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Review

New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish☆

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ABSTRACT

Teleost fishes are able to acclimatize to seawater by secreting excess NaCl by means of specialized "ionocytes" in the gill epithelium. Antibodies against Na^+/K^+-ATPase (NKA) have been used since 1996 as a marker for identifying branchial ionocytes. Immunohistochemistry of NKA by itself and in combination with $Na^+/K^+/2Cl^-$ cotransporter and CFTR Cl^- channel provided convincing evidence that ionocytes are functional during seawater acclimation, and also revealed morphological variations in ionocytes among teleost species. Recent development of antibodies to freshwater- and seawater-specific isoforms of the NKA alpha-subunit has allowed functional distinction of ion absorptive and secretory ionocytes in Atlantic salmon. Cutaneous ionocytes of tilapia embryos serve as a model for branchial ionocytes, allowing identification of 4 types: two involved in ion uptake, one responsible for salt secretion and one with unknown function. Combining molecular genetics, advanced imaging techniques and immunohistochemistry will rapidly advance our understanding of both the unity and diversity of ionocyte function and regulation in fish osmoregulation.

1. Introduction

1.1. Ionocytes

In seawater environments, teleost fishes secrete excess sodium and chloride from the body fluid by means of specialized, mitochondrion-rich cells in the gill epithelium. This cell type was first identified as "chloride secreting cell" in the gills of seawater-acclimated European eel *Anguilla anguilla* (formerly *Anguilla vulgaris*) by Keys and Willmer (1932), and then has often been referred to as "chloride cell" in many teleost species. More recently, the terms "mitochondrion-rich cell" or "ionocyte" have been preferred, because this cell type is known to be involved not only in chloride secretion in seawater, but also in multiple functions such as ion uptake in freshwater, acid-base regulation and ammonia excretion. This review adopts "ionocyte" in place of "chloride cell" or "mitochondrion-rich cell" throughout the text.

There are numerous reviews dealing with the structure and function of ionocytes (e.g. Pisam and Rambourg, 1991; McCormick, 1995; Evans et al., 2005; Hwang and Lee, 2007; Kaneko et al., 2008; Hwang et al., 2011; Marshall, 2011; Dymowska et al., 2012). In contrast to those comprehensive reviews, this article focuses on a morpho-functional approach for the last decade to immunohistochemically identifying ion-transport proteins, such as Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR Cl^- channel, within branchial ionocytes of teleost fishes that are euryhaline (capable of tolerating a wide range of salinities) or diadromous (migrate between freshwater and seawater). For detailed molecular properties of each ion-transport protein, please refer to recent reviews listed above. This review also presents several unique studies on ionocytes in the skin of euryhaline teleost embryos, since cutaneous ionocytes serve as a convenient experimental substitute for branchial ionocytes.

1.2. Apical and basolateral membranes

In general, epithelial tissues are in contact with both the external and internal environments by their apical and basolateral plasma membranes, respectively, and the cells principally function as a physical barrier between the two environments. Furthermore, specialized epithelial cells (including fish ionocytes) transport ions from the internal to the external environments (i.e. secretion) or from the external to the internal environments (absorption or uptake). This vectorial ion transport is performed by combinations of several ion pumps, transporters and channels selectively

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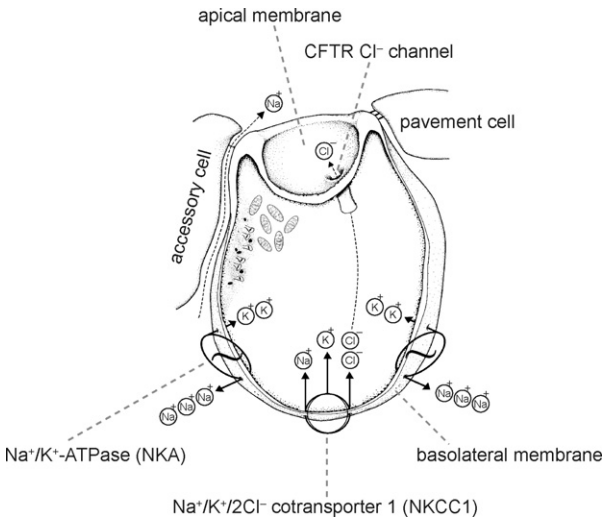

Fig. 1. A schematic diagram of an ionocyte in seawater-acclimated teleost fish. Ionocytes are in contact with the external and internal environments by their apical and basolateral membranes, respectively. Active salt secretion is performed by the cooperative action of three major ion-transport proteins, NKA, NKCC1 and CFTR. NKA and NKCC1 are localized to the basolateral membrane, and CFTR is localized to the apical membrane. From McCormick (2001).

expressed in each of the apical and basolateral membranes. Therefore, determining the localization patterns of the multiple ion-transport proteins at the apical and basolateral membranes is essential for determining the ion-transport functions of the proteins themselves and of ionocytes.

1.3. Three major salt secretory proteins: NKA, NKCC1 and CFTR

The currently accepted model for active NaCl secretion by ionocytes in seawater-acclimated teleosts consists primarily of the cooperative action of three major ion-transport proteins: Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1), and Cl^- channel homologous to human cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2011). In the model (depicted in Fig. 1), a basolaterally-located NKA transports 3 Na^+ outward in exchange for 2 K^+, creating low intracellular Na^+ and a highly negative charge within the cell; the Na^+ gradient is used to transport Na^+, K^+ and 2 Cl^- into the cell through a basolateral NKCC1; Cl^- then leaves the cells down an electrical gradient through an apical CFTR; Na^+ is transported back outside the cells via NKA, and then leaves through a paracellular pathway between ionocytes and adjacent smaller cells known as accessory cells.

2. Immunohistochemical studies on branchial ionocytes

2.1. NKA antibody visualizes ionocytes

Biochemical measurements of NKA enzymatic activity in gill homogenate have been utilized since the 1960s to evaluate the response of gill tissue to external salinity. NKA has a high affinity for ouabain, and ^{3}H-labeled or fluorescent ouabain was used to localize NKA in fish ionocytes (Karnaky et al., 1976; McCormick, 1990). Immunohistochemical detection of NKA within fish ionocytes was first reported relatively late, in 1996, with masu salmon *Oncorhynchus masou* (Ura et al., 1996) and with rainbow trout *Oncorhynchus mykiss* (Witters et al., 1996). Ura et al. (1996) raised rabbit polyclonal antiserum against a synthetic peptide corresponding to a region of the NKA α-subunit that is highly conserved throughout the animal kingdom. Witters et al. (1996) utilized mouse monoclonal antibody against chicken kidney NKA α-subunit

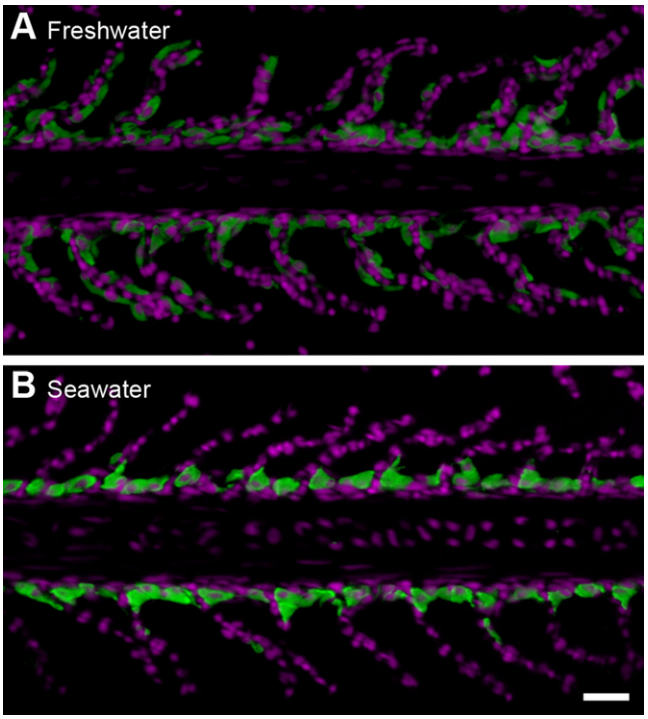

Fig. 2. NKA immunoreactivity (green) in the gills of Atlantic salmon smolts kept in freshwater (A) and those transferred and kept in seawater for 3 weeks (B). The nuclei were counterstained with DAPI (magenta). The primary filament is horizontal, and secondary lamellae are seen perpendicular to the filament. In freshwater, NKA-positive ionocytes are distributed on both the primary filaments and secondary lamellae, whereas lamellar ionocytes disappear and filament ionocytes enlarge and result in stronger staining in seawater. Materials and methods are according to Hiroi and McCormick (2007). Scale bar, 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

(named α5; available from the Developmental Studies Hybridoma Bank; http://dshb.biology.uiowa.edu). The basolateral membrane of ionocytes, in which abundant NKA is located, is invaginated to form an extensive tubular system throughout most of the cytoplasm (see a review by Karnaky et al., 1976; Sardet et al., 1979); immunoreactivity for NKA is therefore detectable throughout the ionocyte except for the nucleus and the apical region (Fig. 2).

