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Differentiation of pigmented tissues in the eyes of medaka (*Oryzias latipes*) embryos: An electron microscope study

Euichi Hirose[1] and Jiro Matsumoto[2]

Department of Biology, Keio University, Hiyoshi, Kohoku-ku, Yokohama 223, Japan.

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Abstract The formation of pigmented tissues in the eye of developing embryos of the wild-type strain of medaka, *Oryzias latipes,* was studied by transmission electron microscopy. Particular attention has been paid to morphological differentiation of pigmentary structures in pigment epithelial cells, choroid melanophores and choroid iridophores. Melanogenesis in pigment epithelium first occurs at stage 26 after formation of the proximal wall of the optic vesicle. Melanosomes, which are the sites of melanogenesis in pigment epithelium, are multivesicular and spherical at the onset of pigmentation; however, as development proceeds up to hatching, they acquire a long, ellipsoidal shape which is typical to this tissue. At the stages at which active melanogenesis occurs, most melanosomes in this tissue are formed by fusion of large and small vesicles into a multivesicular body, whereas the premelanosomes containing a lattice structure of fibrils occur at a limited frequency. The choroid iridophores appear by stage 28 along the outer region of pigment epithelium, and can be identified by the formation of reflecting platelets via multivesicular vesicles. Following the differentiation of these iridophores, the choroid melanophores appear between pigment epithelium and the layer of choroid iridophores. They contain spherical melanosomes, the morphological characteristics of which are similar to those of melanosomes in skin melanophores but different from those of melanosomes in pigment epithelial cells. All these findings indicate that in the eye of medaka, (1) the morphological properties of melanosomes in melanogenic cells are expressed in a tissue-specific manner, even though they are derived from similar multivesicular organelles and (2) melanosomes and reflecting platelets are formed from multivesicular organelles, which supports the suggestion of a common origin of pigment particles in vertebrate chromatophores.

Introduction

A series of combined morphological and biochemical studies on melanogenesis in mammalian and avian tissues have revealed that the melanosomes, cytoplasmic organelles specialized for melanin biosynthesis *in vivo,* are formed within melanocytes or pigment epithelial cells by fusion of two different kinds of vesicles derived from different sources: Golgi-derived small coated vesicles bearing tyrosinase and large vesicles which originate from rough endoplasmic reticulum (Seiji, 1981; Mishima, 1992). Melanin synthesized in these organelles is then deposited on their internal structure in a lattice-like arrangement, yielding the so-called fibrillar premelanosomes or stage II melanosomes (Toda *et al.*, 1976). In integumental melanophores of a teleost species, the goldfish *Carassius auratus*, it has been shown that the melanosomes are formed or mature in a different manner; melanin deposition occurs in the matrix of this organelle, which contains numerous small vesicles, yielding the so-called multivesicular premelanosomes (Turner *et al.*, 1975). A similar observation has also been reported with medaka (Nakajima and Obika, 1986).

In the recent studies on melanogenesis in transgenic orange-colored variants of medaka bearing the gene for mouse tyrosinase, it has been shown that melanin deposition in the melanosomes of their integumental melanophores occurs basically in a form of fibrillar premelanosomes (Matsumoto *et al.*, 1992). This raises questions as to whether the maturation pattern of melanosomes is essentially different between teleosts and mammals, whose phyletic positions are entirely different, and what is a primary factor responsible for expression of the melanosome phenotype such as gene composition or tissue environment. In relation to these questions, Nakajima and Obika (1986) have reported that even in medaka, the fibrillar melanosomes appear at a certain frequency, together with

[1]Present address: Biological Laboratory, College of Agriculture and Veterinary Medicine, Nihon University, Kameino, Fujisawa 252, Japan. [2]To whom all correspondence should be addressed.

the multivesicular ones in the same integumental melanophores during the embryonic to larval stages.

It has also been observed that malformation often occurs in the eyes of transgenic medaka, resulting in extraordinary eyes, larger or smaller compared to normal ones (Matsumoto *et al.*, unpublished data). So far, little is known about what occurs during the formation of pigmented tissues in such an abnormal eye.

The objective of the present study is to clarify the histogenesis and differentiation of pigmentation-associated tissues in the eye of medaka, with particular references to melanosome formation in the pigment epithelium and the choroid, by transmission electron microscopy. The results obtained are expected to provide a clue to the mechanisms by which the melanosome phenotype is determined in a tissue-specific manner in normal development and by which an abnormal histogenesis is caused in a transgenic medaka.

