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Shift of Chloride Cell Distribution during Early Life Stages in Seawater-Adapted Killifish, *Fundulus heteroclitus*

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ABSTRACT—The shift of chloride cell distribution was investigated during early life stages of seawater-adapted killifish (*Fundulus heteroclitus*). Chloride cells were detected by immunocytochemistry with an antiserum specific for Na^+, K^+-ATPase in whole-mount preparations and paraffin sections. Chloride cells first appeared in the yolk-sac membrane in the early embryonic stage, followed by their appearance in the body skin in the late embryonic stage. Immunoreactive chloride cells in the yolk-sac membrane and body skin often formed multicellular complexes, as evidenced by the presence of more than one nucleus. The principal site for chloride cell distribution shifted from the yolk-sac membrane and body skin during embryonic stages to the gill and opercular membrane in larval and later developmental stages. Our observations suggest that killifish embryos and newly-hatched larvae could maintain their ion balance through chloride cells present in the yolk-sac membrane and body skin until branchial and opercular chloride cells become functional.

INTRODUCTION

In adult teleosts, the gills, kidney and intestine are important osmoregulatory organs, maintaining ionic composition and osmolality of the fluid at levels different from external environments. In particular, chloride cells in the gills are responsible for the secretion of Na^+ and Cl^- in fish adapted to seawater (SW) (Zadunaisky, 1984). In embryos and newly-hatched larvae, however, these osmoregulatory organs are not yet developed or not fully functional. Nevertheless, fish embryos and larvae are also able to maintain the hydromineral balance. In several teleost species, a rich population of chloride cells have been demonstrated in the yolk-sac membrane and body skin of embryos and newly-hatched larvae, which may act as ion-secreting cells in place of gill chloride cells in adult fish (Hwang and Hirano, 1985; Alderdice, 1988; Ayson *et al*., 1994; Kaneko *et al*., 1995; Shiraishi *et al*., 1997; Sasai *et al*., 1998; Hiroi *et al*., 1998). Thus, the principal site for chloride cell distribution is expected to shift from the yolk-sac membrane and body skin to the gills as fish grow; however, little information is available on the transition of chloride cell distribution during fish development (Hiroi *et al*., 1998; Sasai *et al*., 1998).

Killifish (*Fundulus heteroclitus*) is a euryhaline species, inhabiting fresh water (FW), brackish water and SW (Griffith, 1974; Hardy, Jr., 1978). Because of the high density of chloride cells, the opercular membrane of killifish has been used as an experimental model for transepithelial ion transport in relation to ionic and osmotic regulation. Although a lot of electrophysiological studies have been performed using the opercular membrane of killifish, only a few studies have focused on gill chloride cells (Marshall *et al*., 1995, 1997; Zadunaisky 1997).

In the present study, to obtain basic information on chloride cells for future studies concerning ion-transporting mechanisms of chloride cells and their involvement in the excellent euryhalinity, we attempted to investigate the changes in chloride cell distribution during early life stages of killifish.

MATERIALS AND METHODS

Fish

Adult killifish of Arasaki strain (Shimizu, 1997) reared in SW were obtained from National Research Institute of Fisheries Science (Yokosuka, Japan) in January 1998. The fish were kept in a 250-*l* indoor tank with recirculating SW, and fed on artificial tilapia pellets, Tilapia 41 S (Shikoku Kumiai Shiryo, Tokushima, Japan), twice a day. In May 1998, to obtain fertilized eggs, two pairs of mature fish were introduced to a 8-mm mesh cage placed in a 15-*l* tank. The water temperature was not controlled, ranging from 20 to 25°C. Naturally spawned and fertilized eggs sank through the mesh to the bottom of the tank. The eggs were collected with a large-mouthed pipette in the morning and evening, and incubated in 1-*l* plastic beakers at 20°C with aeration. Dead eggs were removed to prevent the water from fouling. At the incubation temperature of 20°C, the egg typically hatched at 11 days after fertilization. Larvae were fed on

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brine shrimp (*Artemia salina*) and Tetramin Baby Food (Tetra, Germany) a few times a day.

Sampling

To observe the development of chloride cells in the yolk-sac membrane and body skin during early life stages, embryos and larvae were sampled at 2, 4, 6, 8, 10, 15, 20 and 25 days after fertilization. After anesthesia with 2-phenoxyethanol, the fish were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at room temperature and preserved in 70% ethanol at 4°C. In the case of embryos, the egg shell was removed prior to fixation. For each developmental stage, about 30 samples were fixed. For the examination of chloride cells in adult fish, the gills and operculum were removed from anesthetized fish (body weight, 7.0–13.0 g, n = 5) of both sexes, and fixed and preserved in the same way as described above.