These NKA antibodies serve as a highly-specific marker for ionocytes in fixed gill tissues. In a large number of teleost species examined, including chum salmon *Oncorhynchus keta* (Uchida et al., 1996) and Mozambique tilapia *Oreochromis mossambicus* (Uchida et al., 2000), NKA-positive ionocytes show increases in size and staining intensity following transfer from freshwater to seawater, which are in parallel with increasing gill NKA enzymatic activity. On the other hand, in a relatively small number of species, such as mummichog *Fundulus heteroclitus* (Katoh et al., 2001) and milkfish *Chanos chanos* (Lin et al., 2006), ionocyte size and/or numbers increase in freshwater compared to seawater. The diversisty of responses among morphology of NKA-positive ionocytes, NKA enzymatic activity, protein abundance and mRNA expression level among teleost were recently reviewed by Hwang and Lee (2007).

2.2. Filamental and lamellar ionocytes

NKA immunohistochemistry revealed a clear salinity-induced change in the distributional pattern of ionocytes in the gills of several euryhaline and diadromous teleosts. In freshwater, NKA-immunopositive ionocytes were distributed on both the (primary) filaments and (secondary) lamellae, whereas lamellar ionocytes disappeared and filament ionocytes enlarged following seawater transfer (Fig. 2). This phenomenon was first observed in chum

salmon (Uchida et al., 1996), and has been confirmed in a variety of teleost taxa (Fig. 3, Group A): Japanese eel *Anguilla japonica* (Sasai et al., 1998), belonging to superorder Elopomorpha; American shad *Alosa sapidissima* (Zydlewski and McCormick, 2001) and alewife *Alosa pseudoharengus* (Christensen et al., 2012), superorder Clupeomorpha; milkfish (Lin et al., 2006), superorder Ostariophysi; brown trout *Salmo trutta* (Seidelin et al., 2000) and Atlantic salmon *Salmo salar* (Pelis et al., 2001), superorder Protacanthopterygii; Japanese sea bass *Lateolabrax japonicus* (Hirai et al., 1999), European sea bass *Dicentrarchus labrax* (Nebel et al., 2005) and silver moony *Monodactylus argenteus* (Kang et al., 2012), superorder Acanthopterygii. These observations have led to the hypothesis that lamellar ionocytes are responsible for ion uptake in freshwater, and filament ionocytes are for ion secretion in seawater, respectively. However, in primitive salmonids, lake trout *Salvelinus namaycush* and brook trout *Salvelinus fontinalis* (Hiroi and McCormick, 2007), and in amphidromous Hawaiian goby *Stenogobius hawaiiensis* (McCormick et al., 2003), NKA-immunopositive ionocytes were found in both filaments and lamellae in both freshwater and seawater (Fig. 3, Group B). In addition, European sea bass, which possess lamellar ionocytes in freshwater but not in seawater as stated above (Nebel et al., 2005), were found to possess a rich population of lamellar ionocytes following transfer to two-times concentrated (70 ppt) seawater (Varsamos et al., 2002). The lamellar ionocytes of these species (lake trout, brook trout, Hawaiian goby and European sea bass) are likely to be involved in active ion secretion in hypertonic environments. In the case of Hawaiian goby in seawater, lamellar cells had NKA, NKCC1 and CFTR, indicating that they were functional salt secretory cells. As will be discussed more fully below, NKA is involved in both ion uptake and secretion, so that identifying basolateral NKCC1 and apical CFTR in addition to basolateral NKA has provided more definitive evidence that these ionocytes are involved in active ion secretion.

It is unclear why ionocytes are often present lamellae in freshwater but not in seawater. One possible explanation is the physical requirements for ionocytes differ in freshwater and seawater. Unlike freshwater ionocytes, seawater ionocytes often contact both the external environment and the blood, contain a deep apical pit and an extensive tubular system for NKA. Only a large columnar cell could do this, and the only place that this is possible without large disruption of respiration is the filament (not the lamellae). A possible reason for lamellar ionocytes seen in seawater-acclimated trout and goby is that these are primarily freshwater species, with little selection pressure for seawater adaptation, and thus have not displayed the levels of specialization seen in other, even closely related species (such as salmon in relation to trout). The European sea bass and other marine fish in hyper-saline water may have no place else to put 'extra' ionocytes except on the lamellae.

In contrast to the teleost groups possessing lamellar ionocytes in freshwater (Fig. 3, Groups A and B), there are other species in which NKA-positive ionocytes are found only in the filaments and rarely in the lamellae in both freshwater and seawater (Fig. 3, Group C): mummichog (Katoh et al., 2001), sailfin molly *Poecilia latipinna* (Yang et al., 2009), medaka *Oryzias latipes* and Indian medaka *O. dancena* (Kang et al., 2008), Mozambique tilapia (Uchida et al., 2000), striped bass *Morone saxatilis* (Madsen et al., 2007), southern flounder *Paralichthys lethostigma* (Tipsmark et al., 2008) and spotted green pufferfish *Tetraodon nigroviridis* (Lin et al., 2004). All these species lacking lamellar ionocytes are euryhaline but non-diadromous (not exhibiting large-scale migration between freshwater and seawater), and belong to a higher teleost taxon, superorder Acanthopterygii. In contrast, those species possessing lamellar ionocytes in freshwater (Fig. 3, Group A) are essentially diadromous, including both anadromous and catadromous, and