Materials and Methods

The fish used in this study were the wild-type strain (BBRR) of medaka, *Oryzias latipes*, which were obtained from the breeding stock of its homozygous strain (courteous gift from Dr. H. Tomita, Nagoya University, Nagoya) and then maintained by inbreeding in our laboratory. The mature female fish usually carried fertilized eggs for several hours after mating. These eggs were removed from the abdomen and kept in dechlorinated tap water at room temperature. Through daily observations under a binocular stereomicroscope, we collected several embryos at stages 19–20, 23, 26, 28, 30 and 31–32 for fixation according to the nomenclature of Matsui (1949). The larvae immediately after hatching were also examined.

The embryos and larvae were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4). In the fixative, we removed the egg chorion and decapitated the heads of the embryos or larvae with a razor blade. These isolated head pieces were rinsed in the fixative at 4°C for 1 hr. After brief washing in the buffer, the specimens were postfixed in 1% osmium tetroxide in the buffer, dehydrated through a graded series of ethanol, cleared with methyl glycidyl ether and embedded in epoxy resin. Thick sections were stained with toluidine blue for light microscopic observation. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100 S transmission electron microscope at an accelerating voltage of 80 kV.

Results

The optic vesicles appeared as a spherical cell mass at stages 19–20 (Fig. 1). Little morphological sign of cytodifferentiation was observed at these stages. At stage 23, the proximal wall of the optic vesicle transformed into a thin layer and covered the surface of the optic cup except for the concave sides (Fig. 2). This thin layer was considered to be prospective pigment epithelium of the retina and no melanin deposition was observed in the cells of this layer at this stage. Many fusiform mesenchymal cells loosely circumscribed the optic cup and most of them were considered to be prospective connective tissue covering the eye and choroid. The lens was formed by depression of a thickened portion of the spherical optic cup at stage 26, and some of the pigment epithelial cells of the retina contained a moderate number of melanosomes (Fig. 3). The melanosomes were round to ellipsoidal with ragged surfaces. The external surface of the pigment epithelium was covered with flattened mesenchymal cells.

At stage 28, melanin deposition was commonly observed in pigment epithelial cells (Fig. 4). A few reflecting platelets were recognized in cells adhering to the external surface of pigment epithelial cells. These cells were considered to be iridoblasts or to differentiate into choroid iridophores. Most reflecting platelets in them were rectangular and their matrices appeared as an empty space due to sublimation under the electron beam or solubilization during lead citrate staining as seen in skin iridophores (guanophores) (Kawaguchi and Takeuchi, 1968). Fig. 5 shows parts of pigment epithelial cells at high magnification; a number of round to ellipsoidal maturing melanosomes were distributed in their cytoplasm, most of which were surrounded by or attached to small vesicles, indicating fusion of a larger particle, i.e. the melanosomal body, and small vesicles, presumably suppliers of tyrosinase. Most of the small vesicles undergoing fusion were filled with an electron-dense substance, presumably, melanin, and were considered to be derived from rough endoplasmic reticulum as indicated by Turner *et al.* (1975). Within melanosomes, there were numerous fine hollows (white arrow in Fig. 5). These hollows were considered to be the inner spaces of the fused small vesicles, the outside surfaces of which were inverted to become the inner surfaces upon fusion,

