Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 148 (2007) 479–497
CBP
www.elsevier.com/locate/cbpa

Review

New insights into fish ion regulation and mitochondrion-rich cells

Pung-Pung Hwang [a,*], Tsung-Han Lee [b]

[a] *Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei, 115, Taiwan*
[b] *Department of Life Sciences, National Chung-Hsing University, Taichung, 402, Taiwan*

Received 19 May 2007; received in revised form 18 June 2007; accepted 19 June 2007
Available online 27 June 2007

Abstract

Compared to terrestrial animals, fish have to cope with more-challenging osmotic and ionic gradients from aquatic environments with diverse salinities, ion compositions, and pH values. Gills, a unique and highly studied organ in research on fish osmoregulation and ionoregulation, provide an excellent model to study the regulatory mechanisms of ion transport. The present review introduces and discusses some recent advances in relevant issues of teleost gill ion transport and functions of gill ionocytes. Based on accumulating evidence, a conclusive model of NaCl secretion in gills of euryhaline teleosts has been established. Interpretations of results of studies on freshwater fish gill Na^+/Cl^- uptake mechanisms are still being debated compared with those for NaCl secretion. Current models for Na^+/Cl^- uptake are proposed based on studies in traditionally used model species. Many reported inconsistencies are claimed to be due to differences among species, various experimental designs, or acclimation conditions. Having the benefit of advanced techniques in molecular/cellular biology, functional genomics, and model animals, several new notions have recently been raised concerning relevant issues of Na^+/Cl^- uptake pathways. Several new windows have been opened particularly in terms of molecular mechanisms of ionocyte differentiation and energy metabolite transport between gill cells during environmental challenge.

Keywords: NaCl secretion; Na^+/Cl^- uptake; Fish; Gills; Mitochondrion-rich cells; Differentiation; Energy metabolism

Contents

* Corresponding author.
E-mail address: pphwang@gate.sinica.edu.tw (P.-P. Hwang).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2007.06.416

1. Introduction

Compared to terrestrial animals, fish have to cope with more-challenging osmotic and ionic gradients from aquatic environments with diverse salinities, ion compositions, and pH values. Fishes, the lowest vertebrate group, have adapted to every possible niche in marine and freshwater habitats over a span of 500 million years. Natural selection has led to diverse physiologies in fishes adapted to vast arrays of differences in these aquatic environments, and this may provide very important information for elucidating the mysteries of the early evolution of vertebrates.

Teleosts, the most advanced group of fishes, need to have highly efficient ion/osmoregulatory mechanisms in order to maintain their body fluid homeostasis, which is necessary for normal operation of all biochemical/physiological processes. To compensate for passive water loss, marine teleosts drink seawater and actively secrete salt via the gills as well as kidneys. On the other hand, freshwater (FW) fishes do not drink (or drink very little) water but produce diluted urine via the kidneys for balancing the passive water gain, while actively absorbing salt through the gills from the environment. Gills are a unique and the most-studied organ in research of fish osmoregulation and ionoregulation, and because the directions of ion transport in gills are reversed during acclimation to different salinity environments, they provide an excellent model to study the regulatory mechanisms of ion transport. Mitochondrion-rich (MR) cells (also formerly called chloride cells) are specialized ionocytes, and are the main site responsible for the active transport of ions in gills. Since the pioneering work by (Keys et al., 1932), plenty of literature has been accumulated on the study of the functions of MR cells in fish gills, and several models for gill ion regulation have been proposed. However, there are still many issues that are unclear and still require convincing evidence for their elucidation. Having the benefit of advance techniques in molecular/cellular biology, functional genomics, and model animals, several new notions have recently been raised. Instead of making a detailed overall review, the present review places more emphasis on introducing and discussing some of these advances, in an attempt to extend our understanding of fish gill ion transport and the functions of MR cells.

2. NaCl secretion in seawater fish

The mechanisms of NaCl secretion by seawater (SW) teleost gill epithelium are clearer than those of NaCl absorption by FW ones. The current model proposes that NaCl extrusion is mediated by the basolateral cotransport of NaCl down the electrochemical gradient provided by Na^+–K^+-ATPase (NKA), coupled with the apical exit of Cl^- via channels and the paracellular extrusion of Na^+, both of which are operated down their respective electrochemical gradients. Hence the key transporters associated with the NaCl transport process are thought to be NKA, the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Marshall, 2002; Hirose et al., 2003; Evans et al., 2005).

2.1. Na^+–K^+-ATPase

The membrane-spanning enzyme, NKA, is responsible for the active transport of Na^+ out of and K^+ into animal cells. It is important not only for sustaining intracellular homeostasis but also for providing a driving force for many transporting systems including fish gills. NKA is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex with four α (α1∼4) and three β (β1∼3) isoforms. The molecular weight of the catalytic α-subunit is about 100∼110 kDa, while the smaller glycosylated β-subunit has a molecular weight of approximately 55 kDa (Scheiner-Bobis, 2002). Immunocytochemical studies on gill sections as well as biochemical studies on isolated MR cells have demonstrated that epithelial cells contain the most NKA in the gills (Dang et al., 2000; Lee et al., 2000; Sakamoto et al., 2001; Brauer et al., 2005). Differential distributions of NKA-immunoreactive (IR)/MR cells in gills of euryhaline teleosts have been reported: (1) NKA-IR/MR cells appear only in the filamental epithelia of both FW- and SW-acclimated fish, e.g., tilapia (*Oreochromis mossambicus*) (Lee et al., 1996c; Uchida et al., 2000) and spotted green pufferfish (*Tetraodon nigroviridis*) (Lin et al., 2004b); (2) in addition to filaments, NKA-IR/MR cells occur abundantly in the lamellar epithelia of FW-acclimated individuals, e.g., chum salmon (*Oncorhynchus keta*) (Uchida et al., 1996), Japanese sea bass (*Lateolabrax japonicus*) (Hirai et al., 1999), European sea bass (*Dicentrarchus labrax*) (Varsamos et al., 2002), and milkfish (*Chanos chanos*) (Lin et al., 2003, 2006b; Chen et al., 2004); and (3) NKA-IR/MR cells are found in both gill filaments and lamellae of FW and SW fish, e.g., Hawaiian goby (*Stenogobius hawaiiensis*) (McCormick et al., 2003). Lamellar NKA-IR/MR cells are thought to migrate from the filaments (Dang et al., 1999; Hirai et al., 1999). The occurrence of lamellar NKA-IR/MR cells was suggested to be a mechanism for meeting the functional requirements of ion uptake in some FW teleosts (Uchida et al., 1996; Sasai et al., 1998; Hirai et al., 1999;

Seidelin et al., 2000; Varsamos et al., 2002); however, the partitioning of filament and lamellar NKA-IR/MR cells in the function of ion regulation in fish gills is still unknown, and thus, is a challenging and interesting phenomenon.

Most euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Marshall, 2002; Evans et al., 2005). Increases in NKA activity upon salinity challenge are attributed to (1) the precedence of or accompaniment of increased NKA α-subunit mRNA abundance (Scott et al., 2004; Seidelin et al., 2000; Singer et al., 2002), protein amounts (Tipsmark et al., 2002; Lee et al., 2000; Lin et al., 2003), or both (D'Cotta et al., 2000; Lin et al., 2004a, 2006b); and (2) modulation of the hydrolytic rate of this enzyme, as reported in gills of Atlantic cod (*Gadus morhua*) (Crombie et al., 1996) and striped bass (*Morone saxatilis*) (Tipsmark et al., 2004). A salinity challenge necessitates activation of gill NKA as well as MR cells which is crucial for a fish's acclimation. According to previous studies, however, two major salinity-induced branchial NKA responses were found: (1) higher NKA in hyperosmotic media and (2) higher NKA in hyposmotic media. The first group of euryhaline teleosts (including salmon, eel, and tilapia) all responded to salinity challenge with higher NKA activities as well as MR cell densities, which dominated the mechanisms used for salinity adaptation in the fish gills (Marshall, 2002; Hirose et al., 2003). In fish of this group, apical V-H^+-ATPase is another major primary pump, in addition to NKA, which provides a driving force for Na^+ and/or Cl^- uptake (see Section 3). The other group of euryhaline teleosts (e.g., killifish, striped bass, and milkfish) responds to hyposmotic-medium acclimation with higher NKA activities (Marshall and Bryson, 1998; Kelly and Woo, 1999; Lin et al., 2003, 2006b) as well as NKA-IR/MR cell densities (Lin et al., 2003, 2006b). In fish of this group (e.g., killifish, *Fundulus heterclitus*), V-H^+-ATPase was found to be basolaterally localized in gill NKA-IR/MR cells (Katoh et al., 2003). Without the electrochemical gradient created by the active apical extrusion of protons via V-H^+-ATPase, higher NKA activity may be required for Na^+ and/or Cl^- uptake in gills of the group with higher NKA in the hyposmotic medium, implying the possibility of different mechanisms of gill ion regulation between the two groups of fish. On the other hand, higher NKA responses in either hyperosmotic or hyposmotic medium may be ascribed to stress states of the fish which are inhabiting their respective environments. As a response to osmotic stress due to changes in environmental salinities, cytoprotective proteins belonging to the HSP70 family are induced (Iwama et al., 1998). A positive correlation between the expression of gill NKA and hepatic HSPs was found in teleosts of both the group with higher NKA in hyperosmotic medium and higher NKA in hyposmotic medium (Deane et al., 2002; Deane and Woo, 2004; C. H. Tang and T. H. Lee, unpublished data). Clearly, detailed mechanisms of these two patterns of adaptive changes in gill NKA activity have yet to be elucidated in diverse euryhaline teleosts following salinity challenges.