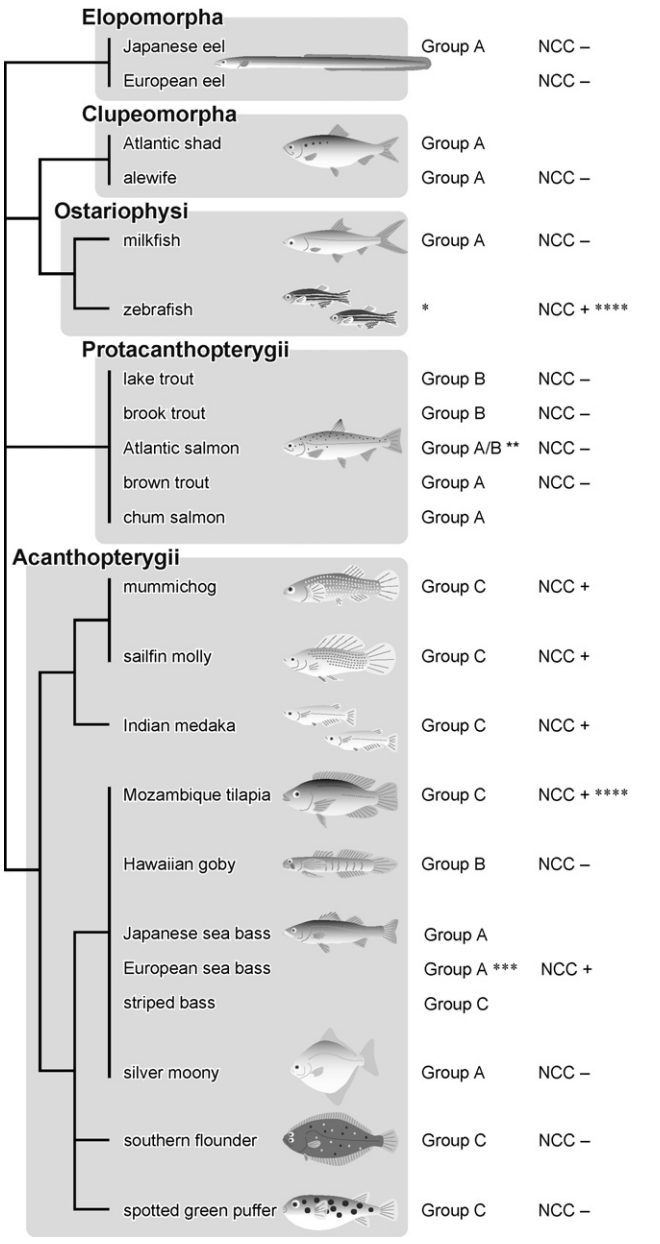

Fig. 3. A phylogenetic tree of teleost fishes which have been employed for immunohistochemical studies on ionocytes. The phylogenetic tree is based on Nelson (2006). All examined species are divided into three groups showing different distributional patterns of ionocytes in the gills: Group A, fishes possessing lamellar ionocytes in freshwater but lacking in seawater; Group B, possessing lamellar ionocytes in both freshwater and seawater; Group C, fishes lacking lamellar ionocytes in both freshwater and seawater. Group A corresponds to basal teleost taxa such as Elopomorpha, Clupeomorpha, Ostariophysi and Protacanthopterygii, whereas Group C belongs to a higher teleost taxon, Acanthopterygii. * Zebrafish is a stenohaline freshwater species and therefore not applicable for grouping A–C. ** Atlantic salmon smolts are Group A whereas parr are Group B (McCormick, et al., unpublished results). *** European sea bass are generally Group A, but have lamellar ionocytes in 2× concentrated (70 ppt) seawater (Varsamos et al., 2002). All species are also divided in two groups according to the localization patterns of immunoreactant with the T4 human NKCC1 antibody: NCC+, fishes showing apical immunoreactivity in freshwater ionocytes; NCC−, fish showing basolateral immunoreactivity in both freshwater and seawater ionocytes. **** The localization of fish-specific NCC (SLC12A10) within freshwater ionocytes was so far only confirmed at the molecular level with Mozambique tilapia and zebrafish (Hiroi et al., 2008; Wang et al., 2009). As is the case with tilapia and zebrafish, the apical immunoreactivity found in other species (mummichog, sailfin molly, Indian medaka and European sea bass) seems to represent NCC rather than NKCC2. It would be interesting to determine whether the common ancestor of Ostariophysi (including zebrafish) and Acanthopterygii (including tilapia) acquired the fish-specific NCC, and then some species conserved but others lost during evolution, or whether the fish-specific NCC was independently acquired in Ostariophysi and Acanthopterygii.

belong to relatively more basal teleost taxa (with the exception of Japanese sea bass, European sea bass and silver moony). Although more immunohistochemical evidence for other species (including stenohaline freshwater and marine species) is needed, maintaining a similar nearly constant distributional pattern of branchial ionocytes might be a more suitable mechanism for euryhaline teleosts to cope with continual salinity fluctuations, and/or might be a more advanced adaptive mechanism which was acquired by a higher taxon during the evolution of teleosts.

2.3. Participation of NKCC1 and CFTR

NKA immunohistochemistry has certainly contributed to understanding the structure and function of branchial ionocytes. However, NKA appears to have a role in both ion uptake and salt secretion in the teleost gill. NKA immunoreactivity is present basolaterally in ionocytes of both freshwater- and seawater-acclimated fish, and is therefore unable to reflect the difference in ionocyte's expected functions, ion absorption in freshwater and secretion in seawater. In order to compensate for this problem, attempts to localize the two other major ion-transport proteins within ionocytes, NKCC1 and CFTR, started in the early 2000s.

Probably the first immunohistochemical study for NKCC1 and CFTR in fish branchial ionocytes was conducted with giant mudskipper *Periophthalmodon schlosseri* in 50% seawater, determining basolateral NKCC1 and apical CFTR using heterologous antibodies, although the study focused on ammonia excretion by ionocytes rather than NaCl excretion (Wilson et al., 2000). Then, in Atlantic salmon, NKCC1 and NKA immunoreactivity was found to colocalize on the basolateral membrane of branchial ionocytes, and the immunopositive ionocytes increased in size and number following transfer from freshwater to seawater, during smolting, and by growth hormone and cortisol treatments (Pelis et al., 2001; Pelis and McCormick, 2001). Similar changes in NKCC1/NKA-positive ionocytes were also confirmed in other salmonids such as brown trout, lake trout and brook trout (Tipsmark et al., 2002; Hiroi and McCormick, 2007). These reports on salmonids strongly suggest that basolateral NKCC1 plays a crucial role in ionocytes during seawater acclimation. Unfortunately, immunocytochemical detection of CFTR in salmonid ionocytes so far has proven difficult, even with the production of homologous antibodies (McCormick, unpublished results). Amphidromous Hawaiian goby was used to localize NKA, NKCC1 and CFTR within ionocytes and to examine the effect of environmental salinity on the three proteins: the goby's branchial ionocytes showed basolateral NKA, basolateral NKCC1 and apical CFTR, and especially apical CFTR immunoreactivity was enhanced with increasing salinity, supporting the currently accepted model for active NaCl secretion, (Fig. 4; McCormick et al., 2003). The salinity-induced enhancement of apical CFTR immunoreactivity was commonly observed in branchial ionocytes of various species, such as European eel (Wilson et al., 2004), alewife (Christensen et al., 2012), milkfish (Tang et al., 2011), mummichog (Katoh and Kaneko, 2003), Mozambique tilapia (embryonic cutaneous ionocytes; Hiroi et al., 2005) and spotted green pufferfish (Tang et al., 2011).

As well as branchial ionocytes, apical CFTR and basolateral NKCC1 were immunohistochemically demonstrated in ionocytes in the opercular membrane of mummichog; low and diffuse CFTR immunoreactivity was observed in freshwater ionocytes, with increasing levels of CFTR in the apical membrane 24 and 48 h following exposure to seawater (Marshall et al., 2002). The opercular membrane of this species contains a rich population of ionocytes and has played a pioneering role in understanding ion-transport mechanisms in ionocytes by electrophysiology (see a review by Marshall, 2011).

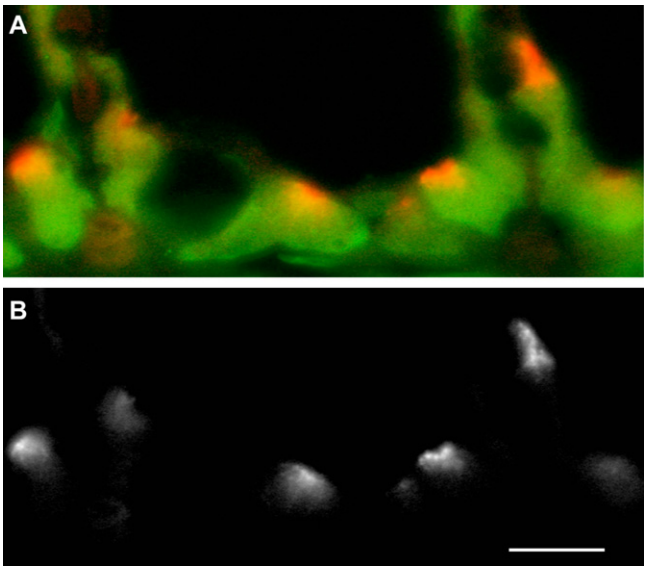

Fig. 4. NKA (green) and CFTR (red) immunoreactivity in gills of Hawaiian goby acclimated to seawater (A). Image B contains only CFTR staining (white). Scale bar, 12 μm. From McCormick et al. (2003). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

2.4. Problem of apical NKCC immunohistochemistry: not NKCC2 but NCC

To detect NKCC in ionocytes, mouse monoclonal antibody against human colonic NKCC1 (named T4; available from the Developmental Studies Hybridoma Bank) has been frequently used with various teleost species. The immunohistochemistry with the T4 antibody is known to divide the examined fishes into two groups (Fig. 3, NCC−/+; NCC, Na^+/Cl^- cotransporter). One group (Fig. 3, NCC−) is defined by similar basolateral immunoreactivity in ionocytes in both freshwater and seawater, although staining intensity is higher in seawater than in freshwater, as first reported in Atlantic salmon (Pelis et al., 2001), and also found in European eel (Wilson et al., 2004), Japanese eel (Seo and Kaneko, unpublished results), alewife (Christensen et al., 2012), milkfish (Tang et al., 2011), brown trout (Tipsmark et al., 2002), lake trout and brook trout (Hiroi and McCormick, 2007), Hawaiian goby (McCormick et al., 2003), silver moony (Kang et al., 2012), southern flounder (Tipsmark et al., 2008) and spotted green pufferfish (Tang et al., 2011). The other group (Fig. 3, NCC+) shows basolateral immunoreactivity in seawater but apical immunoreactivity in freshwater by the T4 antibody, first observed in Mozambique tilapia (Wu et al., 2003), and also confirmed in mummichog (Katoh et al., 2008), sailfin molly (Yang et al., 2011), Indian medaka (Kang et al., 2010) and European sea bass (Lorin-Nebel et al., 2006). These two groups of fishes determined by the immunohistochemical patterns with the T4 antibody show some similarity to the groups distinguished by the distributional patterns of filament/lamellar ionocytes (Fig. 3).