Antiserum

A polyclonal antiserum was raised in a rabbit against a synthetic peptide based on sequences of high homology and the areas of hydrophilicity of Na^+, K^+-ATPase α-subunit as described in Ura *et al*. (1996). The antigen designed was Cys-Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys-Ser. The antigen conjugated with keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) was emulsified with complete Freund's Adjuvant, and immunization was performed in a New Zealand white rabbit.

Western blot analysis

The specificity of the raised antiserum, named NAK121, was confirmed using Western blot analysis. Membrane fractions were prepared from the gills of SW-adapted killifish. The gills were homogenized on ice in homogenization buffer consisting of 25 mM Tris-HCl (pH 7.4), 0.25 M sucrose and a pellet of Complete Protein Inhibitor (Boehringer Mannheim, Mannheim, Germany). The homogenate was first centrifuged at 4,500 × g for 15 min, and the supernatant was subjected to 200,000 × g for 1 hr. The pellet was resuspended in the homogenization buffer. All above procedures were performed at 4°C. Protein content of the sample was quantified by the BCA Protein Assay kit (Pierce, Rockford, IL). The samples (12 μg) were solubilized in a sample loading buffer (0.25M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 30% glycerol, 0.01% bromophenol) and heated at 70°C for 15 min. They were separated by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels. As molecular length markers, Prestained SDS-PAGE Standards (188 – 7.3 kDa, BIO-RAD, Hercules, CA) were electrophoresed in parallel. After electrophoresis, the protein was transferred from the gel to a polyvinyliden difluoride membrane (ATTO, Tokyo).

The membranes were preincubated in 50 mM Tris buffered saline (TBS, pH 7.6) containing 0.05% Triton X-100 and 2% skim milk at 4°C overnight and incubated with the antiserum for 1 hr at room temperature. The antiserum was diluted at 1 : 500 with 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 2% normal goat serum (NGS), 0.1% bovine serum albumin (BSA), 0.02% KLH and 0.01% sodium azide (NB-PBS). The specificity of the immunoreaction was also confirmed by incubating the membranes with normal rabbit serum (NRS) at the same dilution. After rinsing in washing buffer (TBS, 0.05% Triton X-100, 0.2% skim milk), the membranes were stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al*., 1981), using commercial reagents (Vectastain ABC kit, Vector Laboratories, Burlingame, USA).

Whole-mount immunocytochemistry

For the detection of chloride cells in the yolk-sac membrane and body skin of embryos and larvae, whole-mount samples were immunocytochemically stained with the antiserum specific for Na^+, K^+-ATPase, a key enzyme for ion-transporting functions of chloride cells. The specific antibody was affinity-purified and labeled with fluorescein isothiocyanate (FITC) as a fluorescent marker. For the observations on chloride cells in the gills and opercular membrane of adult fish, gill filaments were removed from the gill arch and the opercular membrane was peeled off from the operculum prior to the whole-mount immunostaining.

After washing in 0.01 M PBS, the whole-mount samples were incubated overnight at 4°C with FITC-labeled anti-Na^+, K^+-ATPase diluted 1 : 500 with PBS containing 0.05% Triton X-100, 10% NGS, 0.1% BSA, 0.02% KLH and 0.01% sodium azide. The samples were then washed in PBS at least for 1 hr, placed in a chamber slide, coverslipped, and observed with a conforcal laser scanning microscope (LSM, LSM 310, Zeiss, Germany). The 488 nm line of an argon ion laser was used as the excitation wavelength, and the emission was collected at 515–565 nm. The digital images were stored in a MO disk for quantitative analyses.

Light microscopy

To reveal the development of the gills and gill chloride cells, conventional light microscopic observations were also made in the embryos, larvae and adult fish. After fixation, the samples were dehydrated in ethanol and embedded in paraplast. Serial cross sections (4 μm) of embryos and larvae, as well as gill filaments of adult fish, were divided into two groups and mounted on separate gelatin-coated slides.

To observe the development of the gills, one group of the sections was stained with hematoxylin and eosin. For the specific detection of chloride cells, the other group of the sections was immunocytochemically stained with the antiserum specific for Na^+, K^+-ATPase by the ABC method (Hsu *et al*., 1981) using commercial reagents (Vectastain ABC kit). In brief, deparaffined sections were incubated sequentially with: 1) 0.6% H_2O_2 for 30 min, 2) 2% normal goat serum for 30 min, 3) anti-Na^+, K^+-ATPase serum overnight at 4°C, 4) biotinylated goat anti-rabbit IgG for 30 min, 5) ABC for 1 h, and 6) 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 4 min (Uchida *et al*., 1996). The antiserum was diluted 1:4000 with NB-PBS. The sections were observed under a light microscope (Nikon, Tokyo, Japan) equipped with a differential interference contrast device.