Discrepancies in the salinity-induced NKA responses between these two major groups of teleosts may also be attributed to NKA characteristics of species, because different affinities of NKA for sodium or potassium were found in FW- and SW-acclimated tilapia, pufferfish (the first group), and milkfish (the second group) (Lin and Lee, 2005). Being responsible for the catalytic and transport work of NKA, α-subunit isoforms revealed distinct differences in their affinities to Na^+ and K^+ in the rat brain, kidney (Urayama and Nakao, 1979), and adipocytes (Lytton et al., 1985). In the zebrafish (*Danio rerio*), nine distinct NKA α-subunit genes have been identified by analyzing the expressed sequence tag (EST) and genomic databases; among them, four (α1a.1, α1a.2, α1a.4, and α1a.5) of seven α1 genes were found to be expressed in mucous cells of the skin (Blasiole et al., 2002; Canfield et al., 2002), and are probably associated with ionocytes in skin (Lin et al., 2006a). Different isoforms of the NKA α-subunit were also found in gills of euryhaline teleosts including European eel (*Anguilla anguilla*) (Cutler et al., 1995), salmon/trout (Madsen et al., 1995; D'Cotta et al., 2000; Seidelin et al., 2001; Richards et al., 2003), killifish (Semple et al., 2002), tilapia (Hwang et al., 1998; Feng et al., 2002), and the Antarctic notothenii (*Trematomus bernachii*) (Guynn et al., 2002; Brauer et al., 2005). SW tilapia revealed different levels of increase of α1 and α3 mRNA as well as protein in gills from FW individuals (Lee et al., 1998; Feng et al., 2002). In rainbow trout (*Oncorhynchus mykiss*) gills, two NKA α-isoforms (α1a and α1b) were differentially expressed and accompanied by an elevation in gill NKA activity 10 d after transfer to 80% SW from FW (Richards et al., 2003). Moreover, NKA α1-, α2-, and α3-isoforms were expressed in the gills of the Antarctic notothenii, and the composition of NKA α-subunit isoforms in gill MR cells was thought to change following warm acclimation (Guynn et al., 2002; Brauer et al., 2005). Taken together, differential expressions of various NKA isoforms may lead to different Na^+ or K^+ affinities and fulfill some of the requirements for the subtly altered enzyme behaviors of gills of SW and FW teleosts. The exhibition and switching of NKA α-isoforms in the gills of euryhaline teleosts following salinity transfer provide new insights into the importance of this enzyme in fish ionoregulation and will be the subject of further investigations.

2.2. NKCC

NKCC includes two isoforms, NKCC1 and NKCC2. Because of prominent expression of NKCC1 in the basolateral confinement and its association with the operation of Cl^- secreting epithelia, NKCC1 is considered to be the secretory isoform (Lytle et al., 1995). Cutler and Cramb (2002) first reported isolation of two cDNAs encoding NKCC1a and NKCC1b from the European eel. An increase in the mRNA expression of gill NKCC, reflecting stimulation of Cl^- secretion, was found after transfer to SW of the European eel (Cutler and Cramb, 2002), killifish (Scott et al., 2004), and striped bass (Tipsmark et al., 2004). Using an anti-human colonic NKCC1 antibody (T4; Lytle et al., 1995), the protein abundance of NKCC was found to have been upregulated after SW acclimation in brown trout (*Salmo trutta*) (Tipsmark et al., 2002), tilapia (Wu et al., 2003), killifish (Scott et al., 2004), striped bass (Tipsmark et al., 2004), and several salmonids (Hiroi and McCormick, 2007). Meanwhile, NKCC was only detected in gills of SW rather than FW pufferfish (Tang and Lee, 2007). NKCC1 was localized to the basolateral aspect of NKA-IR/MR cells in the giant mudskipper (*Periophthalmodon schlosseri*) (Wilson et al., 2000), several salmonids (Hiroi and McCormick,

2007), killifish (Marshall et al., 2002), Hawaiian goby (McCormick et al., 2003), tilapia (Wu et al., 2003; Hiroi et al., 2005), and grass pufferfish (*Takifugu niphobles*) (Shen et al., in press). This accumulating evidence confirms the role of NKCC1 in Cl^- secretion mechanisms in SW fish gills.

2.3. CFTR

Cl^- secretion by the apical membrane of MR cells uses an anion channel with characteristics resembling those of the CFTR (Marshall et al., 1995). The first teleost CFTR was cloned by Singer et al. (1998) from SW killifish gills. Following SW exposure, upregulation of CFTR mRNA was reported in gills of the Atlantic salmon (*Salmo salar*) (Singer et al., 1998, 2002), killifish (Scott et al., 2004), and Japanese eel (*Anguilla japonicus*) (Tse et al., 2006). CFTR protein abundances were found in gills of SW-acclimated mudskipper (Wilson et al., 2000), killifish (Marshall et al., 2002; Katoh et al., 2003; Scott et al., 2004), eel (Tse et al., 2006), and pufferfish (Tang and Lee, 2007). Marshall et al. (2005) proposed a model in which the CFTR in killifish is activated by phosphorylation of protein kinase A (PKA), which in turn is stimulated by cAMP. Moreover, different CFTR antibodies applied to diverse species all revealed the same apical distribution of gill NKA-IR/MR cells in SW-acclimated teleosts, e.g., giant mudskipper (Wilson et al., 2000), Hawaiian goby (McCormick et al., 2003), killifish (Katoh and Kaneko, 2003; Marshall et al., 2002), tilapia (Hiroi et al., 2005), and grass pufferfish (Shen et al., in press). Time-course studies of killifish revealed that the apical CFTR occurred 24 h after SW transfer when CFTR expression was elevated, while it disappeared within 24 h of transfer to FW when CFTR expression is significantly decreased (Marshall et al., 2002; Katoh and Kaneko, 2003; Scott et al., 2005). Similarly, in tilapia, SW-subtype MR cells with apical CFTR appeared at 12 h and showed a remarkable increase in number between 24 and 48 h after transfer to SW (Hiroi et al., 2005). Obviously, successful activation of CFTR expression in the apical membrane of gill MR cells is crucial for euryhaline teleosts. In future studies, it will be of great interest to determine the regulation of CFTR activity, e.g., the signaling protein, 14-3-3a, which is potentially important for inactivating Cl^- secretion via CFTR after FW transfer (Kohn et al., 2003).

3. Na^+ uptake mechanisms in freshwater fish

Smith (1930) and Krogh (1937) using early isotopic experiments proposed that in fish gills, the secretion of acids (H^+ and/or NH_4^+) is linked to the transepithelial absorption of Na^+ while the secretion of bases (HCO_3^- and/or OH^-) is independently linked to the transepithelial absorption of Cl^-. Since then, these Na^+/Cl^- linked acid/base transport mechanisms have been examined, and the results are still being debated among different species and various external water conditions to the present (Marshall, 2002; Hirose et al., 2003; Perry et al., 2003; Evans et al., 2005). So far, at least two models have been proposed for the apical transport of Na^+ in fish gill cells: (1) an apical V-type of H^+-ATPase electrically linked with Na^+ absorption via the epithelial Na^+ channel (ENaC) and (2) an electroneutral exchange of Na^+ and H^+ via an apical Na^+/H^+ exchanger (NHE).

3.1. V-H^+-ATPase/ENaC

Since the uptake of Na^+ via passive exchange with H^+ has been questioned on thermodynamic grounds (Kirschner, 1983; Avella and Bornancin, 1989), most later studies favored the model of H^+-ATPase/ENaC. So far, plenty of accumulated data using pharmacological, physiological, immunocytochemical, and molecular approaches have convinced us of the role of V-type H^+-ATPase in the apical Na^+ uptake mechanism. A V-type H^+-ATPase-specific inhibitor, bafilomycin (1–10 μM), impaired over 60% of the Na^+ uptake in tilapia, carp (*Cyprinus carpio*), and zebrafish (Fenwick et al., 1999; Boisen et al., 2003). Accordingly, immunocytochemistry with heterologous or homologous antibodies and in situ hybridization with mRNA probes provided evidence for the existence of V-type H^+-ATPase in fish gills. The distribution of the enzyme in MR and/or pavement cells has been debated, and this has been ascribed to antibody specificities, and/or differences in species, populations, or acclimation conditions (see detailed discussion by Evans et al., 2005). However, recent studies in rainbow trout gills further provided more-integrative and -convincing molecular physiological evidence for this issue. Goss et al. (Galvez et al., 2002) used peanut lectin agglutinin (PNA) to isolate two subpopulations of MR cells, PNA^- and PNA^+ MR cells, and the PNA^- subtype showed a much-higher expression and acid-stimulated response of H^+-ATPase compared to PNA^+ cells. Moreover, only PNA^- cells demonstrated bafilomycin-sensitive acid-activated $^{22}Na^+$ uptake (Reid et al., 2003). Hwang's group (Lin et al., 2006a) identified two subtypes of ionocytes, NKA-rich (NaR) cells and H^+-ATPase-rich (HR) cells, by immunocytochemistry and in situ hybridization in zebrafish embryo skin, and used a non-invasive ion-selective electrode to demonstrate the in vivo function of bafilomycin-sensitive acid secretion from apical membranes of HR cells (but not from those of NaR cells). A subsequent study showed that knockdown of V-type H^+-ATPase translation with a specific morpholino impaired the Na^+ uptake activity in zebrafish morphants, providing molecular physiological evidence for the coupling of V-type H^+-ATPase with the Na^+ uptake mechanism in HR cells, a specific type of gill ionocytes (Horng et al., 2007). At the same time, work by Hirose's group (Esaki et al., 2007) also supported this notion; they used a sodium-green fluorescent reagent to confirm that Na^+ accumulated only in skin HR cells in zebrafish embryos, and this accumulation was inhibited by bafilomycin. On the other hand, a study in killifish provided convincing immuno-electron microscopic localization of V-ATPase in the basolateral membrane of gill MR cells and demonstrated stimulation of expression by low-NaCl fresh water, implying an alternative pathway for the involvement of the enzyme in Na^+ (and/or Cl^-) uptake in that species (Katoh et al., 2003).