Mammalian $Na^+/K^+/2Cl^-$ cotransporter occurs in two isoforms, basolaterally-located NKCC1 involved in salt secretion and apically-located NKCC2 involved in ion aborption, and the T4 antibody is known to react with both isoforms (Lytle et al., 1995). This well-established information on mammalian NKCCs had led fish researchers to assume that the basolateral and apical immunoreactivity in ionocytes represents NKCC1-like protein and NKCC2-like protein, respectively. However, the assumption of apical NKCC2 was later shown to be incorrect; sequencing NCC in tilapia and producing a homologous NCC antibody indicated that the apparent apical "NKCC" immunoreactivity of ionocytes was in fact NCC (Hiroi et al., 2008; details are described in Sections 3.2 and 3.3). There

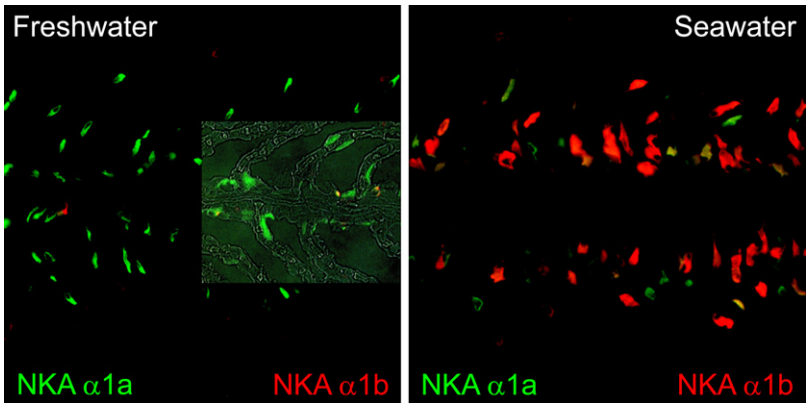

Fig. 5. Co-localization of NKAα1a (green) and NKAα1b (red) in gill tissue of Atlantic salmon parr in freshwater (left) and gradually acclimated to 30 ppt seawater (right). Details of antibody production and specificity can be found in McCormick et al. (2009). Photomicrograph from Arne Christensen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

is currently limited information available on the cation–chloride cotransporter family in teleost species, but the localization of NCC within ionocytes was also confirmed with zebrafish *Danio rerio* (Wang et al., 2009). Furthermore, NKCC2 mRNA is not present in large amount in gills, but is abundant in the intestine and/or kidney of Mozambique tilapia (Hiroi et al., 2008), European eel (Cutler and Cramb, 2008), Indian medaka (Kang et al., 2010) and pufferfishes (*Takifugu rubripes*, *T. obscurus*, *T. poecilonotus* and *T. pardalis*) (Kato et al., 2011). Based on these studies it seems likely that the apical T4 immunoreactivity in ionocytes of mummichog, sailfin molly, Indian medaka and European sea bass (Fig. 3, NCC+) is NCC rather than NKCC2, though further studies will be needed to confirm this.

2.5. Back to NKA: distinguishing freshwater and seawater isoforms

The ability of ouabain, a specific inhibitor of NKA, to stop salt secretion in isolated gill and opercular tissue is one of the observations that led to the formulation of the mechanisms of ion secretion in fish (Silva et al., 1977). Ionocytes were later found to have high levels of NKA activity and ouabain binding. However, ouabain could also affect ion uptake, and ionocytes in freshwater also had high levels of NKA, though for most species not as high as in seawater (McCormick, 1995). Thus, NKA appears to have a role in both ion uptake and salt secretion. There is also evidence that the kinetics of NKA differed in freshwater and seawater (Pfeiler and Kirschner, 1972), suggesting differential function or regulation of this enzyme in different salinities.

The NKA enzyme is composed of two major subunits, α and β. The α-subunit is the main catalytic unit and contains all of the critical binding sites for ATP, Na^+, K^+, and ouabain (which is now recognized not just as an inhibitor but as an endogenous regulator). The β-subunit is a glycosylated polypeptide that assists in folding and positioning of the protein into the basolateral plasma membrane and is essential for its normal activity. A third subunit termed γ, also known as FXYD, is not necessary for the catalytic function of NKA but apparently acts to adapt the kinetic properties of sodium and potassium transport for the functions of different cell types (Garty and Karlish, 2006). There are at least 4 α isoforms that are expressed in vertebrates and have different kinetic properties for Na^+, K^+ and ATP binding, vary in their inhibition by ouabain and calcium, and in their regulation by intracellular second messengers (Blanco and Mercer, 1998). These unique properties along with their cell-specific distribution suggest that the NKA α isoforms have distinct physiological function and regulation.

Recent molecular genetic studies indicate there are several isoforms of the α-subunit expressed in the gill tissue of rainbow trout that are the product of distinct genes. The mRNA levels of the NKAα1a isoform increases after transfer from seawater to freshwater, whereas the α1b isoform increases after exposure to seawater (Richards et al., 2003). Similar effects of salinity on these isoforms have been found in Atlantic salmon (*Salmo salar*), and that transcription of gill NKAα1a decreases and NKAα1b increases in freshwater during the parr-smolt transformation when the salinity tolerance of juveniles increases (Nilsen et al., 2007; Madsen et al., 2009). Salinity-specific isoforms have also recently been found in gill tissue of Mozambique tilapia (Tipsmark et al., 2011), though their presence in various ionocyte types (types I–IV; Section 3.3) has yet to be established.

Although the two salinity dependent isoforms have high sequence similarity, there was sufficient variability in one region of the protein to allow production of antibodies that are highly specific for each isoform and can be used in both immunohistochemistry and western blots (McCormick et al., 2009). These studies determined that the two isoforms are primarily present in gill ionocytes of Atlantic salmon parr (Fig. 5). NKAα1a predominates in freshwater, and is present in both filamental and lamellar ionocytes. NKAα1b is present at low levels in freshwater and is localized to small filamental ionocytes that appear to be below pavement cells and thus not in contact with the external environment. After gradual acclimation of parr to seawater, NKAα1a becomes undetectable by western blots but is present in a small number of small, filamental gill ionocytes. NKAα1b becomes highly abundant in seawater and is present primarily in large filamental ionocytes, but with significant numbers also on the lamellae.

Determining the changes in the protein abundance and localization of the salinity-dependent isoforms during smolt development provides additional information on their role in osmoregulation in freshwater and seawater. Coincident with downstream migration in the spring, Atlantic salmon develop a high level of salinity tolerance that is accompanied by increased NKA activity. Although parr have slightly higher levels of gill NKAα1a relative to smolts, there are no significant changes in protein abundance and only a slight decrease in the number of NKAα1a ionocytes in the gill during smolt development (McCormick, et al., unpublished results). These results contrast with transcription studies that found substantial decreases in gill NKAα1a mRNA levels during smolt development (Nilsen et al., 2007). The increase in salinity tolerance in smolts is accompanied by large increases in the abundance of NKAα1b abundance and cell numbers. A large number of ionocytes with both NKAα1a and NKAα1b are present in smolts in freshwater, suggesting that NKAα1a ionocytes are gradually transforming to

NKAα1b ionocytes. After exposure to seawater NKAα1b abundance increases further, NKAα1a decreases dramatically, there are almost no ionocytes expressing both isoforms and only a small number of NKAα1a ionocytes remain. Interestingly, there are no gill ionocytes present on the lamellae of smolts in seawater, whereas parr gradually acclimated to seawater have significant numbers of lamellar NKAα1b ionocytes, suggesting that the location of ionocytes in response to salinity can also be dependent on developmental stage. Overall, these results indicate that the NKAα1a and NKAα1b isoforms are differentially regulated by salinity, are normally present in only one type of gill ionocyte, and can be used to distinguish functionally distinct cells involved in ion uptake and salt secretion, respectively.

