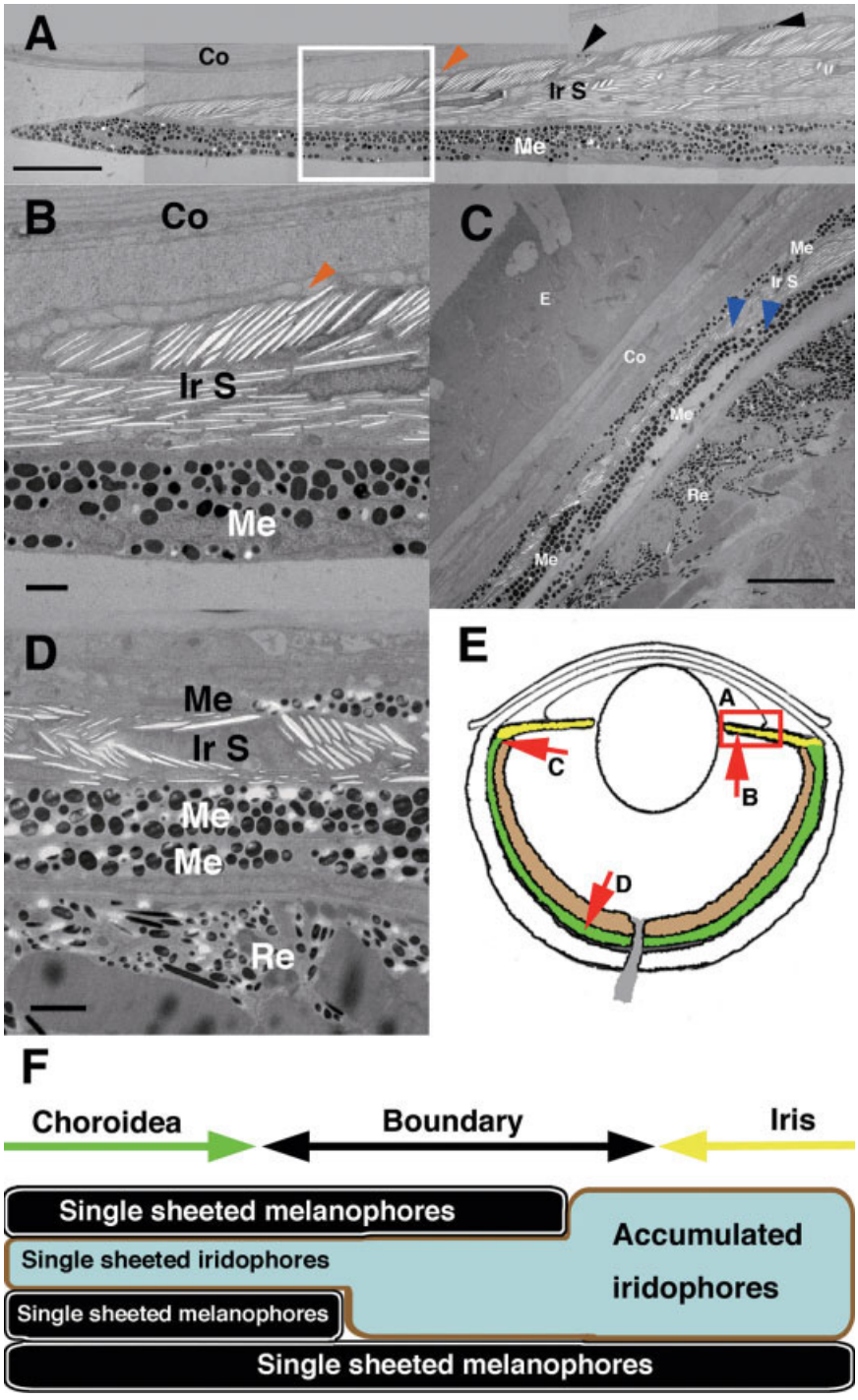

Fig. 6. A-F: Transmission electron micrographs of pigment cells in the eye. **A,B:** Pigment cells in the iris. Outside of the eye is to the top. Lower magnification image (A) and higher magnification image of the white-boxed region in A (B). The iris was composed of single-sheeted melanophores and accumulations of type S iridophores. Thin-scattering cells, which contain melanosome (black arrowheads) or pterinosome (orange arrowheads) -like granules were observed over the iridophore population. The positions and directions of these pictures are indicated by a red box and arrow in E, respectively. **C:** Pigment cells at the boundary between the iris and choroidea. Dorso-lateral is to the top. The direction and position of this picture are indicated by the red arrow in E. Two out of the four sheets of pigment cells in the choroidea are structurally continuous with the iris (blue arrowheads). **D:** Pigment cells in the choroidea at the eyeground level. The direction and position of this picture are indicated by the red arrow in E. The vertical (i.e., along the axis from the orbit to the lens) layer order in choroidea is single-sheeted melanophores/single-sheeted type S iridophores/double-sheeted melanophores (C, D). **E:** Schematic drawing of a cross-section of zebrafish eye. Yellow, green, and brown-colored areas indicate the region of the iris, choroidea, and retinal region, respectively. A red box and arrows indicate the positions and directions of the pictures shown in A–D. Lateral is to the top. **F:** Schematic drawing showing the conformational changes of pigment cells over the region from the iris to the choroidea. Note that the color of the yellow and green arrows corresponds to the yellow and green portions in E, respectively. Outside of the eye (the direction to the orbit) is to the top. Co, cornea; E, (corneal) epithelium; Ir S, type S iridophores; Me, melanophores; Re, retinal region. Scale bars = 2 μm.