Goss et al. (Reid et al., 2003; Parks et al., 2007) demonstrated phenamil-sensitive $^{22}Na^+$ uptake and intracellular acidification in isolated PNA^- MR cells, providing in vitro pharmacological evidence for the involvement of ENaC in fish

gill's Na^+ uptake mechanism. On the other hand, in an in vivo study by Esaki et al. (2007) 10 mM amiloride (an inhibitor of ENaC but not NHE) did not affect the sodium-green fluorescent accumulation in HR cells in zebrafish embryos but did decrease the $^{22}Na^+$ uptake in the whole embryo. A human ENaC polyclonal antibody has been used to localize ENaC in gill MR cells and/or pavement cells in rainbow trout and tilapia (Wilson et al., 2000). However, a counterpart to the human ENaC could not be found in the zebrafish or fugu genomes. In mammals, ENaC and the amiloride-sensitive cation channel (ASIC) belong to the same gene family. According to (Paukert et al., 2004) six zebrafish amiloride-sensitive cation channel genes have been cloned, but in situ hybridization experiments have not indicated the expressions of these genes in fish gills. Apparently, convincing molecular evidence for the existence of ENaC or equivalent channels is still lacking, and is an urgent issue which needs to be resolved.

3.2. NHE

Na^+ uptake via passive exchange with H^+ has been questioned for a long time as discussed above; however, recently accumulating evidence has allowed us to reconsider the role of NHE in the fish gill Na^+/acid transport mechanism. In mammalian renal proximal tubule cells, several isoforms of the Na^+/H^+ exchanger, NHE2, NHE3, and NHE8, have been localized in the brush-border membranes of cells (Wagner et al., 2004). NHE3 was found to account for about 50% of the overall apical NHE activity in renal proximal tubule cells (Choi et al., 2000). Genetic knock-out of NHE2 had no effect on renal function, whereas complete or kidney-specific knock-out of NHE3 resulted in a reduction of proximal tubular Na^+ and water loss (Schultheis et al., 1998; Woo et al., 2003). Therefore, these isoforms of NHE would be expected to be the targets involved in apical NHE's functions in FW fish gills. Using heterologous antibodies, NHE2 and NHE3 immunoreactivities were located in gill MR cells of rainbow trout, blue-throated wrasse (*Pseudolabrus tetrious*), and tilapia (Edwards et al., 1999; Wilson et al., 2000). Hirata et al. (2003) used homologous antibodies and molecular probes to provide the most convincing evidence for the expression of NHE3 in the apical membrane of gill MR cells in a unique freshwater teleost, the Osorezan dace (*Tribolodon hakonensis*). Gill NHE3 expression greatly increased during acclimation to highly acidic water, and therefore was thought to play a major role in acid secretion in that species (Hirata et al., 2003). A recent paper by Hirose's group (Esaki et al., 2007) further demonstrated the involvement of NHE in fish Na^+ uptake mechanisms; the NHE inhibitors, amiloride at 100 mM and EIPA at 10 mM, were found to block over 90% of Na^+ accumulation (estimated from the sodium-green fluorescent reagent) in skin ionocytes of zebrafish embryos, but no molecular evidence for the existence of NHE was available from the same work (Esaki et al., 2007). Nine NHE isoforms could be predicted from the NCBI and Ensembl genome database. We have cloned eight of them, and found that only one of them, NHE3b (EF591980) was specifically expressed in HR cells (Fig. 1). Further molecular physiological experiments are needed to certify

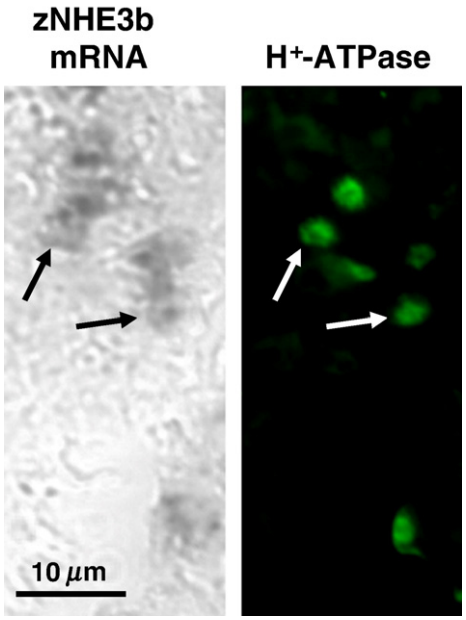

Fig. 1. Specific expression of zNHE3b mRNA in H^+-ATPase-rich cells. The same frozen section of zebrafish gill was double labeled with a specific RNA probe of zNHE3b (EF591980, nt1907 ~ 2745) and an anti-killifish H^+-ATPase antibody (Katoh et al., 2003). Arrow indicates the colocalized signals.

that this NHE isoform is the one specifically responsible for the Na^+ uptake function in zebrafish HR cells.

3.3. Driving force for NHE

In a model for mammal renal proximal tubules (Purkerson and Schwartz, 2007), filtered HCO_3^- and H^+ secreted by the apical NHE3 actively form H_2CO_3, H_2CO_3 is dehydrated by the apical CAIV (carbonate anhydrase IV isoform), thereby enabling the passive diffusion of CO_2 into cells, and then the cytosolic CAII hydrates CO_2 to provide a substrate for NHE3, i.e., CAIV associated with NHE3 facilitates Na^+/H exchange in the apical membranes of cells. CAIV has recently been cloned from dogfish (*Squalus acanthias*) and trout, but CAIV expression was found mainly in non-MR cells in dogfish gills (Gilmour et al., 2007) and never in trout gills (Georgalis et al., 2006). Determining whether apical CAIV provides the driving force for NHE to operate Na^+/H exchange in fish gill ionocytes, like mammal proximal tubule cells, deserves further study. Our preliminary experiments indicated that a CAIV-like isoform (EF591981) was mainly expressed in skin HR cells of zebrafish (Fig. 2A and B), and knockdown of the translation of the zCAIV-like isoform with morpholino (1.5 ng/embryo) caused a decrease about 50% in Na^+ uptake in zebrafish embryos (T. Y. Lin, C. D. Hsiao and P. P. Hwang, unpublished data).

3.4. Basolateral Na^+ exit

Basolateral Na^+ exit has typically been assumed to be achieved via NKA, which is highly expressed in gill MR cells and has even been used as a marker for those cells (Evans et al., 2005). Recent studies have raised the possibility of the involvement of the electrogenic Na^+/HCO_3^- cotransporter (NBC1) in the basolateral

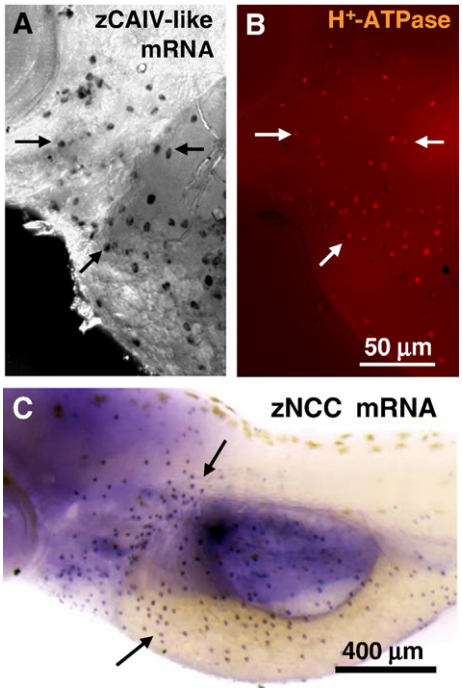

Fig. 2. Transporter expressions in zebrafish 5-day-post-fertilization embryos. A and B, whole-mount double labeling with a specific RNA probe of zCAIV-like (EF591981, nt783 ~ 1277) and an anti-killifish H^+-ATPase antibody. Arrow indicates the co-localization of the two signals in skin ionocytes. C, whole-mount in situ hybridization with a specific RNA probe of zNCCg (EF591989, nt639 ~ 2162). zNCC mRNA signals (arrow) were found in skin ionocytes of zebrafish embryos.