Quantitative analyses of chloride cells

The size of cells stained by the whole-mount immunocytochemistry was measured on stored LSM images by means of an internal program. The area measurement of branchial chloride cells in the paraplast sections stained with hematoxylin and eosin were carried out with an image processor, ARGUS-20 (Hamamatsu Photonics, Hamamatsu, Japan). When chloride cells fused to form multicellular complexes, the area of the complex was measured. Data of the chloride cell size were obtained from 10 cells per individual (n = 3) in early developmental stages and from 20 cells per individual (n = 3) in adult fish.

RESULTS

Western blot analysis

Membrane fractions were prepared from the gills of SW-adapted killifish and analyzed by Western blot using the antiserum against the synthetic peptide based on sequences of high homology of Na^+, K^+-ATPase α-subunit (Fig. 1). The antibody recognized a major protein band with an approximate size of 100 kDa. No band appeared when membranes were incubated with NRS.

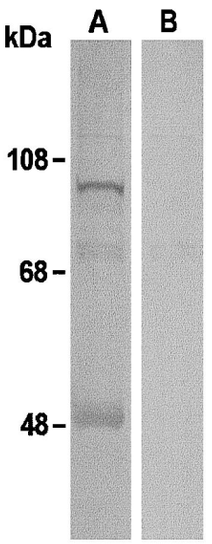

Fig. 1. Western blot analysis for Na^+, K^+-ATPase protein expressed in SW-adapted killifish. The membranes were incubated with anti-Na^+, K^+-ATPase (lane A) and normal rabbit serum (lane B). Positions of molecular weight markers, expressed in kilodaltons, are indicated on the left side of the figure.

Chloride cells in the yolk-sac membrane and body skin

In the yolk-sac membrane, Na^+, K^+-ATPase immunoreactive chloride cells were detected from day 2 (2 days after fertilization) to day 10. In embryos at day 2, chloride cells were small (mean ± SEM, 208 ± 8 μm^2) and sparsely distributed over the yolk-sac membrane (Figs. 2A, 3A). At days 4–10, a rich population of chloride cells were detectable in the yolk-sac membrane, their mean size ranging from 339 μm^2 to 351 μm^2 (Figs. 2B, C and 3B, C). At day 15, however, yolk-sac chloride cells disappeared as the yolk absorption was completed (Fig. 2D).

In addition to the yolk-sac membrane, chloride cells also appeared in the body skin at day 6 and later developmental stages (Fig. 2C, D). Chloride cells in the body skin were rather small (132 ± 6 μm^2) at day 6 (Fig. 4A), increased to the maximal size of 251 ± 13 μm^2 at day 10 (Fig. 4B), and decreased thereafter (150 ± 6 μm^2 at day 15 (Fig. 4C), 160 ± 10 μm^2 at day 20, 169 ± 7 μm^2 at day 25).

Immunoreactive chloride cells in the yolk-sac membrane and body skin during early developmental stages often appeared to be closely associated with each other to form cellular complexes, as evidenced by the presence of

