

Microtubule-Dependent Changes in Morphology and Localization of Chloride Transport Proteins in Gill Mitochondria-Rich Cells of the Tilapia, *Oreochromis mossambicus*

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ABSTRACT The tilapia (*Oreochromis mossambicus*) is a euryhaline fish exhibiting adaptive changes in cell size, phenotype, and ionoregulatory functions upon salinity challenge. Na⁺/Cl⁻ cotransporter (NCC) and Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) are localized in the apical and basolateral membranes of mitochondria-rich (MR) cells of the gills. These cells are responsible for chloride absorption (NCC) and secretion (NKCC), respectively, thus, the switch of gill NCC and NKCC expression is a crucial regulatory mechanism for salinity adaptation in tilapia. However, little is known about the interaction of cytoskeleton and these adaptive changes. In this study, we examined the time-course of changes in the localization of NKCC/NCC in the gills of tilapia transferred from fresh water (FW) to brackish water (20‰) and from seawater (SW; 35‰) to FW. The results showed that basolateral NKCC disappeared and NCC was expressed in the apical membrane of MR cells. To further clarify the process of these adaptive changes, colchicine, a specific inhibitor of microtubule-dependent cellular regulating processes was used. SW-acclimated tilapia were transferred to SW, FW, and FW with colchicine (colchicine-FW) for 96 h. Compared with the FW-treatment group, in the MR cells of colchicine-FW-treatment group, (1) the average size was significantly larger, (2) only wavy-convex-subtype apical surfaces were found, and (3) the basolateral (cytoplasmic) NKCC signals were still exhibited. Taken together, our results suggest that changes in size, phenotype, as well as the expression of NCC and NKCC cotransporters of MR cells in the tilapia are microtubule-dependent. *J. Morphol.* 277:1113–1122, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: colchicine; ionocytes; Na⁺/Cl⁻ cotransporter; Na⁺/K⁺/2Cl⁻ cotransporter; teleost

INTRODUCTION

In most teleosts, mitochondria-rich (MR) cells, also known as chloride cells or ionocytes, are the major ion-transporting cells in the epithelium of gills and are found to be pleomorphic. In hypertonic environments (e.g., seawater [SW]), the MR cells

exhibited deeply invaginated surfaces with smaller orifices and high electron density in the cytoplasm. In hypotonic environments (e.g., fresh water [FW]), however, the MR cells exhibited low electron density in the cytoplasm and only slightly invaginated surfaces, similar to membrane patches with short cellular projections (Hwang and Lee, 2007; Kaneko et al., 2008). Polymorphism in the apical membrane configuration of MR cells is a structural modification of active MR cells (Lin and Hwang, 2004). Because different phenotypes of branchial MR cells are transformable and reversible in response to changing levels of environmental ions, the heterogeneity of MR cell subtypes is to perform different ionoregulatory functions (Hwang and Lee, 2007; Hwang et al., 2011; Hiroi and McCormick, 2012). The switch of MR cell subtypes is a model for the plasticity of ion-transporting epithelial cells, allowing the examination of de novo generation of transporters and their insertion in polarized membrane areas, as well as the redistribution of transporters in existing ionocytes (Marshall, 2002; Hwang and Lee, 2007; Kaneko et al., 2008).

Subtypes of gill MR cells in the euryhaline tilapia (*Oreochromis mossambicus*) categorized

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according to ultrastructures of their apical openings have been observed in previous studies. Three subtypes with distinct apical surfaces, including wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III) were observed in FW tilapia, whereas in SW-adapted tilapia, only subtype III of MR cells with significant apical crypts was found (Lee et al., 1996; Heijden et al., 1997; Wang et al., 2009; Tang and Lee, 2011a). Previous study has shown that when tilapia were transferred from SW to FW, most subtype III (i.e., SW subtype) MR cells were transformed to subtype-I MR cells within 3 h (Lin et al., 2004). Modification of subtypes of tilapia MR cells reached a steady state at 96 h after transfer to a new media. The time-lag and transformations of tilapia MR cell subtypes were similar in artificial water with different Cl^- levels and media of various salinities (Chang et al., 2003; Lin et al., 2004; Wang et al., 2009). Apparently, the transition between different subtypes of MR cells reflected a modulation of functions in response to changes in environmental ion compositions or salinities (Hwang and Lee, 2007; Hwang et al., 2011; Hiroi and McCormick, 2012).

Previous studies have illustrated the ion transporter systems of branchial MR cells (Marshall, 2002; Hwang et al., 2011; Hiroi and McCormick, 2012). In FW fish, multiple functional and structural changes were observed in the branchial MR cells in relation to ion absorption. In the current model of branchial MR cells in SW-acclimated teleosts, salts were secreted by the basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) and Na^+-K^+ -ATPase (NKA), as well as the apical cystic fibrosis transmembrane conductance regulator for Cl^- and the "leaky" tight junction for Na^+ , respectively (Marshall, 2002; Kaneko et al., 2008; Hiroi and McCormick, 2012). Among them, the furosemide- and bumetanide-sensitive NKCC, a member of the chloride-cation cotransporter family (i.e., solute carrier family 12, SLC12), is widely distributed in different vertebrate species (Hebert et al., 2004; Gamba, 2005). It is one of the transmembrane proteins in which the pattern of expression in gill epithelium is ultimately responsible for the adaptation of teleosts to environmental salinity (Kaneko et al., 2008; Hwang et al., 2011; Hiroi and McCormick, 2012). The coupled and electrically neutral movement of Na^+ , K^+ , and Cl^- serves a number of physiological functions in different cell types. NKCC includes two isoforms, the secretory isoform (NKCC1) and the absorptive isoform (NKCC2), located at the basolateral and apical sides of epithelial transporting cells, respectively (Hebert et al., 2004; Gamba, 2005). In certain teleosts, including tilapia, NKCC1 is expressed in most organs and tissues including osmoregulatory organs, whereas NKCC2 appears to be expressed mainly in the kidney and/or intes-