The differential regulation of NKAα1a and NKAα1b in response to salinity suggests that these isoforms may differ in transport kinetics or regulation that is physiologically relevant to ion uptake and secretion, respectively. Previous molecular studies in mammals have determined that NKAα transmembrane regions 4, 5, and 6 are critical for cation binding and transport characteristics of the enzyme (Mobasheri et al., 2000). In their initial sequencing of these isoforms in rainbow trout, Richards et al. (2003) pointed out that the fifth transmembrane region of NKAα1a and α1b differs by 7 of 21 amino acids. Recent mutagenic studies using mammalian NKA as a template found that three amino acid substitutions characteristic of the NKAα1a in transmembrane regions 5, 8 and 9 resulted in promotion of intracellular binding of Na^+ over K^+, which would potentially favor Na/H exchange over Na/K exchange (Jorgensen et al., 2003). These results are consistent with kinetic studies noted above in which lower K^+ requirements for Na^+-dependent ATPase activity were found in freshwater relative to seawater (Pfeiler and Kirschner, 1972; Pagliarani et al., 1991). However, more research with the native proteins will be necessary to determine whether altered ion transport kinetics characterize the NKAα1a and α1b isoforms of teleost fishes. Other possible differences with physiological relevance may exist between the isoforms, including ouabain sensitivity and differential regulation by FYXD and endocrine factors.

3. Tilapia embryonic cutaneous ionocytes

3.1. Sequential observation of individual ionocytes

In embryonic and larval stages of teleosts without functional gills, ionocytes have been found in the epithelia covering the yolk and body, considered to be the major site of ionic regulation during the early developmental stages (Kaneko et al., 2008). The yolk-sac membrane exhibits a simple flat structure, thus serving as a suitable experimental substitute for the gills that have a complex three-dimensional structure that is difficult to use for repeated morphological analyses or analysis of ion fluxes. Sections 3.1, 3.2 and 3.3 review a series of studies on ionocytes in the yolk-sac membrane of euryhaline Mozambique tilapia embryos that sheds light on their function and differentiation.

From both physiological and morphological points of view, it is of great interest to determine whether salinity changes (e.g. transfer from freshwater to seawater or vice versa) cause replacement of pre-existing ionocytes by newly-differentiated ionocytes with a different ion-transport function, or whether the same ionocytes can function in both salinities. An effective way to answer this question is the analysis of sequential changes in individual ionocytes during acclimation to different salinities. Hiroi et al. (1999) examined individual ionocytes in the yolk-sac membrane of tilapia embryos transferred from freshwater to seawater. Ionocytes were visualized in vivo with DASPEI, a fluorescent probe specific for mitochondria, and each ionocyte was sequentially observed for 4 days under a confocal laser scanning microscope, which allowed optical sections that eliminated troublesome autofluorescence from the yolk materials. The sequential observation revealed that 75% of pre-existing ionocytes were able to survive for 4 days following direct transfer from freshwater to seawater, and that each of these ionocytes showed a remarkable increase in size (Fig. 6). In contrast, the cell size did not change in the control embryos kept in freshwater, and the same rate of ionocyte turnover was observed in both freshwater and seawater. Moreover, the combination of differential interference contrast optics and NKA immunohistochemistry with the fixed yolk-sac membrane revealed that single ionocytes enlarged and were indented by accessory cells to form multicellular complexes during acclimation to seawater. These findings indicated that freshwater-type single ionocytes are transformed to seawater-type multicellular complexes, suggesting plasticity in the ion-transport functions of ionocytes. A decade later, functional plasticity in changing from NaCl secretion to NaCl absorption was directly confirmed using a scanning ion-selective electrode technique with cutaneous ionocytes in medaka larvae (Shen et al., 2011). Inward and outward fluxes of Na^+ and Cl^- were detected at the apical opening of ionocytes in intact medaka larvae acclimated to freshwater and seawater, respectively. By sequentially probing individual ionocytes, Na^+ and Cl^- secretion decreased dramatically after transfer from seawater to freshwater and Na^+ and Cl^- uptake was detected at the same ionocytes.

3.2. Seawater-type NKCC1a and freshwater-type NCC

The differential apical and basolateral immunoreactants with the T4 human NKCC1 antibody in ionocytes between freshwater and seawater (Section 2.4) were first detected in the gills of adult Mozambique tilapia (Wu et al., 2003) and also found in the yolk-sac membrane of tilapia embryos (Hiroi et al., 2005). This phenomenon implied that two different cation–chloride cotransporters exist in tilapia ionocytes: "freshwater-type" (apically-located and ion-absorptive) and "seawater-type" (basolaterally-located and ion-secretory).

Accordingly, Hiroi et al. (2008) set goals to identify the genes of freshwater-type and seawater-type cation–chloride cotransporters in tilapia ionocytes by molecular cloning and real-time PCR mRNA quantification, and to demonstrate immunohistochemically the localization patterns of the two cotransporters within ionocytes by homologous antibodies. At first, three full-length cDNAs homologous to human cation–chloride cotransporters were isolated from tilapia gills, designated as tilapia NKCC1a, NKCC1b and NKCC2. Then, the fourth cDNA, tilapia NCC, was obtained after laborious trials and finally by immunoscreening of a cDNA expression library with the T4 antibody. Among the four candidates, the mRNA encoding NKCC1a was highly expressed in the yolk-sac membrane and gills of seawater-acclimated fish, whereas the mRNA encoding NCC was exclusively expressed in the yolk-sac membrane and gills of freshwater-acclimated fish. During freshwater-to-seawater and seawater-to-freshwater transfer experiments with embryos, the NKCC1a mRNA in the yolk-sac membrane was upregulated in seawater and downregulated in freshwater, and the NCC mRNA was downregulated in seawater and upregulated in freshwater. These salinity-dependent changes in their mRNA levels provides evidence that NKCC1a is the "seawater-type" cotransporter involved in ion secretion and that NCC is the "freshwater-type" cotransporter involved in ion absorption.

The tilapia "freshwater-type" cotransporter discovered by Hiroi et al. (2008) was tentatively designated as "tilapia NCC", in accordance with its relatively high amino acid identity to human NCC (52%), rather than to human NKCC1 (39%) and to human NKCC2 (42%). However, phylogenetic analyses revealed that NCC-like cotransporters of vertebrates are clearly divided into two clades, namely, the conventional NCC clade and the fish-specific NCC clade.

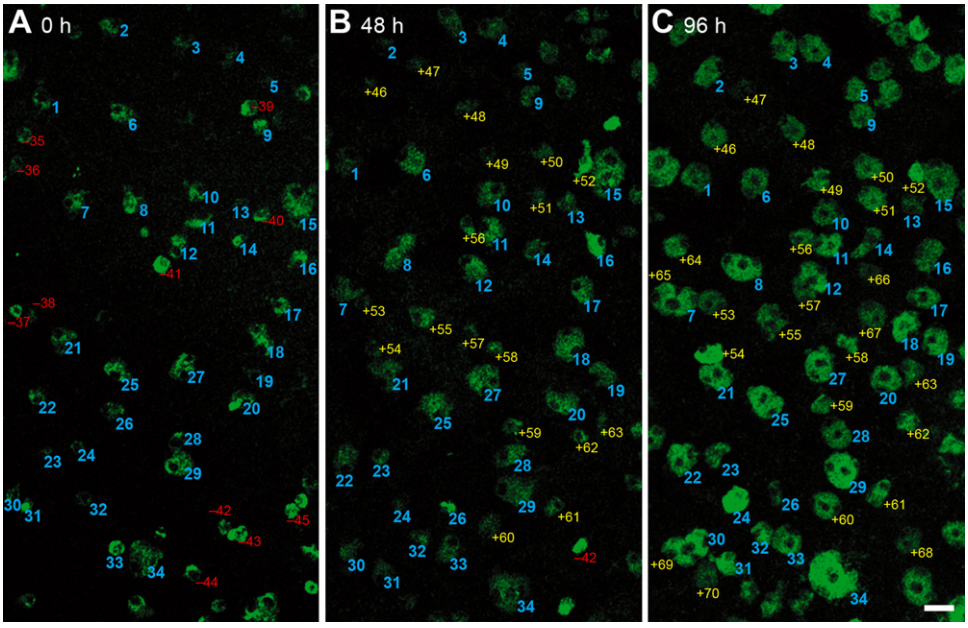

Fig. 6. In vivo sequential images of DASPEI-stained ionocytes in the yolk-sac membrane of a Mozambique tilapia embryo transferred from freshwater to seawater at 0 h (A), 48 h (B) and 96 h (C). Ionocytes detectable throughout 96 h are labeled with blue numbers (1–34). Ionocytes existing at 0 h (in freshwater) and disappeared thereafter are numbered in red (−35 to −45). Newly appearing ionocytes are numbered in yellow (+46 to +70). About 75% of the initial ionocytes survive direct transfer from freshwater to seawater and each ionocyte increases their size markedly (1–34). Scale bar, 10 μm. Modified from Hiroi et al. (1999). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