regions with a striped pigment pattern show conformational differences depending on whether they are in the dark or light stripes, this relationship is not maintained in the fin hypodermis. In striped fins, type L iridophores are never found in dark or light stripes. Rather, only type S iridophores are observed in fins with and without stripes. The number of the cells is much lower than that in the body trunk, and they do not form a sheet structure. We suspect that iridophores are not a core part of the machinery of pigment pattern formation, because at the tip of the striped fins (Fig. 3F), melanophores and xanthophores form a striped pattern with very few iridophores.

TABLE 1. A Summary of the Distribution of Iridophores

Position	Pattern of distribution
Type S iridophores	
Trunk hypodermis	Single-cell sheet
Fin hypodermis	Scattered
Dorsalmost hypodermis at the supraoccipital level	Accumulation
Abdominal wall	Accumulation
Iris	Accumulation
Choroidea	Single-cell sheet
Inner side of the opercula	Single-cell sheet
Tissue around the scales (stratum spongiosum)	Scattered
Type L Iridophores	
Trunk hypodermis	Single-cell sheet
Tissue around the scales (stratum spongiosum)	Scattered

Layer Order of the Pigment Cells

In zebrafish, we found that the layer order of the pigment cell sheets is different among tissues, but that they are consistent within tissues. As the layer order of the pigment cells presumably reflects function (body color, resistance from UV, etc.), the mechanism that gives rise to the layer order must be quite important (Nordlund et al., 1998). Similar pigment cell structures have been reported in adult amphibians. The pigment cells of frogs always form units composed of three classes of dermal pigment cell: xanthophores, iridophores, and melanophores. The vertical order of the pigment cells (from surface to inside) in this unit is xanthophore/iridophore/melanophore (Bagnara et al., 1968; Nordlund et al., 1998). Such a structure is considered to comprise a functional unit called the "dermal chromatophore unit," which enables the amphibian skin to change its color rapidly. Zebrafish have this order of layered structure in the dark stripe region of trunk skin. Therefore, it is possible that there is a common mechanism to form this order of pigment cell layers. In the amphibian, the dermal chromatophore unit is formed during metamorphosis (Yasutomi and Yamada, 1998). It is of great importance to know whether the vertical unit structure and the striped pattern form at the same period of development or not.

Pigment Cells That Organize Into the Striped Pigment Pattern

Our TEM observations described above show that pigment cells involved in striped pattern share the common characteristic of forming sheets that are one cell thick. The number of layers and the order of the different pigment cell types differ between the pigmented pattern region of the trunk and the fins. However, in both tissues, all the cell types form gap-less cell sheets. In stripe-less regions of trunk or fins, we found an absence of such organization. In the stripe-less fins, both melanophores and xanthophores are present in the isolated state without having homophilic or heterophilic contact. At the dorsal-most hypodermis at the supraoccipital level, pigment cells do not form stripes although all types of pigment cells are present. In this region, iridophores form thick accumulations that contain a small number of isolated melanophores. Goda and Fujii (2001) have reported a similar conformation of pigment cells in the hypodermis of the domino damsel (*Dascyllus trimaculatus*), which does not have a striped pigment pattern. In a section across an excised piece of a bright white spot region of this fish, they observed that accumulations of iridophores contained cells with melanosomes.

As our observations described here were obtained from TEM analyses of adult zebrafish, it is difficult to extrapolate from our findings to discuss whether the characteristic structures are directly related to the developmental process of striped pigment pattern formation. There are several different theoretical models that can generate striped patterns (Meinhardt, 1982; Murray, 1993). However, for all of them, the dimensions of the fields should be uniform in order to form the uniform stripe widths observed in zebrafish (Turing, 1952; Asai et al., 1999). Therefore, the sheet structures we found in the hypodermis in the striped regions seem suitable to play the role of the morphogenetic field that instructs pattern formation. Further analyses during the course of development in zebrafish, as well as interspecific studies, are needed. In such situations, it will be important to know the molecular mechanisms as well as the structural information. To understand pigment pattern formation, TEM analysis will play still more important roles in the future.

EXPERIMENTAL PROCEDURES

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, Osaka, Japan).

Transmission Electron Microscopy

TEM analysis was based on Toma et al. (1999). Experimental fish were im-

mersed in a mixture of 3% paraformaldehyde and 3% glutaraldehyde in a 0.1M cacodylate buffer. Specimens were cut into small pieces, immersed in the same fixative overnight at 4°C, and post-fixed in 2% osmium tetraoxide 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 epon812 resin (TAAB) after dehydration. Ultra-thin sections were cut using an Ultracut UCT microtome (Leica, Austria). TEM observation was carried out using a JEM-1010 microscope (JEOL, Japan), and photographs were taken using a Bio Scan Model 792 (Gatan) digital still camera.

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