tine (Cutler and Cramb, 2002, 2008; Hiroi et al., 2008; Kang et al., 2010; Kato et al., 2011). NKCC plays a central role in cell volume homeostasis, maintenance of electrolyte contents, and transepithelial ion and water movement in polarized cells (Hebert et al., 2004; Gamba, 2005). Using a heterologous antibody (T4) known to react with both NKCC isoforms (Lytle et al., 1995), the protein abundance of NKCC was upregulated after SW-acclimation in certain teleosts (Tipsmark et al., 2002; Wu et al., 2003; Tang and Lee, 2007; Kang et al., 2010; Yang et al., 2011). Meanwhile, using the same antibody, NKCC was immunolocalized at the basolateral side of gill MR cells of SW-acclimated teleosts (Tipsmark et al., 2002; Wu et al., 2003; Kang et al., 2010, 2012; Yang et al., 2011; Hsu et al., 2014). After SW-acclimation, the basolateral cell location and increasing abundance of gill NKCC indicate its importance in hypo-osmoregulation through ion secretion in fish (Kang et al., 2010; Yang et al., 2011).

In addition to the basolateral distribution of the SW-subtype MR cells, the antibody T4 also recognizes the apical signals in FW subtypes of MR cells in tilapia (Wu et al., 2003; Hiroi et al., 2005; Inokuchi et al., 2008; Moorman et al., 2014). Recent studies have demonstrated that the apical signal in MR cells of tilapia recognized by the antibody T4 is a thiazide-sensitive Na^+/Cl^- cotransporter (NCC) responsible for Na^+/Cl^- uptake in hypotonic FW (Hiroi et al., 2008; Hiroi and McCormick, 2012). The NCC also belongs to the SLC12 family (Hebert et al., 2004; Gamba, 2005). Therefore, the polarized immunoreaction of the antibody T4 could be used as a marker to identify the SW- or FW-subtype MR cells of certain teleosts, including tilapia (Wu et al., 2003; Inokuchi et al., 2008; Hiroi and McCormick, 2012; Moorman et al., 2014). In addition, recent studies on the SLC12 family in the gills of euryhaline teleosts mainly focused on NKCC (ion secretion) and NCC (ion absorption) groups (Hiroi et al., 2008; Kang et al., 2010; Yang et al., 2011; Hsu et al., 2014).

Ion transporters, including NKCC, are known to be trafficked from staging areas in the Golgi apparatus to the apical or basolateral membranes of epithelial cells by the architecture of cytoskeletal elements, which are crucial for establishing cell polarity and completing certain cellular events (Mays et al., 1995). Electron and confocal microscopic examination of the cytoskeletal structures in gill epithelial cells of the thinlip mullet (*Liza ramada*) and tilapia revealed that numerous microtubules arranged in bundles were centered in the apical regions of epithelial MR cells and expanded toward the base of the cells (Maetz and Pic, 1977; Tsai and Hwang, 1998). The actin filaments, on the other hand, are mainly distributed in the surface pavement cells of the gill epithelia (Tsai and Hwang, 1998). When treated with the

plant alkaloid extract colchicine (Leung et al., 2015), the normal processes of transporting Na^+ , Cl^- , or Ca^{2+} that occurs mainly in MR cells of both SW- and FW-fish were blocked due to the disruption of microtubule assembly of ionocytes and inhibition of processes requiring an intact cytoskeleton for the location of proteins within the cell (Maetz and Pic, 1977; Tsai and Hwang, 1998). In addition, Tresguerres et al. (2006) reported that the movement of H^+ -ATPase from the cytoplasm to the basolateral membrane necessary for increased capacity of base secretion was also microtubule-mediated in the gill epithelial cells of picked dogfish (*Squalus acanthias*) under alkaline stress. These results suggested the interaction between location of ion transporter and microtubules in gill MR cells of teleosts.

The current study was designed to investigate whether the adaptive changes in gill MR cells of tilapia upon osmotic shock were mediated by microtubules. To investigate the correlation between microtubules and adaptive changes in branchial MR cells of fish, a specific inhibitor of microtubule polymerization (i.e., colchicine) (Leung et al., 2015) was used. In the present study, SW-adapted tilapia were transferred to FW with or without colchicine to compare MR cell morphology (cell size and apical surface structures), as well as the NKCC/NCC localization among the treatment and control groups. To our knowledge, this study is the first to examine the relationship between microtubules and changes in subtypes and potential functions of MR cells in fish gills.