Well-known Na^+/Cl^- cotransporters such as human NCC and winter flounder *Pseudopleuronectes americanus* NCC were assigned to the conventional NCC clade, and tilapia NCC was assigned to the fish-specific NCC clade. An ortholog of tilapia NCC was identified in a specific group of ionocytes (NCC cells) in zebrafish, and the newly-recognized fish-specific NCC clade was denominated as the solute carrier family (SLC) 12A10 group (Wang et al., 2009).

3.3. Five-color immunostaining identifies four ionocyte types

The immunolocalization of NKA, NKCC1 and CFTR in ionocytes has been demonstrated in the gills of several teleosts (Wilson et al., 2000; McCormick et al., 2003; Wilson et al., 2004; Tang et al., 2011; Christensen et al., 2012), but there was no attempt to examine the co-localization of all major ion-transport proteins at the single-cell level, not only with fish ionocytes but also with ion-transport epithelial cells in general. Therefore, Hiroi et al. (2008) generated specific antibodies for tilapia NKCC1a and tilapia NCC, and conducted a trial to simultaneously visualize NKCC1a and NCC, together with NKA, CFTR and Na^+/H^+ exchanger 3 (NHE3), within ionocytes in the yolk-sac membrane of tilapia embryos. NHE3 is a critical component of one of the currently proposed ion-uptake models by fish ionocytes (Hwang et al., 2011; Dymowska et al., 2012; Kumai and Perry, 2012). The quintuple-color immunofluorescence staining, in which each primary antibody was directly labeled with different colors of fluorophores by Molecular Probe's Zenon technology, ascertained the apical or basolateral localization of each protein at the single-cell level, and consequently allowed to classify all observable ionocytes into distinct four types – types I, II, III and IV (Figs. 7 and 8): type I, showing only basolateral NKA (Fig. 7A); type II, basolateral NKA and apical NCC (Fig. 7B); type III, basolateral NKA and basolateral NKCC1a, and occasionally with apical NHE3 (Fig. 7C); type IV, basolateral NKA, basolateral NKCC1a and apical CFTR (Fig. 7D). This four-type classification had already been accomplished by the triple-color immunofluorescence staining with antibodies against human NKCC1 (T4 antibody), NKA and CFTR (Hiroi et al., 2005), but the newly generated homologous antibodies clearly distinguished the apically-restricted localization of NCC in type-II ionocytes and the basolaterally-restricted NKCC1a in type-III + IV ionocytes, and demonstrated that the two immunoreactivities did not occur in the same cells.

Type-IV ionocytes were defined by basolateral NKA, basolateral NKCC1a and apical CFTR (Fig. 7D), and this localization pattern was completely consistent with the current accepted model for NaCl secretion by ionocytes in seawater (Fig. 1). Moreover, this cell type was seawater specific: the cells were not observable in freshwater, rapidly (within 12 h) appeared and increased in number following freshwater-to-seawater transfer and disappeared following seawater-to-freshwater transfer (Fig. 8). These facts strongly suggest that type-IV ionocytes are the seawater-type ion-secretory cells, and that the NKCC1a protein at the basolateral membrane cotransports Na^+, K^+ and Cl^- from the internal environment into the ionocyte.

Type-III ionocytes possessed basolateral NKA and basolateral NKCC1a, like type-IV ionocytes, but lacked apical CFTR, and not all but most of type-III ionocytes showed apical NHE3 (Fig. 7C). In contrast to type-IV ionocytes, type-III ionocytes showed freshwater-specific increases in number: they rarely appeared in seawater, rapidly increased following seawater-to-freshwater and disappeared following freshwater-to-seawater transfer (Fig. 8). This inverse salinity-induced relationship between types III and IV suggests that the two types simply represent the same cells without/with apical CFTR. If this were not true, a very high rate of cellular turnover would have to occur following transfer to different salinities: one cell type would almost completely disappear and be replaced by another cell type in a short period of time (within 24 h). Actually, such a high rate of cellular turnover was never determined by the sequential observation (Section 3.1; Hiroi et al., 1999), supporting the identity of type-III and type-IV ionocytes. Based on the lack of CFTR and presence of NHE3 at the apical membrane, type-III ionocytes are considered to be active in ion absorption or acid/base regulation in freshwater, but can be rapidly activated to type-IV ionocytes involved in ion secretion after placement of CFTR at the apical membrane.