Na^+ exit of gill ionocytes. In a study by Perry et al. (2003), a homolog of mammalian NBC1 from rainbow trout was cloned and found to be stimulated at the gill transcript level by respiratory acidosis. In Japanese dace, a homolog of mammalian pNBC1/NBCe1-B and CAII have also been found to be upregulated on acidification; using homologous antibodies, CAII and the NCB1 were localized in the cytosol and the basolateral membrane, respectively, of gill MR cells (Hirata et al., 2003). Based on these results, the electroneutral cotransport of Na^+ and HCO_3^- out of MR cells was proposed to be achieved via NBC1 using the driving force generated by CAII and NKA (Hirata et al., 2003). Recently, Parks et al. (2007) also supported this notion based on evidence that intracellular acidification (assayed with BCECF-AM, a pH-sensitive dye) and membrane potential (with bis-oxonol, a voltage-sensitive dye) depolarization in a subset of isolated trout gill cells were sensitive to NBC (DIDS) and/or a CA inhibitor (acetazolamide). Indeed, at the basolateral membrane of mammal proximal tubule cells, CAII was demonstrated to be associated with kNBC1 (Gross et al., 2002), and CAII hydrates CO_2 to provide a substrate, thereby facilitating the electroneutral Na^+ and HCO_3^- transport by kNBC1 (Purkerson and Schwartz, 2007).

Assuming that a homolog or equivalent of mammalian ENaC exists in a subpopulation of gill ionocytes, both V-H^+-ATPase/ENaC and NHE appear to be involved in the Na^+ uptake mechanism in zebrafish HR cells. Thus, it would be interesting and challenging to determine how these two systems collaborate in the Na^+ mechanisms in HR cells. The partitioning of these two systems may depend on environmental conditions. Indeed, a recent study on zebrafish reported that responses of Na^+ uptake to several inhibitors, bafilomycin, ethoxzolamide, and amiloride, varied in fish acclimated to soft water and hard water (Boisen et al., 2003).

4. Cl^- uptake mechanisms in freshwater fish

In mammalian kidneys several pathways for Cl^- reabsorption exist. In proximal tubules, in addition to paracellular Cl^- transport, Cl^-/anion (formate, oxalate, and bicarbonate) exchangers are the first step in transcellular Cl^- reabsorption (Planelles, 2004). The $Na^+/K^+/2Cl^-$ cotransporter (NKCC2) and Na^+/Cl^- cotransporter (NCC) are responsible for the apical Cl^- uptake in the thick ascending limb and distal tubules, respectively (Lang et al., 2005; Wagner, 2007). On the other hand, pendrin (SLC26a4) was recently found to be involved in the apical Cl^- transport pathway in type B and non-A/non-B intercalated cells within the mammalian connecting tubules and cortical collecting ducts (Wagner, 2007).

4.1. Cl^-/HCO_3^- exchanger

Compared with studies on Na^+ uptake, those for Cl^- uptake mechanisms are much scarcer, and much more remains unelucidated so far. The central mechanism has been proposed as being the absorption of Cl^- linked with the secretion of HCO_3^- via apical anion exchangers (Evans et al., 2005; Tresguerres et al., 2006). However, only a few studies have examined the involvement of Cl^-/HCO_3^- exchangers in this putative pathway. The evident inhibition of Cl^- uptake by the disulfonic stilbene derivatives, SITS (Perry and Randall, 1981) and DIDS (Chang and Hwang, 2004), supports this pathway. In situ hybridization with an oligonucleotide probe, complementary to rat AE1 (anion exchanger, isoform 1) cDNA, indicated mRNA signals in both pavement and MR cells in trout gills (Sullivan et al., 1996). A polyclonal antibody against trout blood cell AE1 was used to localize the transporter in apical membranes of gill cells of tilapia and coho salmon (*Oncorhynchus kisutch*) (Wilson et al., 2000, 2002). In a recent review, Tresguerres et al. (2006) addressed their concerns about the specificity of antibodies used in those previous studies. AE1 mRNA expression (examined by real-time PCR) in gills was not stimulated in low-Cl^- acclimated tilapia (C. W. Yang and P. P. Hwang, unpublished data), the Cl^- uptake of which was enhanced compared to the controls acclimated to high-Cl^- medium (Chang and Hwang, 2004). Recently, Tang and Lee (2007,in press) used antibodies against tilapia AE1 and human CAII to colocalize the 2 proteins with the basolateral membrane NKA in gills of the spotted green pufferfish, and found higher expressions of AEI and CAII in FW gills than in SW ones, indicating the possible role of these proteins in acid/base regulation in FW gills. Taking all these together, there is still no convincing evidence to demonstrate an AE isoform that is specifically expressed in the apical domain of gill ionocytes and functions in apical Cl^- uptake in the cells. On the other hand, Piermarini et al. (2002) used an anti-pendrin (SLC26a4) antibody to indicate immunoreactions in the apical region of stingray (*Dasyatis sabina*) gill ionocytes that are rich in basolateral V-H^+-

ATPase. This shed new light on the apical Cl^- transport pathway in fish gills, since in mammals, pendrin expression in B type intercalated cells of the cortical collecting duct (CCD), and transepithelial voltage and Cl^-net flux in the perfused CCD are highly regulated depending upon the urinary Cl^- excretion and NaCl content in the diet (Quentin et al., 2004; Verlander et al., 2006; Pech et al., 2007).

4.2. Driving force for the Cl^-/HCO_3^- exchanger

CAII and V-H^+-ATPase, which have been proposed to provide a HCO_3^- gradient as a driving force for pendrin-mediated Cl^- absorption (Wagner, 2007), were also colocalized in fish gill ionocytes as described above. Moreover, inhibitors of CA (ACTZ) and V-H^+-ATPase (bafilomycin and NEM) have been also shown to abolish gill Cl^- uptake in several fish species (Maetz and Garciaromeu, 1964; Fenwick et al., 1999; Chang and Hwang, 2004). Acclimation to low-Cl^- FW stimulated Cl^- uptake and protein levels of CA and V-H^+-ATPase in tilapia gills (Chang and Hwang, 2004). All these raise the possibility for fish gill Cl^- uptake mechanisms that are achieved via an apical Cl^-/HCO_3^- exchanger (possibly pendrin) driven by CA and V-H^+-ATPase. However, whether apical or basolateral V-H^+-ATPase is involved is still being debated. Marshall (2002) proposed that local acidification in the apical crypt of gill ionocytes by V-H^+-ATPase would theoretically sufficiently lower HCO_3^- activity at the apical surface to drive the Cl^-/HCO_3^- exchanger for Cl^- uptake. Goss et al. (Tresguerres et al., 2006), on the other hand, addressed how basolateral V-H^+-ATPase is more viable considering the experimentally demonstrated direct 1:1 linkage between Cl^- uptake and HCO_3^- excretion and the light- and electron-microscopic immunocytochemical evidence provided by them. Convincing molecular physiological data to certify these notions are still lacking.

4.3. NKCC/NCC

Two other possible candidates for the apical Cl^- uptake in fish gill cells are NKCC and Thiazide-sensitive Na^+/Cl^- cotransporter (NCC). In tilapia embryonic skin and adult gills, a heterologous antibody (T4) revealed the apical NKCC signals in FW-type MR cells, and the apical NKCC was suggested to be associated with Na^+/Cl^- uptake (Wu et al., 2003; Hiroi et al., 2005). In mammals, NKCC occurs as two major isoforms, a secretory isoform, NKCC1, and an absorptive isoform, NKCC2 (Gamba, 2005), and the anti-NKCC antibody (T4) used was known to react with both NKCC isoforms (Lytle et al., 1995). Therefore, Hiroi's group cloned 3 NKCC isoforms from tilapia, raised isoform-specific antibodies, and found that all of the NKCCs were not apically localized in gill MR cells (J. Hiroi, S. Yasumasu, S. D. McCormick, and T. Kaneko, personal communication). Although Kirschner (2004) proposed that a NKCC-mediated Cl^- uptake pathway may exist in estuarine fish as well as brackish-water crabs, convincing molecular evidence for this pathway in fish gill ionocytes is still unavailable.

Thiazide-sensitive NCC (SLC12A3), a kidney-specific transporter in mammals, is mainly expressed in the apical membrane of distal convoluted tubule (DCT) cells, and is responsible for NaCl reabsorption in DCT cells (Gamba, 2005). NCC has been cloned from several species of fish (NCBI gene bank). Recently, a homologous antibody was used to localize NCC in the apical membrane of MR cells of tilapia embryonic skin and adult gills (J. Hiroi, S. Yasumasu, S. D. McCormick, and T. Kaneko, personal communication), and the expression of NCC was found to be upregulated by low-Cl^- FW (M. Niida, J. Hiroi and T. Kaneko, personal communication). From the NCBI gene bank, two NCC isoforms were predicted and could be sequenced from zebrafish. Our preliminary experiments indicated that only one NCC (EF591989) is the specific form expressed in skin ionocytes in zebrafish embryos (Fig. 2C), and knockdown of the translation of the gill-specific NCC with specific morpholino (2.5 ng/embryo) impaired Cl^- uptake (about 55% decrease) in embryos (Y. F. Wang, Y. C. Tseng, J. J. Yan, J. Hiroi, and P. P. Hwang, unpublished data). These preliminary results opened a new window for examining Cl^- uptake mechanisms in fish gills.