MATERIALS AND METHODS

Experimental Animals and Environments

Mozambique tilapia (*Oreochromis mossambicus* [Peters, 1852]) with 6–10 cm body length and 6–14 g body weight were obtained from laboratory stocks derived from our cooperative laboratory in the Academia Sinica and were reared in our laboratory for many years. Fish were kept in SW (35‰) at $27 \pm 1^\circ\text{C}$ under a 12-h light:12-h dark cycle for at least 4 weeks prior to the experiments. Salt water was prepared from aerated dechlorinated FW (local fresh tap water) by adding standardized amounts of the synthetic sea salt “Instant Ocean” (Aquarium Systems Co., Mentor, OH). Fish were fed daily with commercial pellets ad libitum, water was continuously circulated through fabric-floss filters, and water was changed every 2 days for maintenance of water quality. The protocol employed for the experimental fish was reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 95-82).

Transfer Experiments

Our previous studies revealed that euryhaline tilapia survived in direct transfer from SW to FW (Lin et al., 2004) or from FW to brackish water (BW) (20‰) (Wang et al., 2009), but died within 6 h when transferred directly from FW to SW (Wang et al., 2009). Hence, three regimes of transfer experiments were performed to determine the acute time-course immunolocalization of the NKCC/NCC in gill epithelial cells: (1) SW-transfer: FW-acclimated tilapia were directly trans-

ferred from FW to SW; (2) BW-transfer: FW-acclimated fish were directly transferred from FW to BW; and (3) FW-transfer: SW-acclimated individuals were directly transferred to FW. Fish gills were sampled at 3, 6, 12, 24, 48, 96 h post transfer.

Colchicine Treatment

Fresh colchicine (C9754; Sigma-Aldrich, St. Louis, MO) stock solution (1 mmol l^{-1}) was prepared prior to each treatment. In this study, three treatments were performed. First, colchicine-treatment fish were transferred from SW to a 10-l tank that was treated with colchicine stock solution to prepare a final concentration of $0.2 \mu\text{mol l}^{-1}$ (the colchicine-FW-treatment group). Second, sham control fish were transferred from SW to FW (the FW-treatment group), and third, control individuals were transferred from SW to SW (the SW-treatment group). According to our previous studies, changes in subtypes of MR cells in gills of tilapia when acclimatized to the new environment were completed within 96 h (Lee et al., 1996; Lin et al., 2004; Wang et al., 2009); therefore, all fish (i.e., SW-treatment, FW-treatment, and colchicine-FW-treatment groups) in this study were sampled at 96 h. Samples were used for observations and analyses of MR cell size, subtype, and NKCC/NCC immunolocalization. In addition, because colchicine has a half-life of approximately 1 h in plasma (Moffat, 1986; Tresguerres et al., 2006), colchicine stock solution was added to the tank every 12 h to maintain a concentration of 0.2 mol l^{-1} .

Scanning Electron Microscopic Observation of MR Cells

According to previous studies (Table 2 in Hwang and Lee, 2007), subtypes of tilapia MR cells were identified using electron microscopy. The methods used in this study were modified from our previous studies (Wang et al., 2009; Kang et al., 2012). The first gill arch of tilapia from each side was excised and fixed at 4°C in a fixative consisting of 5% glutaraldehyde and 4% paraformaldehyde (PFA) in 0.1 mol l^{-1} phosphate buffer (PB; pH 7.4) for 12 h. After rinsing with 0.1 mol l^{-1} PB, specimens were postfixed with 1% osmium tetroxide in 0.2 mol l^{-1} PB for 1 h. After rinsing with PB and dehydration in ethanol, specimens were critical point dried using liquid CO_2 in a critical-point dryer (Hitachi HCP-2, Tokyo, Japan) and sputter coated for 3 min with a gold-palladium complex in a vacuum evaporator (Eiko 1B-2, Tokyo, Japan). Coated specimens were examined using scanning electron microscopy (Hitachi S-2500, Tokyo, Japan).

Antibody

A monoclonal antibody (T4) raised against the human colonic NKCC was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) and applied in immunofluorescent staining. This antibody can recognize both NKCC1 (secretory isoform) and NCC (absorptive isoform) in gill MR cells of SW- and FW-acclimated tilapia, respectively (Wu et al., 2003; Inokuchi et al., 2008; Moorman et al., 2014). The secondary antibody was Alexa-Fluor-488 conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR).

Cryosections of Gills and Immunofluorescent Staining of NKCC/NCC

The procedure was performed according to the methods described by Wu et al. (2003) and Yang et al. (2011). Briefly, gills were excised and fixed immediately in 4% PFA in 0.1 mol l^{-1} PB for 10 min at 4°C . Samples were then immersed in acetone followed by ethanol at -20°C for 10 min each. After perfusion in 30% sucrose in phosphate buffered saline (PBS; 137 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4 , 2 mmol l^{-1} KH_2PO_4 , pH 7.4) for 1 h at room temperature ($26\text{--}28^\circ\text{C}$), gills were embedded in optimal cutting temperature compound (Sakura, Tissue-Tek, Torrance, CA). The filament was

cut into cross-sections of 10 μm thickness and mounted on poly-L-lysine-coated slides. To detect the distribution of NKCC/NCC immunoreactive cells, sections were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 30 min at room temperature, washed in PBS, and incubated with the monoclonal antibody (T4; 100 \times dilution) for 2 h at room temperature. After washing with PBS, slides were incubated with the secondary antibody (100 \times dilution) for 1 h at room temperature. Negative control experiments were conducted using mouse whole serum (Sigma-Aldrich) instead of the primary antibody to confirm the above positive results (data not shown).