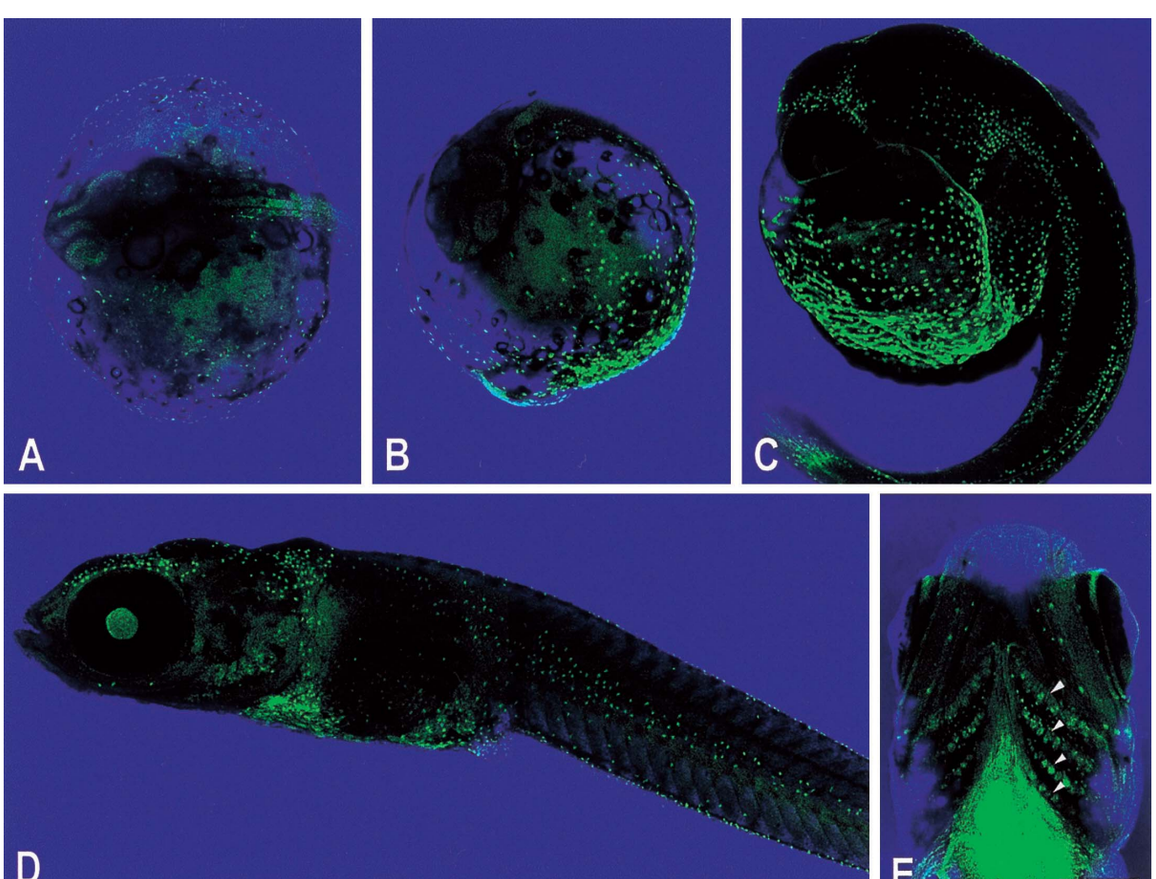

Fig. 2. Chloride cells in the yolk-sac membrane and body skin of killifish embryos and larvae at 2 (A), 4 (B), 8 (C) and 15 days (D, E) after fertilization. Chloride cells were detected by whole-mount immunocytochemistry with FITC-labeled anti-Na^+, K^+-ATPase, and observed by conforcal laser scanning microscopy. The ventral view of a larva (E) shows the arrays of chloride cells (arrowheads) in the developing gills. Bar: 500 μm.

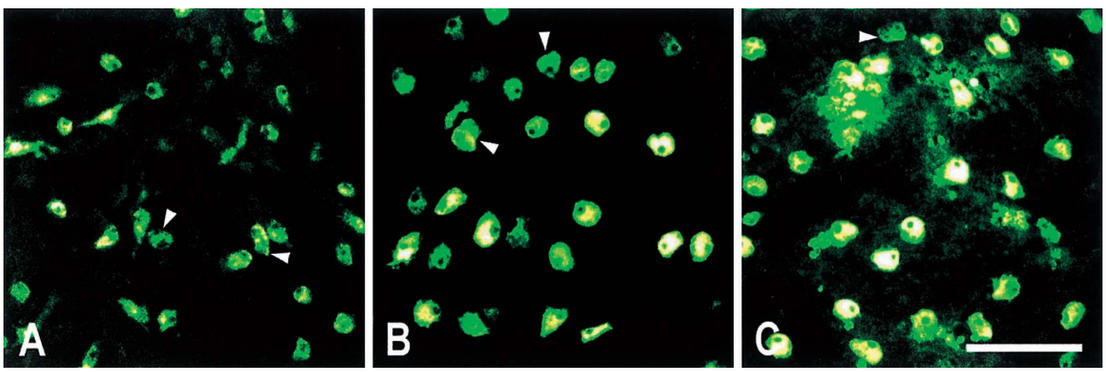

Fig. 3. Magnified views of chloride cells in the yolk-sac membrane of killifish embryos at 2 (A), 4 (B) and 6 (C) days after fertilization. Arrowheads indicate chloride cell complexes. Bar: 100 μm.