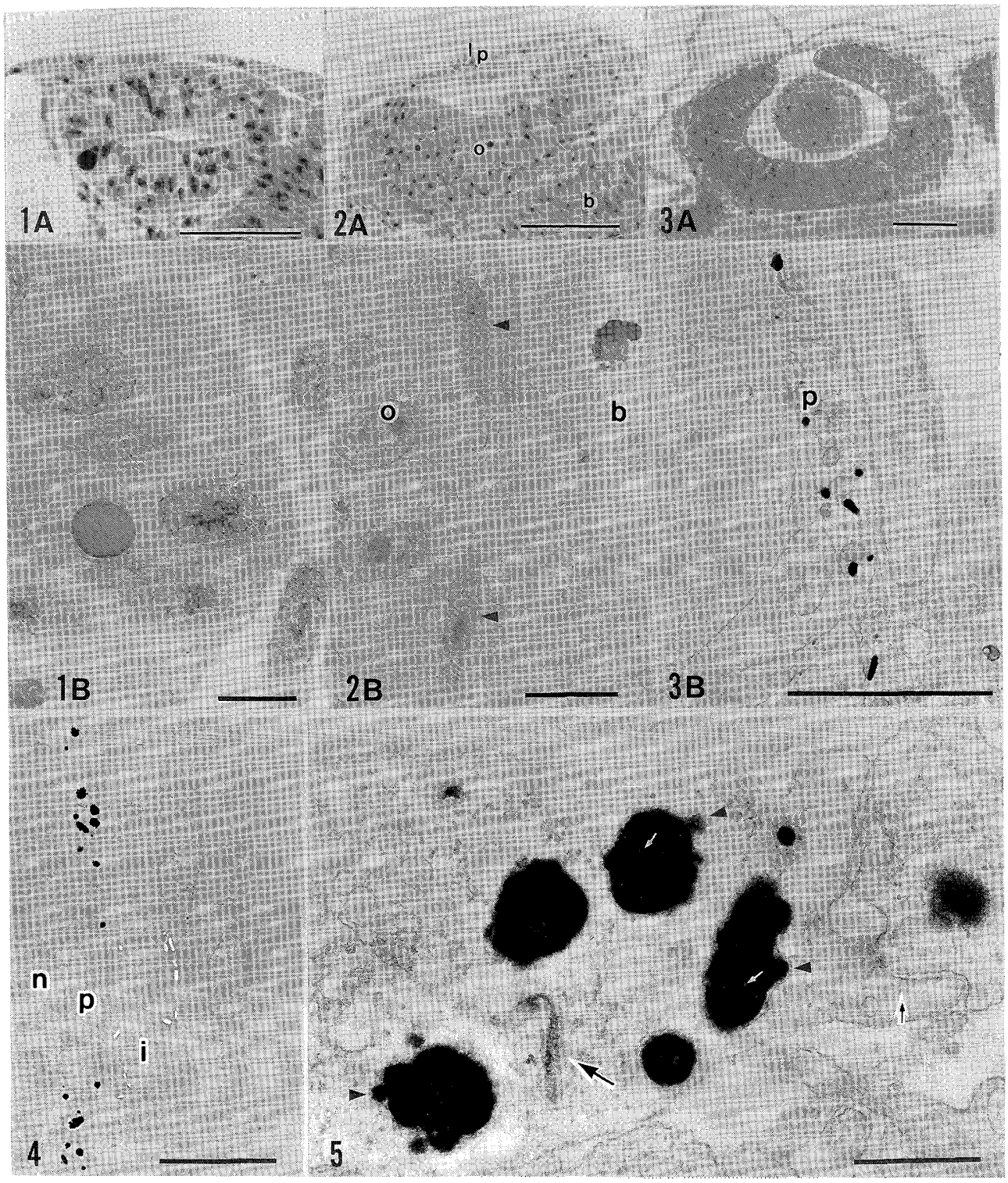


Plate 1. Light and electron micrographs showing the formation of pigmented tissues in the eye of medaka (wild-type strain) embryos at varying stages. Fig. 1. Stage 19–20. Fig. 2. Stage 23. Fig. 3. Stage 26. In these figures, the areas in the light micrographs (A) of the cortical region of the optic vesicle or optic cup in the embryo at a given stage are enlarged in the electron micrographs (B) in the second row. Note that (1) presumptive pigment epithelial cells becomes flattened (arrowhead), together forming a thin sheet covering the outer surface of the optic cup at stage 23, and (2) melanin deposition is observed in pigment epithelial cells at stage 26. Scale bar: 50 μm for A and 5 μm for B. Fig. 4. Stage 28. Appearance of choroid iridophores in the outer surface of pigment epithelium. Note the presence of small reflecting platelets in their cytoplasm. Scale bar: 5 μm. Fig. 5. Enlarged portions of pigment epithelial cells in the embryo at stage 28. Note that most melanosomes are undergoing fusion with small vesicles as revealed by their rugged boundary (arrowheads) and numerous electron-lucent fine spots (white arrows). The presence of fibrillar premelanosome is also observed (large arrow). Interdigitation of cell membranes of pigment epithelial cells in the area of their opposition is commonly seen at this stage (small arrow). Scale bar: 0.5 μm. Abbreviations: b, brain vesicle; i, iridophore; lp, lens placode; n, neural layer; o, optic cup; p, pigment epithelial cell.

thus being absent of melanogenic activity. These observations would indicate that melanosomes in pigment epithelial cells are formed from multivesicular particles. Besides these melanosomes, flattened cisternae were found which contained a lattice-like structure of thin fibril and might have been fibrilar premelanosomes (large arrow in Fig. 5). Along the junction of pigment epithelial cells, interdigitation of cell membranes was often observed between the facing tips of adjacent cells (small arrow in Fig. 5).