Whole-Mount Immunocytochemistry of MitoTracker and NKCC/NCC

The protocols used herein were modified from our previous studies (Tang and Lee, 2007, 2011a). For the determination of the size of MR cells in tilapia of different groups, MitoTracker fluorescent staining was used and confocal micrographs were taken for comparison among groups. For double staining of NKCC/NCC and mitochondria, MitoTracker Deep Red (as mitochondria marker; M22426; Molecular Probes) stocks were prepared in dimethyl sulfoxide. Dissected gill filaments were fixed with 4% PFA and 0.5% glutaraldehyde in 0.1 mol l^{-1} PBS for 1 h at 4°C. After rinsing with PBS, the filaments were stained with MitoTracker Deep Red in a final concentration of 1 $\mu\text{mol l}^{-1}$ in PBS for 30 min. The filaments were then permeabilized with 70% ethanol at -20°C for 10 min after rinsing with PBS. Subsequently, after washing with PBS, the samples were incubated with 5% BSA for 1 h at room temperature to block non-specific binding. The filaments were then incubated overnight at 4°C with T4 monoclonal antibody (50 \times dilution). After rinsing with PBS for 20 min, samples were further incubated in the secondary antibody (150 \times dilution) for 2 h at room temperature.

Confocal Laser Scanning Microscopy and Quantitative Analysis of MR Cells

Observation and image acquisitions were performed using a Zeiss LSM 510 inverted laser scanning microscope (Hamburg, Germany) equipped with appropriate lens and filter sets for simultaneous monitoring of various fluorescence intensities. The micrographs of immunocytochemistry were software controlled (Zeiss LSM Image Browser, version 3.5.0.223). The micrographs taken from each photomultiplier were subsequently merged such that the different-colored labels could be simultaneously visualized. According to a previous study (Tang and Lee, 2011b), cell size was determined as the greatest linear diameter of MR cells, and obtained from 10 cells per individual ($n = 5$), which were randomly selected from the gill filaments.

Statistical Analyses

The values are expressed as the means \pm SEM (standard error of the mean). The results were compared via one-way analysis of variance tests using Tukey's pairwise test. $P < 0.05$ was set as the significance level.

RESULTS

Transfer Experiments

To illustrate the process of NKCC/NCC localization during the acclimation to hyperosmotic environments, transfer experiments from FW to SW or BW were performed (Fig. 1). When tilapia were transferred directly from FW to SW, all fish died within 6 h following transfer, and NCC was found in the apical region of some branchial epithelial cells within 6 h post-transfer (arrowheads in Fig.

1A). On the other hand, when fish were transferred from FW to BW, no mortality was observed in the 96-h acclimation period. After the first 12 h, apical localization of NCC was found in gill epithelium (arrowheads in Fig. 1B). At 24 and 48 h post-transfer, both apical NCC (arrowheads) and basolateral NKCC (arrows) were observed in epithelial cells (Fig. 1B). At 96 h post-transfer, basolateral NKCC (arrows) instead of apical NCC appeared in all stained epithelial cells of tilapia gills (Fig. 1B). In contrast to hyperosmotic challenge, Figure 2 indicates the process of NKCC/NCC localization when tilapia were transferred directly from SW to FW. Similarly, during the first 12 h after transfer, all stained epithelial cells exhibited basolateral NKCC (arrows in Fig. 2). At 24 and 48 h post-transfer, however, both basolateral NKCC (arrows) and apical NCC (arrowheads) were observed. At 96 h post-transfer, only apical NCC (arrowheads) appeared in epithelial cells of tilapia gills (Fig. 2).

Colchicine Treatment

Figures 1 and 2 demonstrate that upon salinity challenge, expression of ion transporters (i.e., NKCC/NCC) in gill epithelial cells of tilapia altered completely in 96 h. Therefore, the following experiments were performed to compare the size, phenotype, and NKCC/NCC localization of branchial MR cells among tilapia transferred directly from SW to SW, FW, and colchicine-FW for 96 h. Using the fluorescent stain of mitochondria (MitoTracker) as a marker of MR cells in gills of tilapia, average sizes of MR cells were determined and compared (Fig. 3). Significant differences were found between average sizes of MR cells in tilapia transferred from SW to SW, FW, and colchicine-FW after 96 h (Fig. 3D). MR cells of SW-treatment fish were the largest (Fig. 3A,D), were slightly, yet significantly larger than that of the colchicine-FW-treatment group (Fig. 3C,D), and almost 2-fold larger than MR cells of the FW-treatment group (Fig. 3B,D). Scanning electron micrographs of the three groups (96 h post-transfer to SW, FW, and colchicine-FW from SW) of tilapia gill epithelium revealed typical responses of MR cells in SW- (Fig. 4A; only MR cell subtype III appeared) and FW- (Fig. 4B; MR cell subtype I, II, and III appeared) treatment fish. In the colchicine-FW-treatment group, however, only subtype-I MR cells with greater apical surfaces were observed (Fig. 4C). Moreover, double labeling of MitoTracker and NKCC/NCC (Fig. 5) showed that in the FW-treatment group only apical NCC were localized to smaller MR cells in gill epithelium (arrowheads in Fig. 5A–C). Meanwhile, in the colchicine-FW-treatment group, although it is difficult to identify the display of apical NCC signals, the distinct immunosignals from FW group