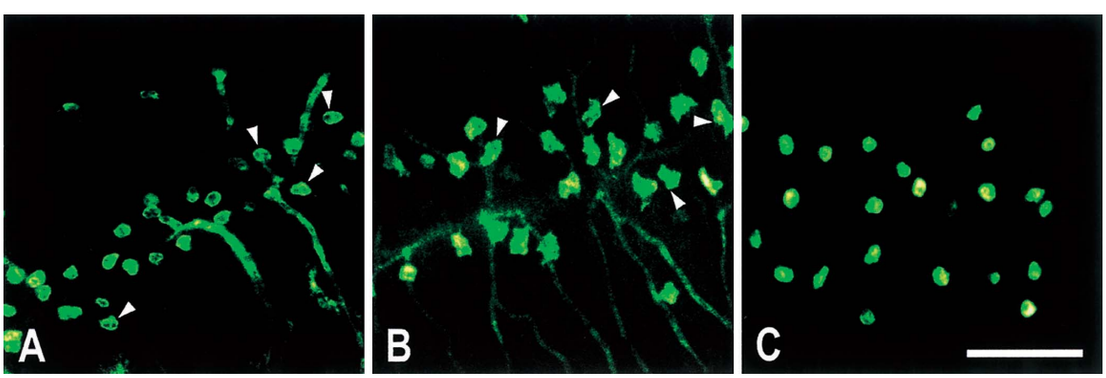

Fig. 4. Magnified views of chloride cells in the body skin of killifish embryos and larvae at 6 (A), 10 (B) and 15 (C) days after fertilization. In some cases, blood vessels show non-specific reaction. Arrowheads indicate chloride cell complexes. Bar: 100 μm.

more than one immunonegative nucleus (Figs. 3, 4). Although precise discrimination between dispersed cells and chloride cell complexes was difficult in the whole-mount preparations because of low resolution, dispersed cells were usually round in shape while large cellular complexes appeared polygonal. The cell boundary within the complex was indistinct.

Branchial and opercular chloride cells in embryos and larvae

The gills were not detectable in embryos at days 2 and 4. Four pairs of gill arches first appeared at day 6, although gill filaments and lamellae were not yet developed. Gill filaments started developing at day 10 (Fig. 5A), followed by the development of gill lamellae at day 15 (Fig. 5C).

Eosinophilic chloride cells of spherical shape were first observed in the gill filaments at day 10, when gill lamellae were not yet formed (Fig. 5A). However, these cells lacked apical pits and faintly immunoreactive to the anti-Na^+, K^+-ATPase (Fig. 5B). Eosinophilic and intensively immunoreactive chloride cells equipped with apical pits were evident in the gill filaments in larvae at days 15–25, although chloride cells were absent from gill lamellae (Figs. 2E, 5C-F). During these developmental stages, a rich population of immunoreactive chloride cells were also observed in the opercular membrane, with which the operculum was lined (Fig. 5). The chloride cells incorporated in the thin epitherium of the opercular membrane were flat in shape, which contrasted with the spherical shape of the gill chloride cells. Similar to those in the yolk-sac membrane and body skin, chloride cells in the gills and opercular membrane often formed multicellular complexes, which shared a common apical pit. During the period from day 10 to day 25, when chloride cells were detected in the gills, the cell size remained rather constant, the mean size ranging from 77 μm^2 to 94 μm^2.

Branchial and opercular chloride cells in adult fish

In adult fish, four pairs of gills were well developed. Each gill consists of a gill arch, filaments and lamellae. Two rows of gill filaments radiated posteroventrally from the arch. The gill lamellae extended laterally from both sides of the filament.

A large number of immunoreactive chloride cells (81 ± 2 μm^2) were detected in the gill filaments (Fig. 6). These cells were round or columnar in shape, and mostly distributed along a flat region of the trailing edge (afferent vascular side) and between lamellae in the filament. No chloride cells were detectable in the gill lamellae. In addition to the gills, a large number of chloride cells were also present in the