4.4. Basolateral Cl^- exit

Nine CLC Cl^- channels have been identified in humans: ClC-1 is a skeletal muscle-specific isoform, while the rest (ClC-Ka, ClC-Kb, ClC-2~7) are expressed in the kidneys (Uchida and Sasaki, 2005). The final step for human renal Cl^- reabsorption, i.e., basolateral Cl^- exit, is mainly achieved via Cl^- channels composed of the pore-forming unit, ClC-Kb, and the beta-subunit, barttin (Lang et al., 2005). Very few studies have investigated ClC Cl^- channels in fish. In tilapia, ClC-3 and -5 were cloned and found to be expressed in various organs including gills, but they were suggested to function as intracellular Cl^- channels based on an in vitro functional analysis (Miyazaki et al., 2002). Subsequently, in the same species, ClCK was also cloned and found to be a kidney-specific isoform (Miguel Mancera et al., 2002). Recently, Western blot with an anti-rat ClC-3 antibody indicated a higher protein expression in FW pufferfish gills than in SW ones (Tang and Lee, 2007). On the other hand, a cystic fibrosis transmembrane regulator (CFTR), another Cl^- channel family, was also proposed to be a candidate for basolateral Cl^- exit based on its basolateral localization in pavement and MR cells in killifish operculum (Marshall 2002). Much remains to be investigated before we can draw definitive conclusions on the mechanisms for basolateral Cl^- exit in fish gill ionocytes.

5. Ca^{2+} uptake in freshwater fish

With regard to the Ca^{2+} uptake mechanism of fish MR cells, Flik et al. (1995) proposed a model similar to that for Ca^{2+} reabsoption in mammalian kidneys (Hoenderop et al., 2005). In that model, following entry of Ca^{2+} through epithelial Ca^{2+} channels (ECaC) in the apical membranes of cells, Ca^{2+} bound to calbindin diffuses to the basolateral membrane. At the basolateral membrane, Ca^{2+} is extruded via an ATP-dependent plasma membrane Ca^{2+}-ATPase (PMCA) and a Na^+/Ca^{2+} exchanger (NCX). In this way, there is net transepithelial Ca^{2+} absorption. In an early physiological study, stanniocalcin (a

hypocalcemic hormone) and lanthanum (a voltage-independent Ca^{2+} channel blocker) were shown to inhibit Ca^{2+} transport through apical membranes of fish gills (Perry and Flik, 1988). Flik et al. conducted a series of biochemical and physiological studies on PMCA and NCX in fish gills. They detected the activities of both PMCA and NCX in basolateral membranes of fish gills (Schoenmakers and Flik, 1992; Flik et al., 1997). A stanniectomy was found not to cause a significant effect on the activities and kinetics of either PMCA of NCX in eel gills (van der Heijden et al., 1999). Considering the kinetic properties of the PMCA and NCX, extrusion mechanisms were proposed as operating far below their maximum capacity in fish gills (Flik et al., 1997). However, there had been no convincing molecular evidence to support the current Ca^{2+} uptake model in fish gill cells until recently.

Qiu and Hogstrand (2004) cloned a homolog of the mammalian ECaC from pufferfish (*Takifugu rubripes*), and demonstrated a Ca^{2+} uptake function in Madin-Darby canine kidney cells expressing the fugu ECaC. Pan et al. (2005) for the first time provided molecular physiological evidence for the role of ECaC in a Ca^{2+} uptake mechanism in fish. They cloned an ECaC from zebrafish (zECaC), and found that zECaC was expressed in a portion of MR cells in the skin and gills of developing zebrafish. zECaC mRNA expression was closely correlated with the profile of Ca^{2+} uptake in developing zebrafish, and acclimation to low-Ca^{2+} FW induced upregulation of zECaC mRNA expression and the Ca^{2+} uptake capacity in zebrafish embryos. Subsequently, Perry et al. also studied the expression and regulation of ECaC in rainbow trout gills (Shahsavarani et al., 2006; Shahsavarani and Perry, 2006). The experiments with gill sections and isolated gill cells indicated that ECaC mRNA and/or protein were expressed in both pavement and MR cells in rainbow trout (Shahsavarani et al., 2006; Shahsavarani and Perry, 2006), which is inconsistent with the prevailing view that MRCs are the predominant site for branchial Ca^{2+} uptake (Evans et al., 2005). Treatments with exposure to soft water or hypercapnia, implantation with cortisol, and infusion with $CaCl_2$ all affected ECaC mRNA and/or protein expressions in trout gills, thus affecting the Ca^{2+} uptake capacity (Shahsavarani and Perry, 2006). In a recent study on isolated trout gill cells, PNA^+ MR cells showed an over 3-fold higher $^{45}Ca^{2+}$ uptake capacity compared to either PNA^- MR or pavement cells (Galvez et al., 2006). Those studies on trout indicated the ECaC-mediated Ca^{2+} transport through membranes of pavement cells, but did not necessarily demonstrate the involvement of those cells in transepithelial Ca^{2+} uptake, because of the lack of molecular or physiological data to demonstrate the transport pathway across the basolateral membrane. Our recent experiments indicated that PMCA2 (EF591990) and NCX1b (Genbank: NM_001039144) are specifically expressed in MR cells in gills of zebrafish (Fig. 3) and tilapia (data not shown), indicating that gill MR cells co-expressing ECaC (Pan et al., 2005), PMCA2, and NCX1b may play major roles in transepithelial Ca^{2+} uptake mechanisms. Moreover, acclimation to low-Ca^{2+} FW, which should induce upregulation of Ca^{2+} uptake, stimulated only ECaC mRNA expression, but did not affect those of PMCA2 or NCX1b (Liao et al., 2006). Taking all these observations together, it appears that entry of Ca^{2+} from water via the apical ECaC serves as the gatekeeper of transepithelial Ca^{2+} transport in fish gills, similar to that in mammalian kidney (Hoenderop et al., 2005).

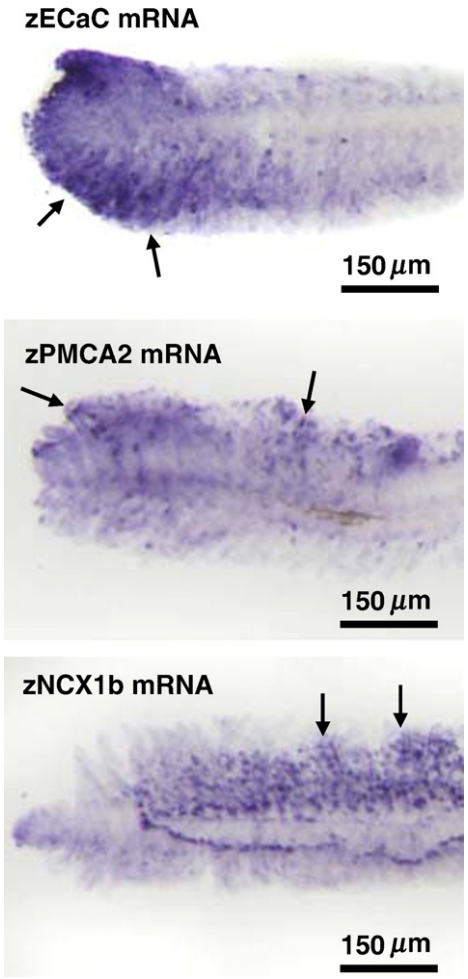

Fig. 3. mRNA expressions of zECaC, zPMCA2 and zNCX1b in zebrafish gills. Whole-mount in situ hybridization of zebrafish gills with specific RNA probes of zECaC (GenBank: NM_001001849, nt349 ~ 696), zPMCA2 (EF591990, nt4067 ~ 5017) and zNCX1b (GenBank: NM_001039144, nt2968 ~ 4270), respectively. mRNA signals (arrow) were found in ionocytes of zebrafish gills.

6. Different subtypes of mitochondrion-rich cells (or ionocytes)

6.1. Identification and functional analysis of subtypes of ionocytes

As discussed above, gills are known to be involved in the transepithelial transport of various ions and in acid/base regulation, and these functions are differentially regulated depending on the environments to which the fish are acclimated. Determining how gill ionocytes carry out such diverse functions has been a challenging and curious topic for researchers in relevant fields.

MR cells, the major ionocytes in fish gills, were documented to be pleomorphic in much-earlier studies (Shirai and Utida,

1970; Hossler et al., 1979; Hwang and Hirano, 1985; Hwang, 1988). Scanning electron microscopy (SEM) was used in earlier studies to identify and quantify functional MR cells including FW and SW subtypes in numerous species (Table 1) such as mullet (*Mugil cephalus*) (Hossler et al., 1979), killifish (Hossler et al., 1985), striped bass (King and Hossler, 1991), brown trout (Brown, 1992), tilapia (Kültz et al., 1995), and goldfish (*Carassius auratus*) (Lee et al., 1996a). In SEM micrographs, the FW subtype of MR cells generally showed flat or slightly invaginated surfaces like membrane patches with short cellular projections on them, while the SW subtype exhibited deeply invaginated surfaces with smaller orifices. In addition to SEM, transmission electron microscopy (TEM) was used to observe the ultrastructures of MR cells in the gill epithelium. Different levels of electron densities are usually described as a criterion for distinguishing subtypes (i.e., α or β subtype) of MR cells (Pisam and Rambourg, 1991; Table 1).