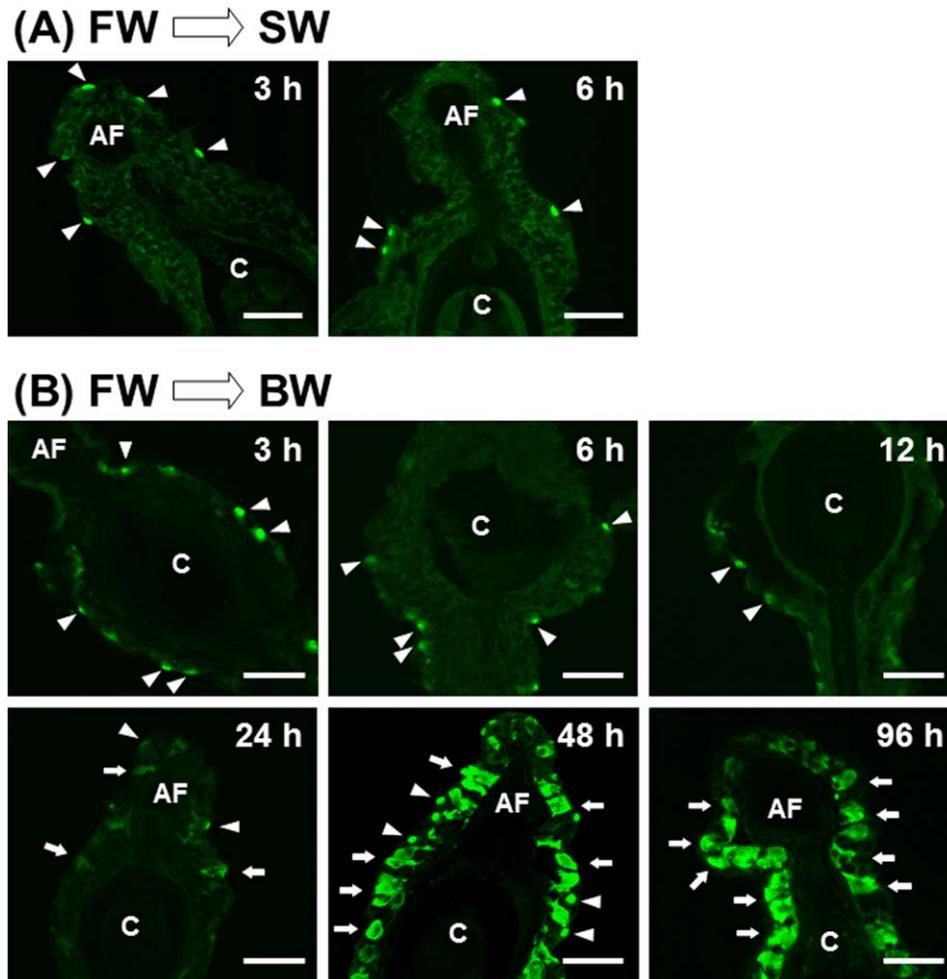


Fig. 1. *Oreochromis mossambicus*, time course of immunolocalization of basolateral NKCC (arrows) and apical NCC (arrowheads) in epithelial cells of cross cryosections of gills transferred from FW to SW (A) and BW (B) during 96 h. AF, afferent artery; C, cartilage. Bar = 60 μm .

were still found in larger MR cells that were supposed to be NKCC (arrows in Fig. 5D–F).

DISCUSSION

The microtubule in the modulation of morphology and function in diverse epithelial cells is generally important for protein sorting, organelle formation, and other microtubule-dependent functions (Müsch, 2004). In addition, microtubules have been demonstrated to interact directly with numerous membrane proteins such as ion channels, transporters, ion pumps, and others (Goswami and Hucho, 2008). In tilapia, microtubules were mainly distributed in branchial MR cells (Tsai and Hwang, 1998). Changes in membrane transport proteins and morphology (i.e., cell size and apical surface structures) to adjust the ion-transporting functions of branchial MR cells in euryhaline tilapia acclimated to environments with different salinities have been widely exam-

ined (Lee et al., 1996, 2003; Wu et al., 2003; Inokuchi et al., 2008, 2009; Tang and Lee, 2011a; Moorman et al., 2014). Moreover, colchicine is a well-known specific inhibitor of microtubule polymerization caused by the formation of tubulin-colchicine complexes (Leung et al., 2015). The present study revealed the effects of colchicine (i.e., dysfunction of microtubule formation) on modulating morphological changes in branchial MR cells of tilapia.