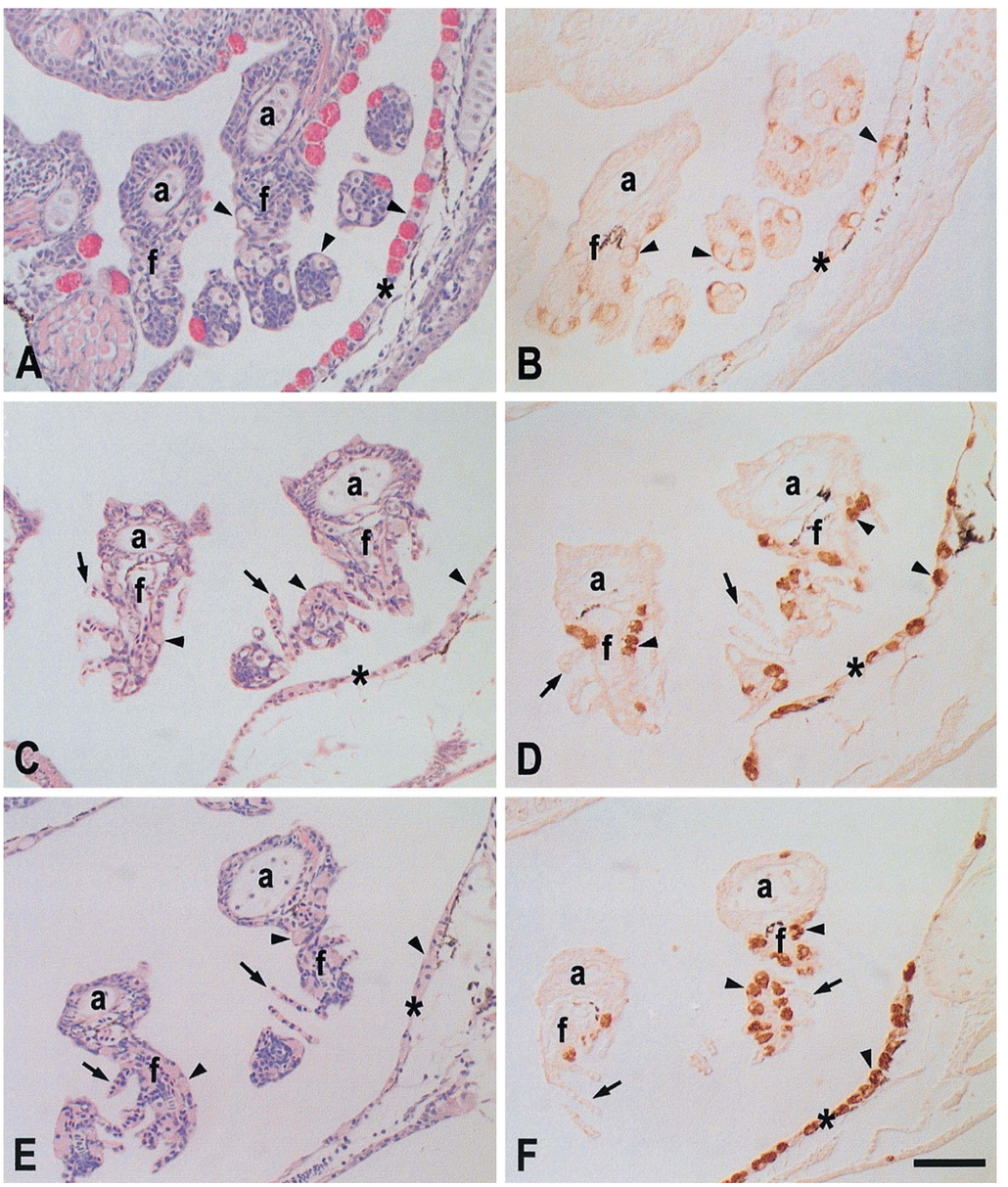

Fig. 5. Cross sections of killifish embryos and larvae at 10 (A, B), 15 (C, D) and 25 (E, F) days after fertilization, showing the development of the gills. A, C, E, Hematoxylin and eosin staining. B, D, F, Immunocytochemical detection of chloride cells with anti-Na^+, K^+-ATPase. Chloride cells (arrowheads) are observed in the gill filaments (f) and the opercular membrane (asterisks), but not in the gill lamellae (arrows). a, gill arch. Bar: 50 μm.

opercular membrane (Fig. 7). Some chloride cells in the gills and opercular membrane of adult fish also formed multicellular complexes.

DISCUSSION

The chloride cells in the yolk-sac membrane and body skin during embryonic and larval stages have been demonstrated in some teleost species by whole-mount immunostaining with antiserum specific for Na^+, K^+-ATPase (Shiraishi *et al.*, 1997; Sasai *et al.*, 1998; Hiroi *et al.*, 1998). In Japanese flounder (*Paralichthys olivaceus*), Hiroi *et al.* (1998) have revealed the shift of chloride cell distribution from the yolk-sac membrane and body skin to the gills during early developmental stages. In the present study, the application of LSM in combination with conventional light microscopy enabled us to observe clear pictures of the chloride cell distribution in the yolk-sac membrane and body skin during early life stages, as well as in the gills and opercular membrane in adult fish. As summarized in Figure 8, our observations clearly showed the transitional process of chloride cell distribution during the development of killifish.

In this study, the antiserum was raised against a synthetic peptide of Na^+, K^+-ATPase α-subunit, which was