In the euryhaline tilapia, different types (subtypes) of MR cells were reported based on the methods of observations (Table 2). Three subtypes of MR cells were exhibited in FW tilapia: wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III). On the other hand, in SW-adapted tilapia, only one subtype of MR cell with significant apical crypts (subtype III) was found in tilapia gill epithelium (Table 2). The polymorphism of the apical membrane compartment of MR cells represents structural modifications of active MR cells. Rapid and reversible changes among MR cell subtypes and numbers occur in order to accommodate abrupt environmental ionic changes (Lee et al., 1996c, 2000; Hiroi et al., 1999; Chang et al., 2001, 2003; Lin et al., 2004a). Lin et al. (2004a) found that branchial wavy-convex MR cells occurred within 3 h when tilapia was transferred from SW (higher Cl^-) to FW (lower Cl^-). Accordingly, when tilapia were transferred from artificial high-Cl^- to low-Cl^- media, most deep-hole (subtype III) MR cells were transformed to wavy-convex cells within 3 h; while wavy-convex (subtype I) MR cells disappeared within 3 h when transferred from artificial low-Cl^- to high-Cl^- media (Chang et al., 2003). Moreover, tilapia gill MR cells were found to respond to brackish water (BW) hyperosmotic shock by the disappearance of wavy-convex cells at 3 h post-transfer and the appearance of deep-hole cells at 48 h post-transfer (C. L. Huang, P. J. Wang, C. H. Lin, T. H. Lee, and P. P. Hwang, unpublished data). The time-lag and transformation of MR cell subtypes were similar to those described in previous studies of Chang et al. (2003) and Lin et al. (2004a). The mechanism of acute wavy-convex MR cell disappearance was demonstrated to be an effort of adjacent pavement cells to cover over the apical surfaces of MR cells (Lin and Hwang, 2004) as reported in gills of the mudskipper (*Periophthalmus modestus*) (Sakamoto and Ando, 2002) and killifish (Daborn et al., 2001; Katoh and Kaneko, 2003). Apparently, the transition between different subtypes of MR cells may reflect modulation of the functions in response to changes in environmental ion compositions or salinities.

As described above, the heterogeneity of MR cell subtypes has been proposed as performing different ionoregulatory functions, and reversible alterations of MR cell phenotypes of tilapia provide indirect evidence for the hypothesis that major functions of

Table 1
Different subtypes of MR cells in gill epithelia of teleosts

Teleosts		Media	Types of MR cells	References
Common name	Scientific name			
Stenohaline				
Loach	*Cobitis taenia*	FW	α cell/β cell	Pisam et al. (1990) (T)
Gudgeon	*Gobio gobio*	FW	α cell/β cell	″
Turbot	*Scophthalmus maximus*	SW	α cell	″
Gold fish	*Carassius auratus*	FW	A cell/B cell	Lee et al. (1996a) (S)
Carp	*Cyprinus carpio*	FW	A cell/B cell	″
Medaka	*Oryzias latipes*	FW	A cell/B cell	Lee et al. (1996b) (S)
Euryhaline				
Japanese eel	*Anguilla japonica*	FW/SW	FW: A cell/B cell; SW: A cell	Shirai and Utida (1970) (T)
″	″	FW/SW	FW: F2 cell/F1 cell; SW: S1 cell/S2 cell	Wong and Chan (1999a,b) (P)
Atlantic salmon	*Salmo salar*	FW/SW	FW type/SW type	Pisam et al. (1988) (T)
Brown trout	*Salmo trutta*	FW/SW	FW: α cell/β cell; SW: α cell/β cell	Brown (1992) (S)
Rainbow trout	*Oncorhynchus mykiss*	FW	PNA^+cell/PNA^-cell	Goss et al. (2001) (P)
Killifish	*Fundulus heteroclitus*	FW/SW	FW: shallow openings; SW: deep openings	Hossler et al. (1985) (s)
″	″	FW/SW	FW: disks with microvilli; SW: pits	Katoh et al. (2001) (S)
Guppy	*Lebistes reticulatus*	FW/SW	FW: α cell/β cell; SW: α cell	Pisam et al. (1987) (T)
Mullet	*Mugil cephalus*	FW/SW	FW: shallow openings; SW: deep openings	Hossler et al. (1979) (S)
Striped bass	*Morone saxatilis*	FW/SW	FW type/SW type	King and Hossler (1991) (S)
Tilapia	*Oreochromis niloticus*	FW	Light cell/Dark cell	Maina (1990) (S/T)
″	″	DW/FW/SW	DW/FW: α cell/β cell; SW: α cell	Pisam et al. (1995) (T)
″	*Oreochromis mossambicus*	FW/SW	FW type/SW type	Kültz et al. (1995) (S)
″	″	FW/SW	Wavy convex/shallow basin/deep hole	Lee et al. (1996c), 2003 (S)
″	″	FW/SW	FW: Type I/II/III cell; SW: Type III cell	van der Heijden et al. (1999) (S/C)
″	″	FW/SW	FW: Type II/III cell; SW: Type IV cell	Hiroi et al. (2005) (C)

FW, freshwater; SW, seawater; DW, deionized water; S, scanning electron microscoy; T, transmission electron microscopy; P, percoll gradient centrifugation; C, confocal laser scanning microscopy.

Table 2
Different types (subtypes) of MR cells of euryhaline tilapia (*Oreochromis mossambicus*)

Environments	Pisam et al. (1995)[a]	Kültz et al. (1995)[b]	Lee et al. (1996c,2003)[b]	van der Heijden et al. (1999)[b]	Tsai and Hwang (1998)[c]	Hiroi et al. (2005)[c]
FW	α cell	–	Deep hole (subtype III)	Type III	WGA^- cell	Type III
	β cell	FW type (apical patches)	Wavy convex (subtype I), Shallow basin (subtype II)	Type I/II	WGA^+ cell	Type II
SW	α cell	SW type (apical cryps)	Deep hole	Type III	–	Type IV

[a] Observed by transmission electron microscopy.
[b] Observed by scanning electron microscopy.
[c] Observed by immunofluorescent staining for wheat germ agglutinin (WGA) or ion transporters.

subtypes of MR cells differ. According to studies on the positive correlations between Cl^- influx and compositions of MR cell subtypes in artificially low- or high-Cl^- media, wavy convex (subtype I) and deep hole (subtype III) MR cells are thought to play major roles in Cl^- uptake and secretion, respectively (Chang et al., 2001, 2003); while subtype II MR cells with shallow-basin apical surfaces are suggested as being responsible for Ca^{2+} uptake because of the apparent relationship between ion influx and cell density (Tsai and Hwang, 1998). Moreover, using immunofluorescent staining, Hiroi et al. (2005) identified four types of tilapia MR cells with different distributions of NKA, NKCC, and CFTR. Among them, type II ion absorptive cells with a wide apical opening are thought to be identical to wavy-convex cells in which NKCC is localized in the apical membrane (Wu et al., 2003; Hiroi et al., 2005). Meanwhile, type IV ion secretory cells in gills of SW tilapia (Hiroi et al., 2005) seem likely to be the deep-hole MR cells (Kültz et al., 1995; Lee et al., 2000; Uchida et al., 2000; Hiroi et al., 2005) with basolateral NKA and NKCC, and apical CFTR, and they participate in Cl^- secreting activity (Wu et al., 2003).

Subtypes of gill ionocytes showing different ion regulatory functions were also proposed in other species like killifish (Katoh et al., 2001), Japanese eel (Wong and Chan, 1999b), rainbow trout (Goss et al., 2001; Galvez et al., 2002; Reid et al., 2003; Parks et al., 2007), and zebrafish (Pan et al., 2005; Lin et al., 2006a, Horng et al., 2007). Recent studies on rainbow trout and zebrafish have provided convincing molecular physiological evidence for this notion. As described above (in Section 3) in rainbow trout, isolated PNA^- and PNA^+ MR cells were found to exhibit different components of ion transporters and show in vitro Na^+ and Ca^+ uptake, respectively (Reid et al., 2003; Galvez et al., 2006; Parks et al., 2007). In zebrafish embryos, two subtypes of ionocytes, HR cells and NaR cells, were identified with immunocytochemistry and demonstrated in vivo with electrophysiological and loss-of-function approaches to be responsible for H^+ secretion/Na^+ uptake and Ca^{2+} uptake, respectively (Pan et al., 2005; Lin et al., 2006a, Esaki et al., 2007; Horng et al., 2007).