The apical NCC and basolateral NKCC of tilapia MR cells are crucial for Cl^- absorption and secretion, respectively (Hwang et al., 2011; Hiroi and McCormick, 2012). It has been verified that both proteins can be recognized by the monoclonal antibody, T4 (Wu et al., 2003; Inokuchi et al., 2009; Choi et al., 2011; Moorman et al., 2014). Therefore, in gills of tilapia and other fishes, the antibody T4 is not only used for labeling the NCC and/or NKCC but also used as a convenient marker for distinguishing the FW- and SW-subtype MR cells

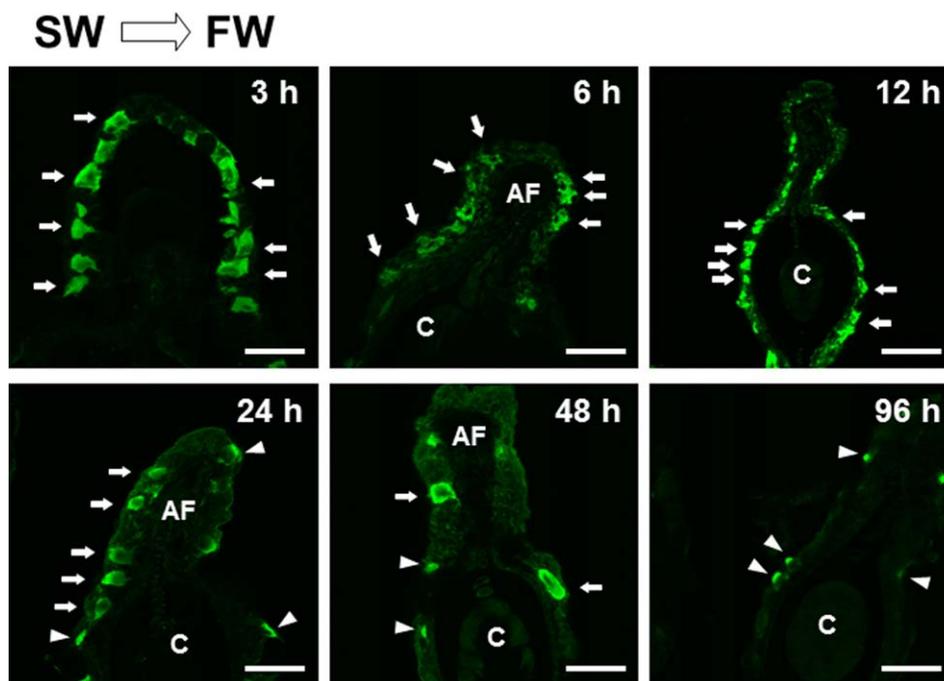


Fig. 2. *Oreochromis mossambicus*, time course of immunolocalization of basolateral NKCC (arrows) and apical NCC (arrowheads) in epithelial cells of cross cryosections of gills transferred from SW to FW during 96 h. AF, afferent artery; C, cartilage. Bar = 40 μ m (12 h) and 60 μ m (3, 6, 24, 48, and 96 h), respectively.

(Kang et al., 2010, 2012; Yang et al., 2011; Hsu et al., 2014; Moorman et al., 2014). The time-course experiments in this study revealed that the apical NCC-immunoreactivity was completely replaced by the immunoreactivity of basolateral NKCC at 96 h post-transfer or vice versa when tilapia were transferred from FW to hyperosmotic BW or from SW to FW, respectively. In the colchicine-FW-treatment group, however, T4 signals seem to be found in whole MR cells rather than in the apical region only. These results were similar to SW-subtype MR cells, showing that the basolateral (cytoplasmic) distribution of NKCC was observed in these MR cells. Nevertheless, the possibility of these basolateral-NKCC MR cells with apical-NCC signals cannot be completely excluded. This subtype MR cells with both signals might be a kind of transitional type when tilapia were transferred from SW to FW. Our results revealed differences in functional switching between the gills of FW- and colchicine-FW-treatment tilapia, showing that the effects of colchicine may block the transformations/changes in MR cells from SW-subtype to FW-subtype. Thus, these results might be attributable to the disruption of microtubule-dependent exocytic and endocytic pathways (Müsch, 2004). Future studies will focus on (i) distinguishing the apical NCC from basolateral NKCC signals by applying different antibodies for observations of fluorescent or confo-

cal microscope and (ii) investigating the changes in mRNA levels and physiological responses (e.g., plasma osmolality and ion concentrations).

Previous studies have revealed that euryhaline species alter the cell size and/or density of their gill MR cells to adjust their ion-transporting functions upon salinity challenge (Lee et al., 2003, 2006; Tang and Lee, 2011b; Kang et al., 2012, 2013). In tilapia, the ion-transporting functions of MR cells were modulated through not only regulation of ion transport protein expression, but also alteration of cell size and apical surface structures (Heijden et al., 1997; Lee et al., 2003; Sardella et al., 2008; Tang and Lee, 2011a; Inokuchi and Kaneko, 2012). The size of MR cells was evidently enhanced in association with increase of the loaded capacity of NKA per MR cell, which resulted in successful acclimation of tilapia to SW (Heijden et al., 1997; Uchida et al., 2000; Lee et al., 2003; Sardella et al., 2008). Therefore, reduced cell size was necessary for acclimation when tilapia were exposed to hyposmotic FW. In tilapia, the average size of SW MR cells is about twofold larger than the average sizes of all subtypes of FW MR cells (Uchida et al., 2000; Lee et al., 2003; Inokuchi et al., 2008), but the density of gill MR cells decreases with elevated environmental salinity (Heijden et al., 1997; Uchida et al., 2000; Sardella et al., 2008). Like the results of previous studies, in this study, the size of branchial