By stage 30, choroid melanophores appeared between pigment epithelial cells and choroid iridophores (Fig. 6). Since the choroid iridophores were in direct contact with pigment epithelial cells at the preceding stages, it was presumed that these choroid melanophores probably migrated into the space between the layers of these two different types of cells. It remains uncertain whether melanogenesis of choroid melanophores occurred before or during their presumed migration.

Part of a developing choroid iridophore is enlarged in Fig. 7. Besides rod-shaped reflecting platelets of varying length, there were many multivesicular bodies in the cytoplasm (arrowheads), and some of them contained a small reflecting platelet (arrow). This observation indicates that these reflecting platelets (or iridosomes) are also derived from multivesicular body-type organelles.

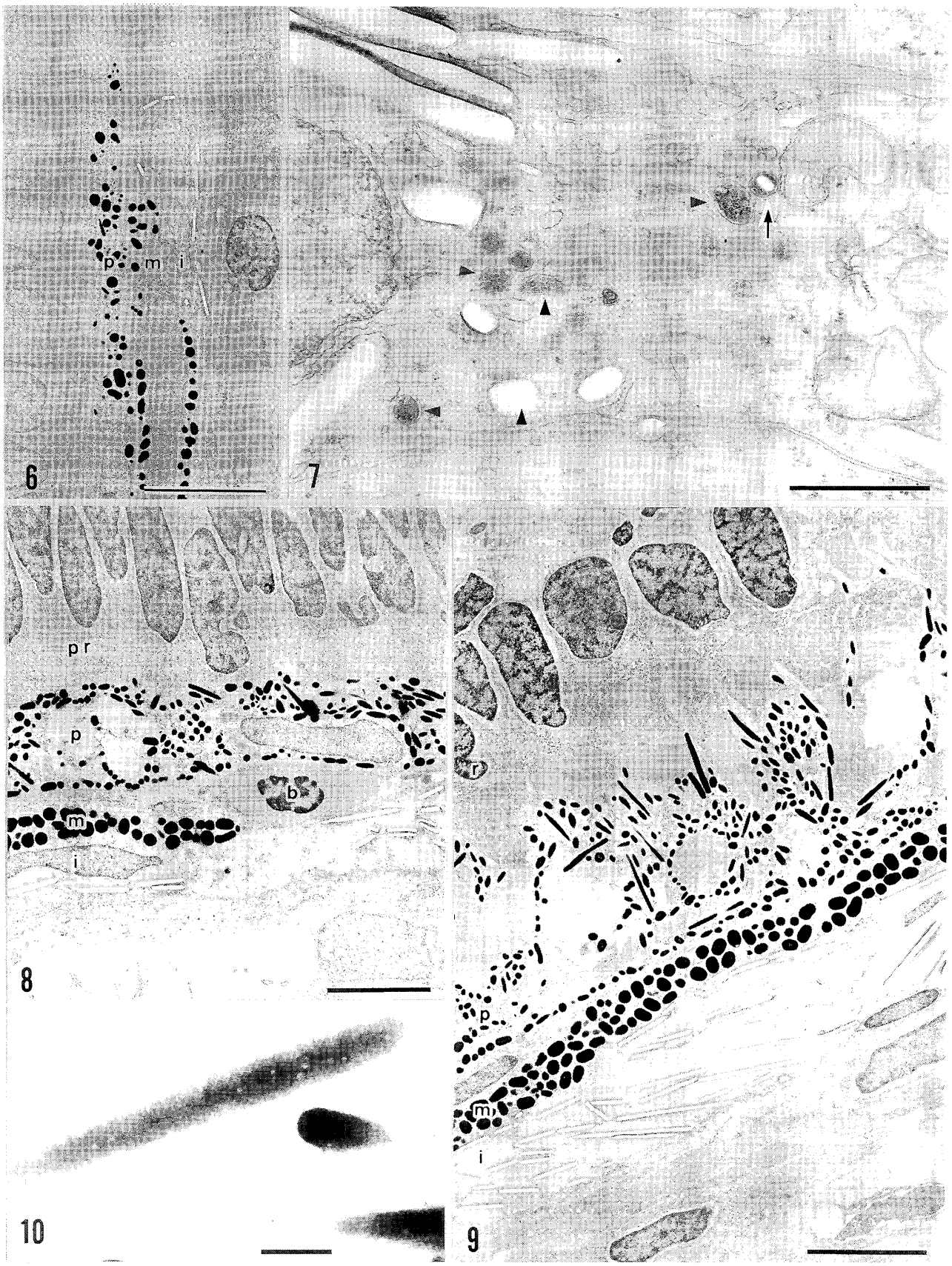
At stages 31–32, the density of choroid melanophores markedly increased but was not sufficient to cover the pigment epithelium completely (Fig. 8). Little evidence was found of morphological differentiation of prospective receptor cells located along the inner surface of the pigment epithelial cells. Blood cells were occasionally found between pigment epithelial cells and choroid cells.