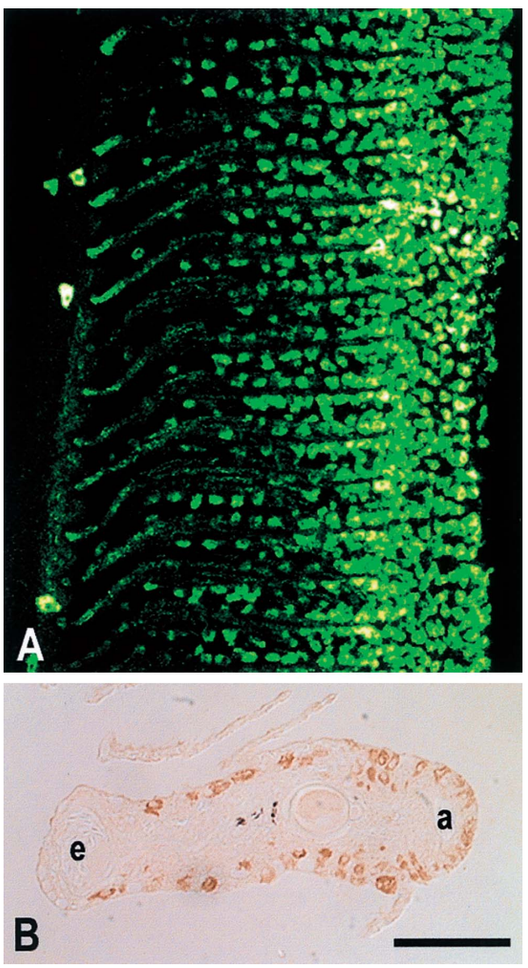

Fig. 6. Immunocytochemical detection of gill chloride cells in adult killifish. A, Whole-mount staining of a gill filament with FITC-labeled anti-Na^+, K^+-ATPase. B, Cross section of a gill filament stained with anti-Na^+, K^+-ATPase. a, afferent filament artery; e, efferent filament artery. Bar: 100 μm.

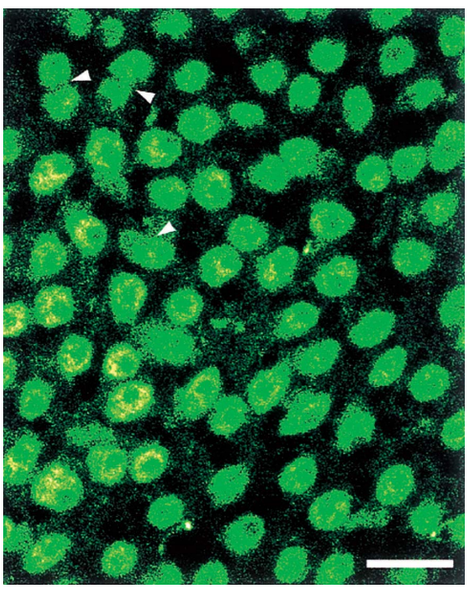

Fig. 7. Chloride cells in the opercular membrane of adult killifish, detected by whole-mount immunocytochemistry with FITC-labeled anti-Na^+, K^+-ATPase. Arrowheads indicate chloride cell complexes. Bar: 25 μm.

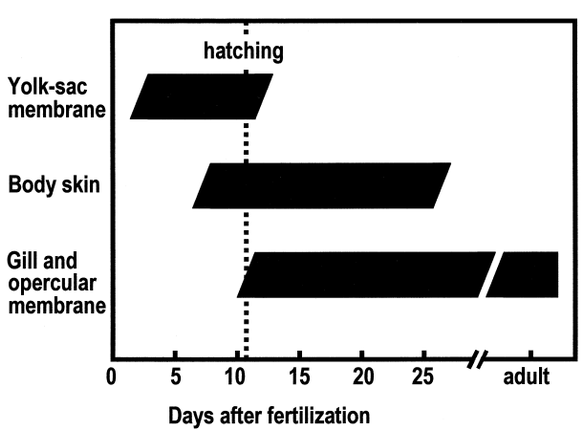

Fig. 8. Changes in chloride cell distribution during early life stages. Chloride cells first appear in the yolk-sac membrane in the early embryonic stage, followed by their appearance in the body skin in the late embryonic stage. The principal site for chloride cell distribution then shifts to the gills and opercular membrane as fish grow.

based on a highly conserved sequence in different vertebrates and invertebrates (Ura *et al.*, 1996). The specificity of the antiserum was evaluated by Western blot analysis. Na^+, K^+-ATPase is composed of two subunits; that is, α-subunit with a molecular weight of about 100 kDa and β-subunit with a molecular weight of about 50 kDa. The antiserum reacted with a single, major protein band of about 100 kDa, corresponding to a predicted molecular mass of Na^+, K^+-ATPase α-subunit (Hwang *et al.*, 1998; Lucu and Flik., 1999). Therefore, the result strongly suggest the high specificity of the antiserum.

In embryos at days 2 and 4, immunoreactive chloride cells were observed only in the yolk-sac membrane. From day 6 to day 10, embryonic chloride cells were distributed both in the yolk-sac membrane and in the body skin. In embryos at day 10, just before hatching, chloride cells first appeared in the filaments of developing gills, although these cells were eosinophilic but faintly immunopositive to the anti-Na^+, K^+-ATPase. Immunopositive chloride cells were consistently observed in the gill filaments from larval to adult stages, whereas cells in the yolk-sac membrane disappeared by day 15. These observations indicate that chloride cells first appear in the yolk-sac membrane in the early embryonic stages, followed by their appearance in the body skin in the late embryonic stage. The principal site for chloride cell distribution shifts from the yolk-sac membrane and body skin during embryonic stages to the gills and opercular membrane in larval and later developmental stages.