6.2. Differentiation of ionocytes

Effects of environmental factors and hormones on cell renewal and proliferation of fish gill MR cells have been another important issue in fish osmoregulation for a long time (Conte and Lin, 1967; Mackinnon and Enesco, 1980; Chretien and Pisam, 1986; Laurent et al., 1994; Tsai and Hwang, 1998). Mackinnon and Enesco (1980) used ^{3}H-thymidin labeling to demonstrate that gill epithelial cells of *Barbus conchonius* originate at the base of the gill arch and migrate to both the filaments and lamellae. In the guppy (*Poecilia reticulata*), the number of nuclei incorporating ^{3}H-thymidine was found to be three times higher in SW fish than in FW ones (Chretien and Pisam, 1986). Similarly, the turnover rate of MR cells in chum salmon gill filaments was about 3 times greater in SW than in FW (Uchida and Kaneko, 1996). On the other hand, with BrdU labeling, tilapia were found to develop WGA-positive MR cells in the first 2–4 d post-labeling which then transformed into WGA-negative MR cells 6 ~ 8 d later, indicating the transition of different subtypes or different differentiation stages of MR cells (Tsai and Hwang, 1998). In those studies, data were collected from different individuals, and therefore do not necessarily reflect the fates of the same cells.

DASPEI vital staining and confocal microscopy were used to trace the in vivo sequential changes in individual MR cells in tilapia larval skin after transfer from FW to SW (Hiroi et al., 1999); this sophisticated study demonstrated that most of the FW-type small MR cells possess the ability to survive after direct transfer from FW to SW and to transform to SW-type large MR cells, and both types of MR cells are renewed from the presumed undifferentiated cells with the same turnover rate. Lin and Hwang (2004) used the same approaches to demonstrate that over 90% of the original MR cells survived and modulated their apical membrane compartments within several hours for regulating their Cl^- uptake capacity after transfer from high- to low-Cl^- FW, and fewer then 10% of the cells were newly recruited. Wong and Chan (1999a) employed flow cytometry on isolated eel gill cells to identify several subtypes of FW and SW MR cells based on cell size, cellular granularity, and cell autofluorescence, and to find the transitions between subtypes during SW acclimation; they also proposed a population of non-chloride (non-MR) cells with mitotic activity as stem cells. Those studies provide convincing evidence for the morphological and functional transitions between subtypes of MR cells and their origins from undifferentiated cells or stem cells. In the model proposed by McCormick (2001), the primary mode of action of cortisol is cytogenic, mediating the growth and differentiation of gill ionocytes in both FW and SW teleosts; cortisol appears to work in concert with prolactin in FW, but with growth hormone and insulin-like growth factor I (IGF-I) in SW. On the other hand in an elegant study by Shiraishi et al. (2001), yolk balls of tilapia larvae were separated from the embryonic body, and the wound had healed 3 h after the surgical operation; they further examined the effects of SW

transfer and cortisol administration on NKA immunocytochemical reactions in yolk-ball MR cells, and concluded that the differentiation of ionocytes in the yolk-sac membrane appears to be independent of embryonic endocrine and nerve systems. The pathway to regulate the MR cells' differentiation in embryos and larvae may differ form that in adult.

Nothing was known about the molecular mechanisms behind the differentiations of MR cells and their subtypes until recently (Fig. 4). In mammals, by gene-knock-out approaches, a forkhead transcription factor, *foxi1*, was demonstrated to function as a master regulator for ionocytes' terminal differentiation in the inner ear and kidney (Hulander et al., 2003; Blomqvist et al., 2004). In zebrafish embryos, two duplicated forkhead transcription factor, *foxi3a* and *foxi3b*, were identified as forming a positive regulatory loop for specification and differentiation of epidermal ionocytes (Hsiao et al., 2007). The process of epidermal development is evolutionarily conserved among vertebrates and relies on balanced signaling gradients secreted from non-neural (BMPs) and neural ectoderm (chordin and noggin) (De Robertis and Kuroda, 2004; Moreau and Leclerc, 2004). Under stimulation by BMPs, downstream targets of *p63* are activated in epidermal stem cells, and they function during the subsequent process of terminal differentiation (Bakkers et al., 2002). In zebrafish embryos, *foxi3a* and *foxi3b* are initially expressed in a subgroup of P63-positive epidermal stem cells at the tail-bud stage, and P63 expression in *foxi3a*/*b*-expressing cells was sharply downregulated later at the 14-somite stage; indicating that *foxi3a*/*b*-expressing cells (i.e., ionocyte progenitors, see below) originate from epidermal stem cells (with P63 as a marker) (Hsiao et al., 2007). By triple-labeling for immunocytochemistry and in situ hybridization, *foxi3a* and *foxi3b* were found to be expressed within a subdomain (the epidermal ionocyte domain) of the ventral ectoderm at around the 90% epiboly to the tail-bud stages; thereafter, *atp1b1b* and *ca2a* (markers for NaR and HR cells, respectively) were differentially expressed in these *foxi3a*- and *foxi3b*-positive ionocyte progenitors (Hsiao et al., 2007). In the same study, Delta-Notch-mediated lateral inhibition was found to play a vital role in singling out epidermal ionocyte progenitors from epidermal stem cells based on the results that the entire epidermal ionocyte domain of genetic mutants and morphants, which failed to transmit the DeltaC-Notch1a/Notch3 signal from sending cells (epidermal ionocytes) to receiving cells (epidermal stem cells), differentiated into epidermal ionocytes. The low Notch activity in epidermal ionocyte progenitors is permissive for activating *foxi3a* and *foxi3b* (Hsiao et al., 2007). Through gain- and loss-of-function assays, the *foxi3a-foxi3b* regulatory loop was demonstrated to function as a master regulator which mediates a dual role of specifying epidermal ionocyte progenitors as well as of subsequently promoting differentiation of HR cells and NaR cells in a concentration-dependent manner (Hsiao et al., 2007).

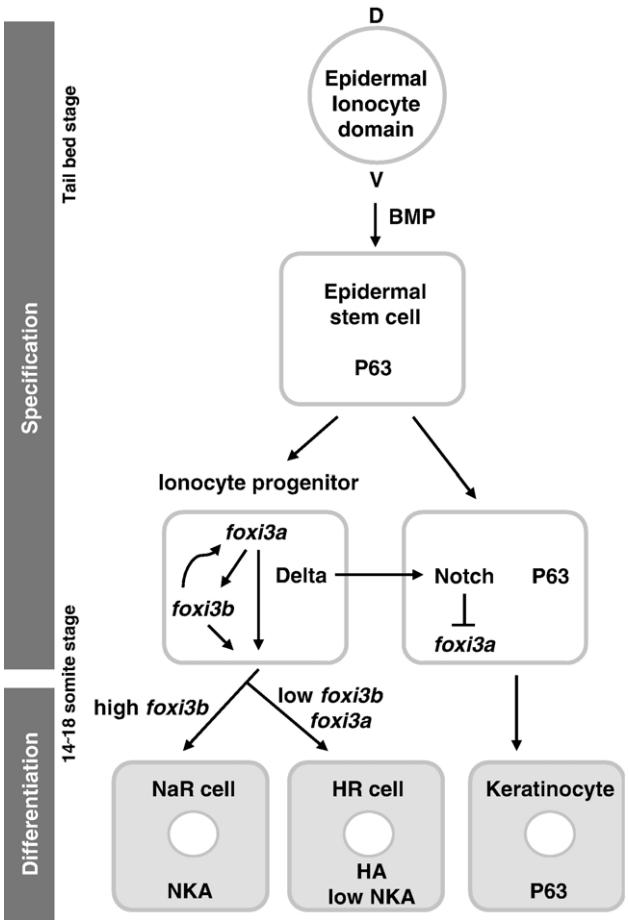

Fig. 4. A proposed model for the molecular mechanisms controlling epidermal ionocyte specification and differentiation in zebrafish (modified from Hsiao et al., 2007). See details in "Section 6.2". At the cell fate specification stage of epidermal ionocyte development, Bmp-Delta/Notch signals play an essential role in setting epidermal ectoderm competence and singling-out epidermal ionocyte progenitors from the epidermal stem cell pool. At the epidermal ionocyte differentiation stage, the *foxi3a/foxi3b* regulatory loop is activated, promoting the subsequent Na^+, K^+-ATPase-rich (NaR) cell and H^+-ATPase-rich (HR) cell differentiation program. D, dorsal; HA, V-H^+-ATPase; NKA, Na^+–K^+-ATPase; V, ventral.

Knockdown of *foxi3a* and *foxi3b* translations was found to inhibit the terminal differentiations of both NaR and HR cells, and as a result, the ionic and osmotic balances were impaired (Hsiao et al., 2007). Esaki et al. (2007) also reported the decreased Na^+ uptake in zebrafish morphants with knockdown of *foxi3a*. Apparently, knocking-down translation of the upstream regulatory gene (like *foxi3a* and *foxi3b*) for ionocyte differentiation provides a convincing molecular physiological approach for studying the in vivo functions of ionocytes. Therefore, it will be challenging and important to determine other transcription factors, which may regulate the differentiation of different subtypes of ionocytes, and to study the functions of the various subtypes of ionocytes.

7. Energy metabolism for ion transport

As discussed above, many transporters and enzymes, which require energy to operate, have been known to be involved in ionic, osmotic and acid/base regulation mechanisms in fish gills. Acclimation to fluctuating environments has been well documented to induce gill MR cells to sufficiently and timely modulate and/or activate the operations of the related transporters and enzymes (Evans et al., 2005), and this requires prompt and extra energy supplies (Boeuf and Payan, 2001). Energy metabolism for fish osmoregulation has been for a traditional and important issues not

only for scientific significance but also for aquaculture industry because it is closely relevant to fish growth (Boeuf and Payan, 2001).