In the newly hatched fry, each of the three layers of different types of pigment cells, *viz* pigment epithelial cells, choroid melanophores and choroid iridophores, became recognized as a continuous solid layer (Fig. 9). The iridophores in the outermost layer contained numerous rod-shaped reflecting platelets, about 60 nm in width, which were sparsely distributed in the cytoplasm. The choroid melanophores in the middle layer and the pigment epithelium in the inner layer contained numerous melanosomes filled with melanin. The size and shape of these melanosomes were quite different between pigment epithelial cells and choroid melanophores; melanosomes in the former were elongated, ellipsoidal and 0.2 μm in width, whereas those in the latter were round to ellipsoidal and about 0.8μm in length. Within the melanosomes of pigment epithelial cells, as those of choroid melanophores, many hollows or electron-lucent spots were found in the matrices and these were probably remnants of the inner spaces of the inverted small vesicles as mentioned above (Fig. 10). This indicates that melanogenesis in ellipsoidal melanosomes proceeded via formation of a multivesicular structure, suggesting a supply of tyrosinase by small coated vesicles. By this stage, structures similar to brush borders or laminated membranes were observed in the photoreceptor cells in their outer segment adjacent to pigment epithelial cells.

Discussion

It is known that in vertebrate melanogenic cells, melanosomes are formed by fusion of two kinds of cytoplasmic particles of different origins, *viz,* an element of endoplasmic reticulum as a vehicle and coated vesicles conveying tyrosinase from Golgi apparatus (Seiji, 1981; Mishima 1992), and that during maturation of these organelles, there exist at least two morphologically different types of immature melanosomes, the internal structure of which is composed of either numerous fine vesicles (multivesicular premelanosomes) or fibrous materials in a lattice-like arrangement (fibrillar premelanosomes) (Turner *et al.*, 1975; Seiji 1981).

Plate 2. Electron micrographs of cortical regions of the eye of medaka embryos after pigmentation. Fig. 6. Stage 30. Appearance of choroid melanophores between pigment epithelial cells and choroid iridophores. Scale bar: 5 μm. Fig. 7 An enlarged view of choroid iridophore appearing in the embryo at stage 30. Note the presence of many multivesicular organelles (arrowheads), one of which contains minute reflecting platelet (arrow). Scale bar: 1 μm. Fig. 8. Stages 31–32. Alignment of pigment epithelial cells, choroid melanophores and choroid iridophores into three distinct layers. Scale bar: 5 μm. Fig. 9 At hatching. The completion of histogenesis. Note that receptor cells, pigment epithelial cells, choroid melanophores and choroid iridophores exhibit their respective morphological characteristics, indicating establishment of cytodifferentiation. It is apparent that pigment epithelial cells and choroid melanophores differ morphologically. Scale bar: 5 μm. Fig. 10. An enlarged view of long, ellipsoidal melanosomes found in the pigment epithelial cells. Note the presence of many fine hollow spaces within their matrices, indicating that melanogenesis occurred via formation of a multivesicular structure. Scale bar: 1 μm. Abbreviations: b, blood cell; i, choroid iridophore; m, choroid melanophore; p, pigment epithelial cell; pr, presumptive receptor cell; r, receptor cell.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The former most often appear as spherical organelles whereas the latter do so as long, ellipsoidal ones. So far, it is accepted that the former type of melanosome which occurs in a variety of species of fish is formed via the so-called multivesicular bodies, which are formed by fusion of two types of elements (Turner *et al.*, 1975; Nakajima and Obika, 1986), whereas the latter which occur commonly in mammalian melanocytes and pigment epithelial cells is taken as a standardized prototype of melanosome formation (Toda *et al.*, 1976).