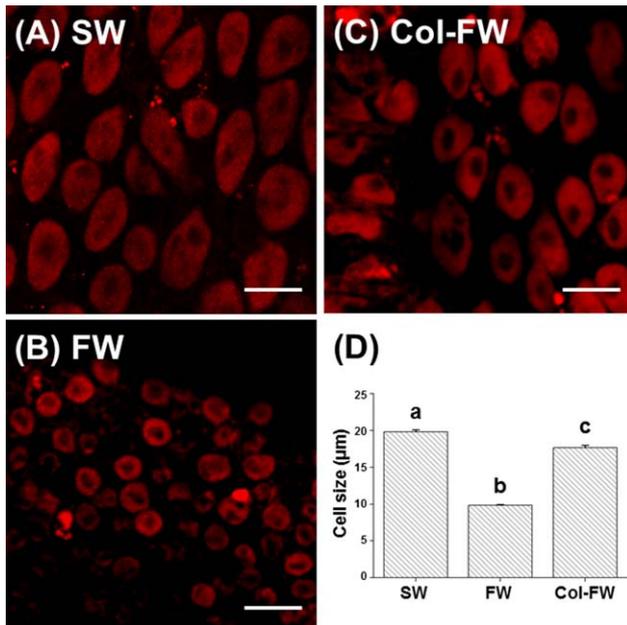


Fig. 3. *Oreochromis mossambicus*, confocal scanning micrographs (A–C) of MitoTracker stained MR cells and their average sizes (D) in gills of tilapia transferred from SW to SW (A), FW (B), or colchicine-FW (Col-FW; C) for 96 h. Average size of MR cells in the FW-treatment tilapia was the smallest among three groups of fish (D). Different letters indicate significant differences. Bar = 20 μm .

MR cell was significantly larger in SW-acclimated than FW-acclimated fish. However, when tilapia exposed to FW with colchicine treatment, the average sizes of branchial MR cells were significantly different from those of the FW-acclimated group. This finding indicated that the modulation of cell size was microtubule-dependent. These changes may have been caused by microtubules arranged in bundles that expanded toward the surface of MR cells in tilapia (Tsai and Hwang, 1998).

The apical membrane is in direct contact with the external environment. Hence, the apical membrane structure is closely related to the ion-transporting activities of MR cells (Marshall, 2002; Kaneko et al., 2008). The apical structures of branchial MR cells in tilapia can be classified into three subtypes: subtype I, wavy-convex; subtype II, shallow-basin; and subtype III, deep-hole (Lee et al., 1996, 2003). The deep-hole subtype with NKCC exhibition in basolateral membrane is the SW type MR cells responsible to Cl^- secretion, whereas the other three subtypes with major, minor, and least proportions simultaneously exist in the gills of FW-acclimated tilapia (Lee et al., 2003; Hiroi et al., 2008; Choi et al., 2011). Among them, the subtype-II MR cells in which Na^+/H^+ exchanger-3 is labeled in the apical membrane may play the role in Na^+ and/or Ca^{2+} uptake (Chang et al., 2001; Chang et al., 2003; Inokuchi et al., 2009; Choi et al., 2011). In subtype-I MR

cells, on the other hand, previous studies reported that the NCC expressed specifically in their apical surfaces for branchial Na^+ and Cl^- uptake (Horng et al., 2009; Inokuchi et al., 2009; Choi et al., 2011). In this study, however, the colchicine treatment led to the increase of the apical surface area of subtype-I MR cells. Interestingly, the shallow-