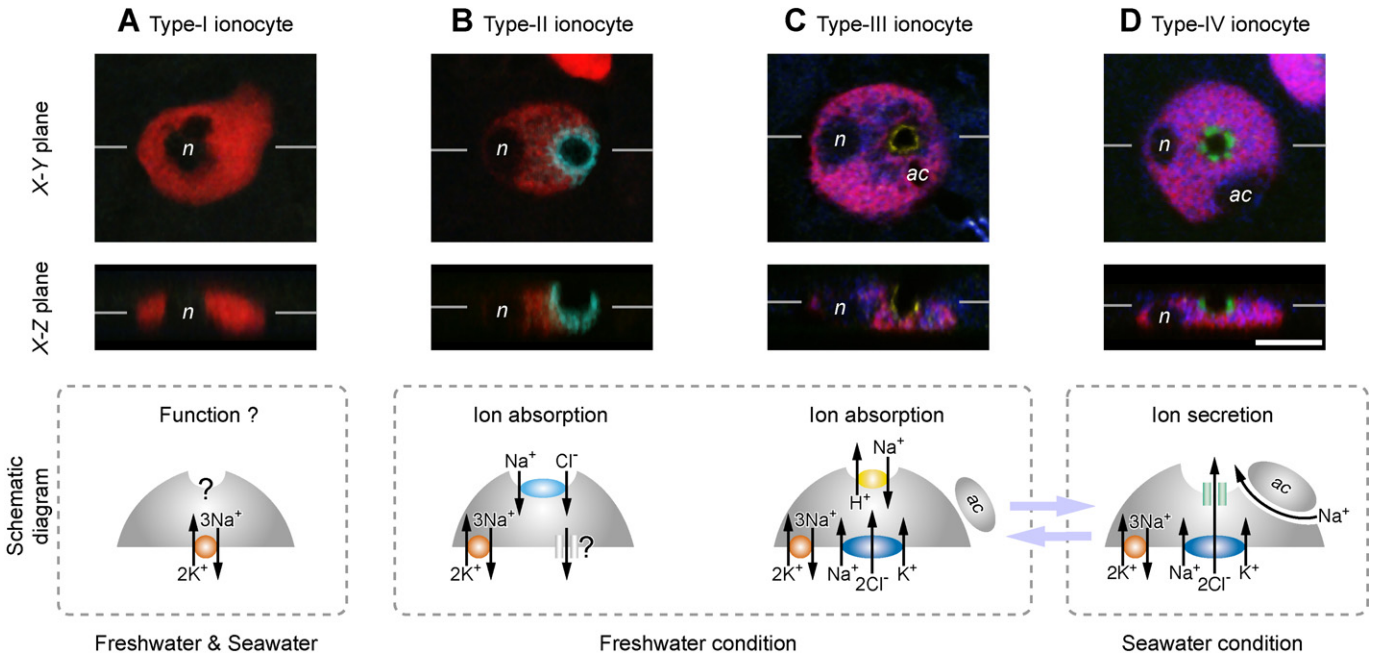

Fig. 7. Classification of ionocytes in the yolk-sac membrane of Mozambique tilapia embryos into four types, by means of quintuple-color immunofluorescence staining: type-I (A), type-II (B), type-III (C) and type-IV (D). Five channels for NKA (red), NKCC1a (blue), NCC (cyan), NHE3 (yellow) and CFTR (green) are merged and shown in *X–Y* and *X–Z* planes. Type-I ionocytes show only basolateral NKA. Type-II ionocytes possess basolateral NKA and distinct apical NCC. Type-III ionocytes are defined by basolateral NKA and basolateral NKCC1a (red for NKA and blue for NKCC1a are merged into magenta), and occasionally with apical NHE3. Type-IV ionocytes are provided with the three major ion-transport proteins, basolateral NKA, basolateral NKCC1a and apical CFTR. *X–Z* plane, the *X–Z* optical section cut transversely at the horizontal lines indicated in *X–Y* plane. *X–Y* plane, the *X–Y* optical section cut at the lines indicated in *X–Z* plane. *n*, nucleus; *ac*, accessory cell. Scale bar, 10 μm. Schematic diagrams of each of the four cell types are presented in the bottom row, showing the apical or basolateral localization patterns of NKA (red), NKCC1a (blue), NCC (cyan), NHE3 (yellow) and CFTR (green). Modified from Hiroi et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

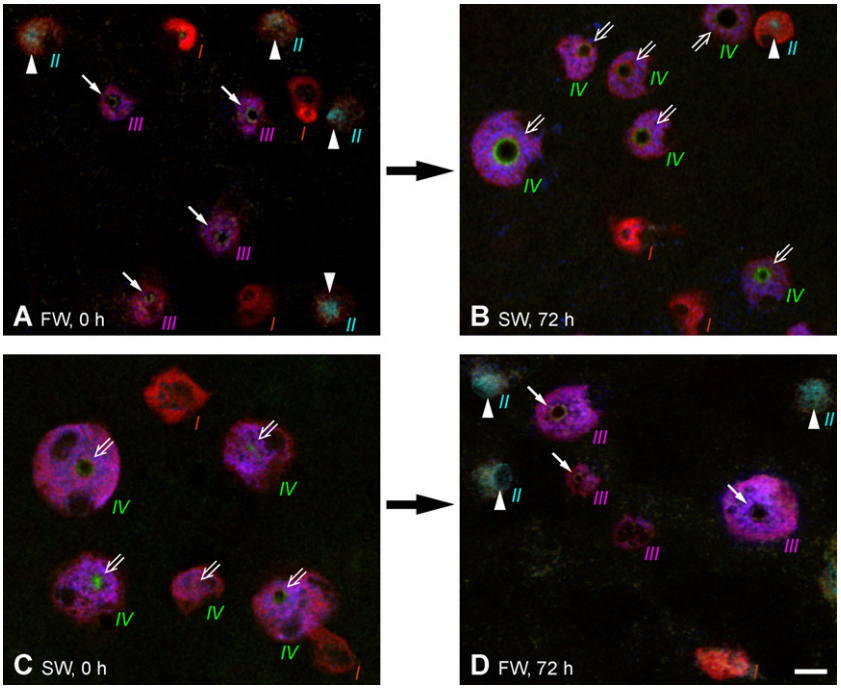

Fig. 8. Four types of ionocytes (types I, II, III and IV) determined by quintuple-color immunofluorescence staining for NKA (red), NKCC1a (blue), NCC (cyan), NHE3 (yellow) and CFTR (green) in the yolk-sac membrane of Mozambique tilapia embryos during freshwater-to-seawater and seawater-to-freshwater transfer experiments. In freshwater, three types of ionocytes, types I, II and III are observed (A). Following transfer to seawater, type-III ionocytes start to develop apical CFTR, consequently regarded as type-IV ionocytes (B, 72 h after transfer). Conversely, embryos developing in seawater possess only type-I and type-IV ionocytes (C), and transfer to freshwater induces differentiation of type-II ionocytes and the disappearance of apical CFTR in type III–IV ionocytes (D, 72 h after transfer). Arrowheads, arrows and double-lined arrows indicate the apical immunoreactivity for NCC, NHE3 and CFTR, respectively. Scale bar, 10 μm. Modified from Hiroi et al. (2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

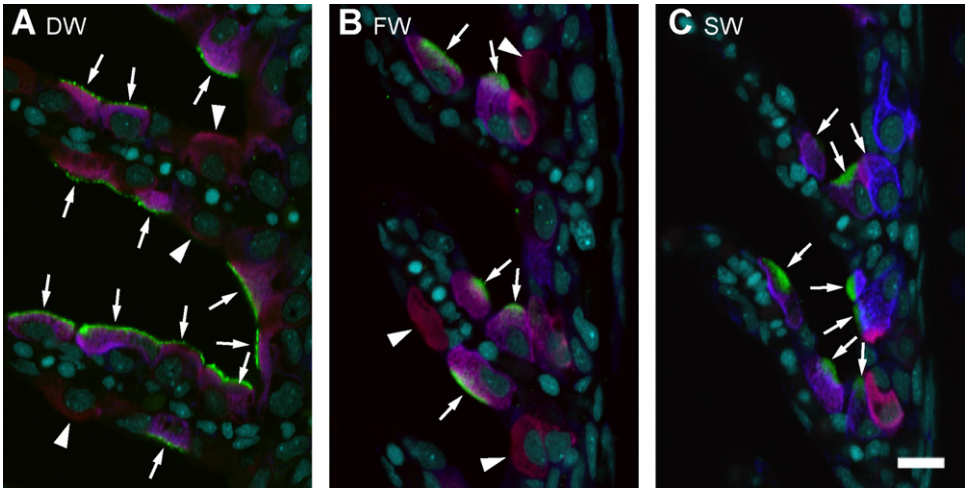

Fig. 9. Immunoreactivity of NKA (red), NKCC1 (blue) and NHE3 (green) in the gills of rainbow trout acclimated to deionized water (A), freshwater (B) and seawater (C). The nuclei were counterstained with DAPI (cyan). Ionocytes are classified into at least two types: cells possessing apical NHE3, basolateral NKA and basolateral NKCC1 (indicated by arrows, red for NKA and blue for NKCC1 are merged into magenta); cells showing only basolateral NKA (indicated by arrowheads). Several cells indicated by arrow appear to lack apical NHE3 in seawater (C), but apical NHE3 immunoreactivity is detectable by changing focus of a confocal microscope. NKA and NKCC1 are detected by α5 and T4 monoclonal antibodies, respectively, and NHE3 is detected by newly-generated antibody against rainbow trout NHE3b (GenBank ID: FJ376630). The trout NHE3b antibody can be used to detect NHE3 in other species, such as alewife (Christensen et al., 2012) and Japanese eel (Seo and Kaneko, unpublished results). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

Although basolateral NKCC1a is considered to be involved in NaCl secretion in seawater, the immunoreactivity was found not only in type-IV ionocytes in seawater but also in type-III ionocytes in freshwater, and accompanied with apical NHE3. As mentioned in the Section 2.4, several teleosts (eels, alewife, milkfish, salmonids, Hawaiian goby, silver moony, southern flounder and spotted pufferfish) showed basolateral NKCC1 immunoreactivity in branchial ionocytes in both freshwater and seawater, and the colocalization of basolateral NKCC1 and apical NHE3 was confirmed in alewife (Christensen et al., 2012), Japanese eel (Seo and Kaneko, unpublished results) and rainbow trout (Hiroi and McCormick, unpublished results; Fig. 9) as well as tilapia. Recent advanced cellular/molecular physiological studies proposed that apical NHE3 and Rh protein link together to achieve ammonia-dependent Na^+ uptake, and that basolateral NKCC1 may be involved in basolateral NH_4^+ transport (see a review by Wright and Wood, 2012). To confirm immunohistochemically the colocalization of those apical ion-transport proteins and basolateral NKCC1 at the single-cell level would provide more convincing evidence for the newly proposed model for ammonia secretion and Na^+ uptake.

Type-II ionocytes possessed basolateral NKA and distinct apical NCC immunoreactivity (Fig. 7B), and showed freshwater-specific increases: the cells were absent in seawater, appeared following seawater-to-freshwater and tended to decrease following freshwater-to-seawater transfer (Fig. 8). Therefore, it seems likely that type-II ionocytes are the freshwater-type ion-absorptive cells and that the apically located NCC protein should cotransport Na^+ and Cl^- from the external environment into the ionocyte.