The mechanisms for melanosome formation from mutlivesicular bodies have been established based on observations summarized as follows: 1) the fusion of small vesicles with a large vesicle which acts as the vehicle for melanosomes development, 2) the presence of fine hollows in the matrices of melanosomes which correspond to the inner spaces of the small vesicles, the limiting membranes of which are inverted upon fusion, and 3) an absence of fibrous structure with a regular arrangement (Turner *et al.*, 1975). The present study has shown that in the eye of medaka, melanogenesis first occurs in the pigment epithelial cells at stage 26, in which the prospective pigment epithelium as a thin layer covers the optic cup entirely, and that multivesicular premelanosomes are primarily responsible for melanogenesis. It is also shown that the fibrillar premelanosomes appear in this tissue at a limited frequency. Although little is known about the intrinsic difference between mutlivesicular and fibrillar premelanosomes, the concomitant formation of these two different types of premelanosomes is also reported in pigment epithelial cells of the eye of *Rana pipiens* tadpoles (Eppig, 1970) and in skin melanophores of the young fry of medaka (Nakajima and Obika 1986). The observations reported herein indicate that this concomitant formation also occurs in the pigment epithelium of developing embryos of the medaka.

In spite of the concomitant appearance of two types of premelanosomes, pigment epithelial cells in the eye of adult medaka fish contain mostly long, ellipsoidal melanosomes, almost all of which contain numerous fine hollows, suggesting formation via the mechanism proposed for the formation of multivesicular premelanosomes. With respect to the morphological properties of fully pigmented melanosomes, an apparent difference is observed between pigment epithelial cells and choroid melanophores, despite the similar tissue location. This may result mainly from the difference in their developmental origin (Hörstadius, 1950; Weston, 1970, 1986). In this connection, it would be interesting to determine the factors regulating the shape and size of the melanosomes.

In the present study, it is shown that the formation of reflecting platelets in choroid iridophores begins at stage 28 or slightly earlier, at which those cells are in close contact with pigment epithelial cells. These observations would imply that pigment epithelial cells play a role in inducing reflecting platelet formation in the iridoblasts or their precursor cells as terminal differentiation. As indicated herein, the developing iridoblasts contain an abundance of multivesicular bodies, some of which contain small reflecting platelet-like structures. This suggests that reflecting platelets are formed through a multivesicular body as multivesicular premelanosomes in melanophores and pterinosomes in xanthophores of this species (Obika 1993). A similar observation was reported in the dermal iridophores of *Pachymedusa dacnicolor* (Bagnara *et al.*, 1979). All these findings essentially support the presumption that three different types of skin pigment cells present in lower vertebrates have a common basis for expression of pigment phenotypes.

The present study also shows that choroid melanophores appear at stage 30 in the boundary where the two layers of pigment epithelial cells and choroid iridophores closely face each other and that they then form a thin continuous membrane layer covering the entire pigment epithelium. Such a pattern of histogenesis would explain well the gross appearance of the eye of this fish around hatching, at which stage it is iridescent on the outside and black inside.

It is apparent that the number of melanophores must increase to form a continuous layer in the choroid. As to the source of these melanophores, there are two possibilities: (1) a limited number of melanoblasts firstly migrate to this location, then proliferate to a sufficient number to cover the surface of the pigment epithelium and finally differentiate into melanophores, or (2) melanoblasts or melanophores successively migrate to this location from areas possibly among nearby mesenchymal cells. Thus far, several investigators have studied histogenesis of the retina in fish (Hollyfield, 1972; Sharma and Ungar, 1980; Johns, 1981; Negishi *et al.*, 1990); however, no studies on the choroid tissue have been reported. It is apparent that in medaka, the size of the eye and

thickness of the choroid increase with progress of post embryonic development. It is also observed that a thin layer of choroid melanophores covers the outer surface of the preexisting iridophore layer. Such a histogenesis of the eye in the wild-type strain of medaka would provide a basis for understanding sporadic or experimentally produced pigmentation mutants of this species.

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