In eel, however, the chloride cells were detected in the yolk-sac membrane, but rarely observed in the other part of

the body surface in a late embryonic stage (Sasai *et al*., 1998). In Japanese flounder, newly-hatched larvae possessed a number of complexed chloride cells in the body skin, whereas branchial chloride cells were not detected until 8 days after hatching (Hiroi *et al*., 1998). Although the distributional shift of chloride cells from the yolk-sac membrane and body skin to the gills seems to be common to teleost species, the timing of the shift may differ in different species.

In killifish embryos at day 2, chloride cells in the yolk-sac membrane were rather small. At days 4–10, these cells became larger and more immunoreactive, and the formation of multicellular complexes became evident. In the body skin, the chloride cell size increased until the appearance of branchial chloride cells at day 10, but decreased thereafter. The formation of cellular complexes concomitant with increase in cell size is considered to be characteristics of SW-type chloride cells responsible for the secretion of Na^+ and Cl^-. In embryos and larvae of tilapia (*Oreochromis mossambicus*), Ayson *et al*. (1994) observed that chloride cells in the yolk-sac membrane were larger in SW than in FW. Enlarged chloride cells in the yolk-sac membrane of SW tilapia result from the formation of multicellular complexes. A chloride cell complex consists of a main chloride cells and one or two accessory cells, which are considered as immature chloride cells (Shiraishi *et al*., 1997). Chloride and accessory cells in the complex share a common apical pit. Such structures of the complex may be advantageous to Na^+ extrusion, since Na^+ is probably secreted through a paracellular pathway down its electrical gradient in SW (Marshall, 1995; McCormick, 1995).

The chloride cell size differed in the different distributional sites. One notable morphological difference between chloride cells in the yolk-sac membrane and body skin and in the gill filaments was in the shape of the cell. Chloride cells in the yolk-sac membrane and body skin were flattened, whereas branchial chloride cells were spherical or columnar in shape. In addition to this morphological difference, the method of area measurement of chloride cells was different in the site of chloride cell distribution. In the yolk-sac membrane and body skin, the cell size was measured in the samples stained by the whole-mount immunocytochemistry. In the gills, however, the cross-sectional area of chloride cells in the paraplast sections was measured. Therefore, it would not be appropriate to simply compare measured chloride cell size among the different distibutional sites.

In killifish, although gill arches were already recognizable at day 6, gill filaments and lamellae were not yet developed. At the late embryonic stage of day 10, buds of the gill filaments occurred on the gill arch, where eosinophilic chloride cells were detected. However, these chloride cells may not be functional, since these branchial chloride cells were faintly immunoreactive to the antiserum specific for Na^+, K^+-ATPase, a key enzyme for ion-transporting functions of chloride cells (McCormick, 1995). This is also supported by our observation that apical pits of branchial chloride cells at day 10 were not open to the external environments.

Chloride cells intensively immunoreactive to the anti-Na^+, K^+-ATPase first appeared in the gills of larvae at day 15, when gill lamellae started developing. The development of gill lamellae markedly enlarges the area of the respiratory surface in the gills. Thus, the gills may start functioning both as a respiratory organ and as an osmoregulatory organ in larvae around day 15, corresponding to 4 days after hatching. Hiroi *et al*. (1998) reported, on the other hand, that the primary function of the gills during the early development of Japanese flounder might be ion regulation rather than gas exchanges, because branchial chloride cells first appeared in gill filaments before the differentiation of lamellae.

Intensively-immunoreactive chloride cells appeared in the opercular membrane at day 15, when functional chloride cells appeared in the gills. In adult fish, chloride cells were densely distributed in the opercular membrane. These observations suggest that, together with the gills, the opercular membrane is the major site for chloride cell distribution in larval and later developmental stages of killifish. To examine transepithelial ion transport through chloride cells, the opercular membrane has been used as an experimental model. According to our observations, chloride cells in the opercular membrane and gills seem to be identical at least in terms of developmental and morphological features, suggesting that the opercular membrane can be utilized for investigation of the chloride cell function in place of the gills.

In the present study, we demonstrated the developmental shift of chloride cell distribution in SW-adapted killifish. The occurrence of chloride cells in the yolk-sac membrane and body skin before the development of the gills indicates the physiological importance of chloride cells throughout the life stages of killifish. Since chloride cells are the principal site for ion excretion in hyperosmotic environments, killifish could maintain their ion balance in the body fluid through chloride cells present in the yolk-sac membrane and body skin during the early life stages in place of gill and opercular chloride cells in adult fish.

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