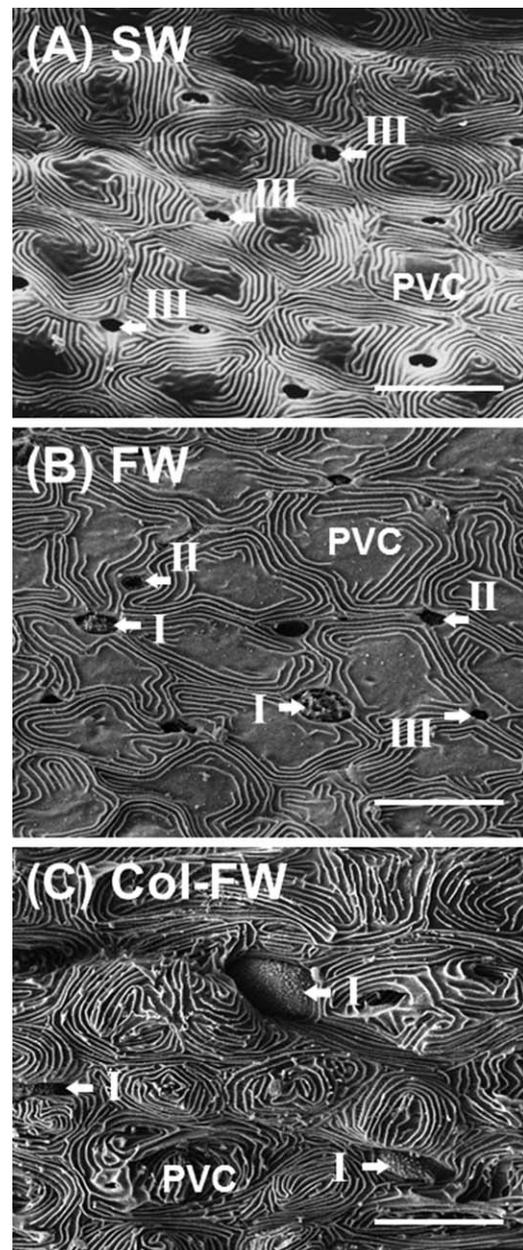


Fig. 4. *Oreochromis mossambicus*, representative scanning electron micrographs of the afferent epithelium of gills of SW-acclimated tilapia after transfer to SW (A), FW (B), or colchicine-FW (Col-FW; C) for 96 h. (A) In the SW-treatment fish, only subtype-III MR cells were found. (B) In the FW-treatment water, three subtypes (I, II, and III) were exhibited, whereas in colchicine-FW-treatment fish (C), only subtype-I MR cell with a large apical surface was observed. PVC, pavement cells. Bar = 10 μm .

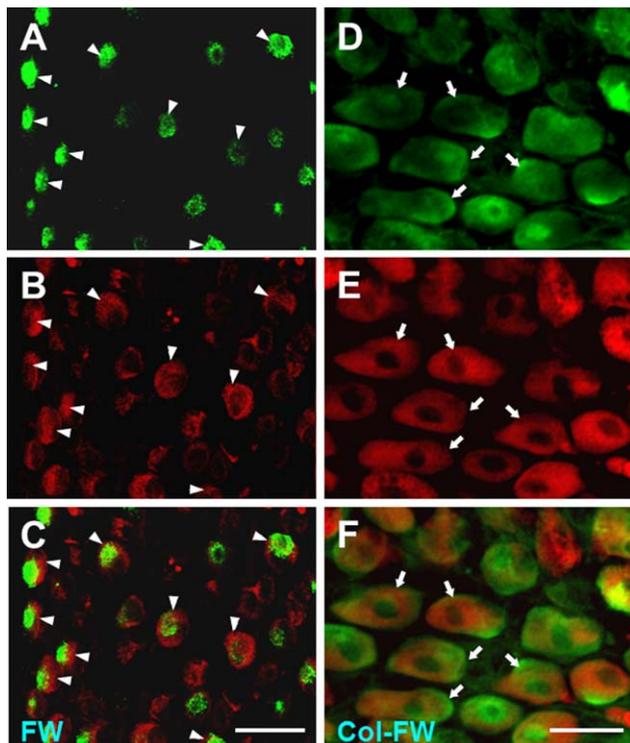


Fig. 5. *Oreochromis mossambicus*, confocal micrographs of double staining of NKCC/NCC immunoreactive cells (A, D) and MR cells (B, E) in gills of tilapia transferred from SW to FW (A–C) or colchicine-FW (Col-FW; D–F) for 96 h. (A, D) Immunofluorescent staining of NKCC/NCC; (B, E) MitoTracker staining of MR cells; (C, F) merged images of NKCC/NCC and MitoTracker double staining indicated that apical NCC (arrowheads) and basolateral NKCC (arrows) were exhibited in branchial MR cells of tilapia transferred to FW or colchicine-FW, respectively. Bar = 20 μ m.

basin and deep-hole subtypes were not found in the colchicine FW-treatment group, which may be a reason for the resultant reduction in the Ca^{2+} influx rate (Tsai and Hwang, 1998). In the MR cells of colchicine-FW-treatment tilapia only subtype-I existed, whereas the transformation of ion transporters was different from the control group. Thus, the functions of these MR cells may be distinct from those of normal subtype-I MR cells and need further investigations in future works.

The cytoskeleton is well investigated in mammals (Coticchio et al., 2015). Although changes in morphology and ion transporting proteins correlated closely with the ion-transporting functions of MR cells were broadly investigated (Hwang and Lee, 2007; Kaneko et al., 2008; Hiroi and McCormick, 2012), less attention has been paid to the study of the correlations between morphological plasticity, trafficking of membrane transport proteins, and cytoskeleton. Maetz and Pic (1977) demonstrated that plasma Na^+ and Cl^- concentrations were disturbed when the thinlip mullet were exposed to SW with colchicine. The imbalanced

plasma ion concentrations might have been caused by the microtubule-dependent trafficking pathways of ion transport proteins, cell size adjustment, and transformation of apical structures inhibited by colchicine, which disrupted the ion-transporting functions of gill MR cells.

In conclusion, this is the first study to directly demonstrate the relationship between changes in morphology and function of MR cells and microtubules observed following the addition of colchicine (a specific inhibitor of microtubule polymerization). The transformation of MR cells was influenced by colchicine treatment when tilapia were transferred from SW to FW. The size, phenotype, as well as expression of ion transporters (i.e., NCC and NKCC) of MR cells in tilapia gills were different between colchicine-FW-treatment and FW groups. The results suggested that the changes in gill MR cells in euryhaline tilapia are microtubule-dependent.

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AUTHOR CONTRIBUTIONS

C.H.T. and T.H.L. designed the experiments. C.H.T. and Y.C.W. performed the experiments. W.K.Y. and C.H.T. analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

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