Taking account of the presence of NCC-positive type-II ionocytes in freshwater, Hiroi et al. (2008) proposed a novel model for active Na^+/Cl^- absorption by teleost ionocytes in freshwater, consisting of apical NCC and basolateral NKA. Although apical cation–chloride cotransporters historically had not been suggested to be involved in ion absorption by ionocytes in freshwater (e.g. there was no description of apical cation–chloride cotransporters in ionocytes in the review by Evans et al., 2005), recent studies on tilapia and zebrafish NCC indicate that NCC is upregulated in low Cl^- conditions and involved in Cl^- uptake in freshwater (Inokuchi et al., 2008, 2009; Wang et al., 2009; Horng et al., 2009).

Type-I ionocytes showed only basolateral NKA (Fig. 7A), were relatively smaller in size and were constant in number during both freshwater-to-seawater and seawater-to-freshwater transfer experiments (Fig. 8). This cell type was first assumed to be immature ionocytes developing into other types of ionocytes (Hiroi et al., 2005), but later, more careful morphological observation (no transitional type-I ionocytes to other types were found; newly emerging type-II, III and IV ionocytes were clearly distinguishable from type-I ionocytes) suggested that type-I ionocytes are not immature ionocytes but rather an independent cell type (Hiroi et al., 2008). Although type-I ionocytes showed only basolateral NKA in these studies, they might be involved in certain ion-transport functions in accordance with other undetermined ion-transport proteins (e.g. epithelial Ca^{2+} channel).

3.4. How many ionocyte types in other fishes?

Ionocyte types with distinct morphological features have been identified in various teleost species, especially by using transmission or scanning electron microscopy (e.g. Pisam and Rambourg, 1991; Hwang and Lee, 2007). However, most of these morphological classifications of ionocytes have not been linked to specific ion transporters or physiological studies that would help determine their relationship to ionocyte function. This Section 3.4 attempts to compare tilapia ionocyte types with those of other species, zebrafish and salmonids.

In the last decade zebrafish have become one of the most extensively studied species for determining ionocyte function. In their gills and skin, four types of ionocytes were determined by combinations of in situ hybridization and immunocytochemistry: H^+-ATPase-rich (HR) cell; NKA-rich (NaR) cell; NCC cell; K^+ secreting (KS) cell (see reviews by Hwang et al., 2011; Dymowska et al., 2012). Although the same 4 types of ionocytes have been identified in tilapia and zebrafish, not all types of both species seem to have a one-to-one correspondence. Among the 4 tilapia and 4 zebrafish types, it is fairly certain that tilapia type-II ionocytes and zebrafish NCC ionocytes are identical, because both cells are defined by coexpression of NCC and NKA. Their identity is also supported by the specific localization of Na^+/HCO_3^- cotransporter 1 (NBC1) within tilapia type-II ionocytes (Furukawa et al., 2011) and

zebrafish NCC ionocytes (Lee et al., 2011), which is considered to be responsible for the basolateral exit of Na^+ in these cells. Tilapia type-III ionocytes and zebrafish HR ionocytes also show some similarity, as both cells possess apical NHE3. However, H^+-ATPase, after which zebrafish HR ionocytes were named, was not localized in any tilapia embryo ionocytes, but found in respiratory pavement cells (Hiroi et al., 1998). Examining localization of other zebrafish-HR-ionocyte-specific transporters (Rh proteins, carbonic anhydrases, anion exchangers and NKA isoforms) with tilapia would help to further compare tilapia type-III ionocytes and zebrafish HR ionocytes. Tilapia type-III ionocytes and zebrafish KS ionocytes might possibly be comparable, because renal outer medullary K^+ channel (ROMK) was localized to the apical membrane of tilapia type-III ionocytes (Furukawa et al., 2012) and also found in zebrafish KS ionocytes (Abbas et al., 2011). Type-IV ionocytes, which are involved in ion secretion in euryhaline tilapia, appear to be absent in the stenohaline zebrafish. Thus, zebrafish HR ionocytes may only be similar to tilapia type-III ionocytes in their characteristics in freshwater, and incapable of switching to a salt secretory function. Indeed, the capability to change from a type-III to type-IV ionocyte may be a hallmark of euryhalinity and deserving of further study.

Rainbow trout and salmonids in general have been widely used as model species in the study of ion transport physiology. By examining the specific binding of peanut agglutinin (PNA) to the apical membrane of ionocytes, which was originally used for identifying HCO_3^--secreting intercalated cells in the mammalian renal collecting duct, trout ionocytes were clearly classified into two types, PNA^+ and PNA^- ionocytes (Goss et al., 2001; Dymowska et al., 2012). These PNA^+ and PNA^- ionocytes are considered to correspond to α and β ionocytes, respectively, named after electron microscopic studies on several teleosts by Pisam and colleagues (Pisam and Rambourg, 1991). Basolateral NKA immunoreactivity was found in both PNA^+ and PNA^- ionocytes, but NHE3 immunoreactivity was restricted to the apical membrane of PNA^+ ionocytes in the trout gills (Ivanis et al., 2008). Similar results can be seen with triple-color immunofluorescence staining for NKA, NKCC1 and NHE3b (trout gill NHE3 isoform) in which two freshwater ionocyte types can be seen in the trout gills, those with and without apical NHE3b; after seawater exposure, apical NHE3b is present in cells with basolateral NKA and NKCC1 (Hiroi and McCormick, unpublished results; Fig. 9). These cells are analogous to the type-IV ionocytes of tilapia, but in addition to their likely salt secretory function, the presence of NHE3b suggests they are also involved in acid/base regulation. Colocalization of NKA, NKCC1 and NHE3 was confirmed in gill ionocytes in seawater-acclimated adult tilapia (Inokuchi et al., 2008), although apical NHE3 immunoreactivity was not observable in tilapia embryonic type-IV ionocytes in seawater (Hiroi et al., 2008). Whether all type-IV ionocytes possess NHE3 or just a subset remains to be determined. As noted above, it appears that all seawater teleosts, including salmonids possess a type-IV ionocyte with basolateral NKA, NKCC1 and apical CFTR. In Atlantic salmon and likely other salmonids and tilapia, the type-IV ionocyte is also characterized by the presence of a seawater-specific isoform, the NKAα1b (McCormick et al., 2009). It will be interesting to determine how widespread the possession of salinity-specific isoforms of the sodium-potassium pump is among euryhaline teleosts.

4. Conclusion

Immunohistochemical approaches to the study of ionocytes, started firstly with NKA and then in combination with NKCC1 and CFTR, have subtantially increased our understanding of the mechanisms of salinity tolerance in teleosts. The NaCl secretory mechanism by ionocytes with NKA, NKCC1 and CFTR seem to be conserved among teleost species, but immunohistochemistry with antibodies identifying isoforms with different functions (e.g. Atlantic salmon NKAα1a and α1b; Mozambique tilapia NKCC1a and NCC) suggests that there are distinct types of ionocytes with different ion-transport functions in freshwater (Section 3.4). Therefore, it seems likely that there is more than 1 type of ionocytes in most teleost fishes adaptable to freshwater, while ion-secretory ionocytes in seawater seem to be only 1 type in all teleosts, but that the latter are capable of both salt secretion and acid-base regulation. It will be important to understand both the similarities and differences of ionocyte types among a variety of teleost species.

Currently, advanced cellular/molecular physiological approaches are available with model animals such as zebrafish (Hwang et al., 2011), and integrated genome databases are available with several fish species (e.g. genome databases of coelacanth *Latimeria chalumnae*, sea lamprey *Petromyzon marinus*, zebrafish, Atlantic cod *Gadus morhua*, three-spined stickleback *Gasterosteus aculeatus*, medaka, Nile tilapia *Oreochromis niloticus*, spotted green pufferfish and fugu *Takifugu rubripes* are available on the Ensembl web site, http://www.ensembl.org). Use of ion-sensitive fluorescent dyes and microprobes have also become powerful tools in ion transport studies, though has only rarely been applied to work with fish. Accompanied with these cellular and molecular techniques, immunohistochemical approaches should be increasingly informative for understanding the diversity of functions and regulation of gill ionocytes among fishes.

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