7.1. Oxygen consumption

Whole-fish oxygen consumption was used to examine the effects of different environmental salinities, and the energy requirement for acclimation to a salinity condition could be derived from the changes of oxygen consumption. Many studies indicated that acclimations to different salinities did cause changes in oxygen consumption, although the results differed depending on species, duration of acclimation, experimental designs and the details of measurement methodology (Febry and Lutz, 1987; Moser and Hettler, 1989; Morgan and Iwama, 1991, 1998; Ron et al., 1995; Woo and Kelly, 1995; Haney and Nordlie, 1997; Morgan et al., 1997; Swanson, 1998; Plaut, 2000; Sardella et al., 2004; Gracia-Lopez et al., 2006). As summarized by Boeuf and Payan (2001), ranging from 20 to 68% of the total energy expenditure was estimated for the consuming of osmoregulation in different species. However, Morgan and Iwama (1991) addressed that estimation of osmoregulation cost based on the whole-fish oxygen consumption should consider the effects by other metabolic processes which respond to changes in salinity.

Whole-fish oxygen consumption could not distinguish the partition of energy cost to gill, which is the major organ responsible for fish osmoregulation. Some studies using isolated/perfused gill arch preparations measured the metabolic rates specific for gills in FW/SW cutthroat trout (*Oncorhynchus clarki clarki*) (Morgan and Iwama, 1999), Atlantic cod (Johansen and Pettersson, 1981) and European flounder *Platichthys flesus* (Lyndon, 1994). Assuming the majority of H^+-ATPase and NKA to be relevant to the active NaCl uptake, the ouabain-sensitive and bafilomycin-sensitive oxygen consumption in the isolated trout gill (Morgan and Iwama, 1999) was found to be similar to the theoretical value determined by Eddy (1982) and Kirschner (1995) who used thermodynamic or molecular approach based on ATP/O_2 consumption by ionic fluxes. As addressed by Morgan and Iwama (1999), the contribution of total gill arch oxygen consumption appears to be relatively small (<4%) to the whole animal oxygen uptake.

7.2. Energetic metabolism

Salinity acclimation, as discussed above, obviously requires additional energy for the modulation and stimulation of ion transport mechanisms in fish gills. Metabolic reorganization and alterations in intermediary metabolic pathways may occur to meet the increased energy demands associated with acclimation to new environmental salinities. Some studies provided basic information on this point, although some of their results were inconsistent depending upon the species, acclimation conditions, and experimental designs. Most of those studies revealed that carbohydrate metabolism plays a major role in the metabolic reorganization during salinity acclimation. In a study (Sangiao-Alvarellos et al., 2005) on gilthead sea bream (*Sparus auratus*), acclimation from 38- to 55-ppt SW for 14 d enhanced the mobilization of glycosyl units from glycogen stores, and as a result, a higher capacity to export glucose in the liver and increased use of exogenous glucose in gills were noted, while the pattern was reversed or was less extensive during acclimation to 6 ppt seawater. In a subsequent study (Polakof et al., 2006), 14 to 20 metabolites and enzymes related to carbohydrates, proteins, and lipids were further examined, and the results indicated that the higher energy consumption of the liver during osmotic acclimation was mainly based on carbohydrates, whereas increased importance of amino acids and lactate appeared in the gills. Apparently, most of those studies also addressed the major role of carbohydrate metabolism in metabolic reorganization during salinity acclimation (Woo and Fung, 1981; Nakano et al., 1998; Kelly and Woo, 1999; De Boeck et al., 2000).

Glycogenolysis and the translocation of subsequent metabolites during an emergency situation have been investigated in detail in the mammalian central nervous system. In the rat brain, glycogen phosphorylase (GP) and glycogen mainly exist in astrocytes, astroglial cells, and ependymal cells of ventricles, but never in neurons (Pfeiffer-Guglielmi et al., 2003). During energy deprivation in the central nervous system, glycogen is degraded to lactate, which is shuttled from astrocytes to highly energy-requiring neurons (Ransom and Fern, 1997; Brown et al., 2003). In the very early literature, glycogen granules were electron microscopically reported in the cytoplasm of gill MR cells (Philpott and Copeland, 1963), but no convincing evidence was available to support this until recently. Changes in GP activities

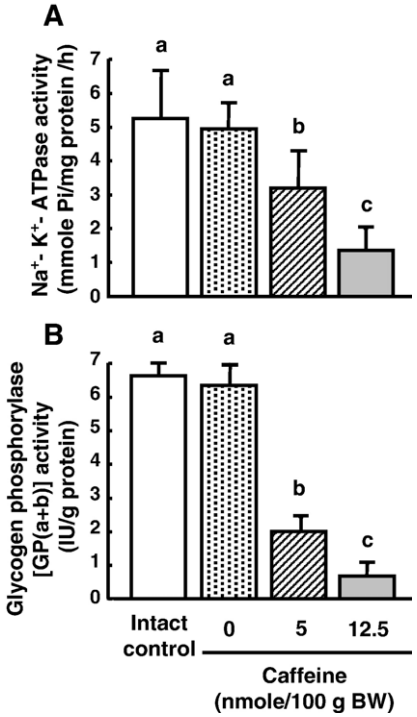

Fig. 5. Effects of in vivo intraparietal injection of caffeine (dissolved in DMSO) on the Na^+–K^+-ATPase activity (A) and the total glycogen phosphorylase activity (B) in FW tilapia gill cells. Caffeine showed dose-dependant inhibitions on both enzyme activities. Injection dose: 0 (DMSO only), 5, and 12.5 nmole/100 g body weight. Intact control was without any injection. Different letters indicate significant differences between treatments (One-way ANOVA, Tukey's pairwise comparisons).

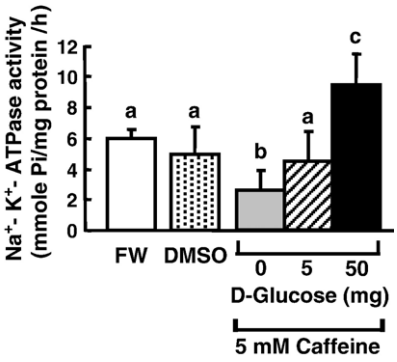

Fig. 6. Effects of in vitro caffeine (dissolved in DMSO) incubation on the cultured FW tilapia gill cells. 5 mM of caffeine inhibited the Na^+–K^+-ATPase activity, but the addition of 5 or 50 mg D-glucose rescued the enzyme activities. FW, control incubated in FW; DMSO, control incubated in FW added with DMSO only. Different letters indicate significant differences between treatments (One-way ANOVA, Tukey's pairwise comparisons).

were also found in fish gills after acclimation to salinity changes (Sangiao-Alvarellos et al., 2005). These results raise the possibility that glycogenolysis in the gills themselves may also be involved in the energy supply during salinity acclimation. In our recent study (Tseng et al., 2007), a novel gill GP isoform (tGPGG) was identified in tilapia gill; double in situ hybridization and immunocytochemistry demonstrated that tGPGG mRNA and glycogen were colocalized in a specific group of gill cells, glycogen-rich cells (GRCs), which surround MR cells and share the same apical opening with the neighboring MR cells; acclimation to seawater stimulated tGPGG mRNA/protein expression levels, enzyme activities, and glycogen contents in tilapia gills. Our preliminary experiments further indicated that in vivo injection of fish (Fig. 5A and B) and in vitro incubation of cultured gills (Fig. 6) with caffeine (an inhibitor of GP) inhibited both the GP activity in GR cells and the NKA activity in MR cells; the addition of D-glucose rescued the NKA activity of cultured gills (Fig. 6). Based on these results, we proposed that the spatial and functional relationships between mammalian neurons and astrocytes for rapid mobilization of local energy stores may also occur in gill MR and GR cells; tGPGG expression in GR cells is stimulated by an acute salinity challenge, and this may catalyze initial glycogen degradation to provide the adjacent MR cells with energy to carry out iono- and osmoregulatory functions (Tseng et al., 2007). In a subsequent experiment of acute salinity transfer, following the rapid increase in gill NKA activity, glycogen depletion initially appeared 1 ~ 3 h post-transfer in gills (i.e., GR cells) and thereafter at 6 ~ 12 h in the liver; moreover, GP activities in the gills and liver were also accordingly stimulated (Chang et al., in press). Much remains to be confirmed; however, these results outline a model for the local (gill GR cells) and systemic (liver cells) partitioning of glycogen metabolism, which provides emergency energy for osmoregulation by gill MR cells.

8. Conclusions and perspectives

The molecular remodeling and functional plasticity of subtypes of ionocytes (or MR cells) are essential to the ion transport process of euryhaline teleosts upon challenge with altered salinities and ionic compositions. A conclusive model of NaCl secretion in gills of euryhaline teleosts depicts apical localized CFTR Cl^- channel as well as basolaterally distributed NKA, NKCC, and an inward rectifier potassium channel (eKir) (Suzuki et al., 1999; Tse et al., 2006) in SW-subtype MR cells. In the future, it will be of great interest to determine the regulatory mechanisms of these ion transporters, e.g., FXYD1 (the regulatory protein of NKA) (Geering, 2006) and 14-3-3a (the

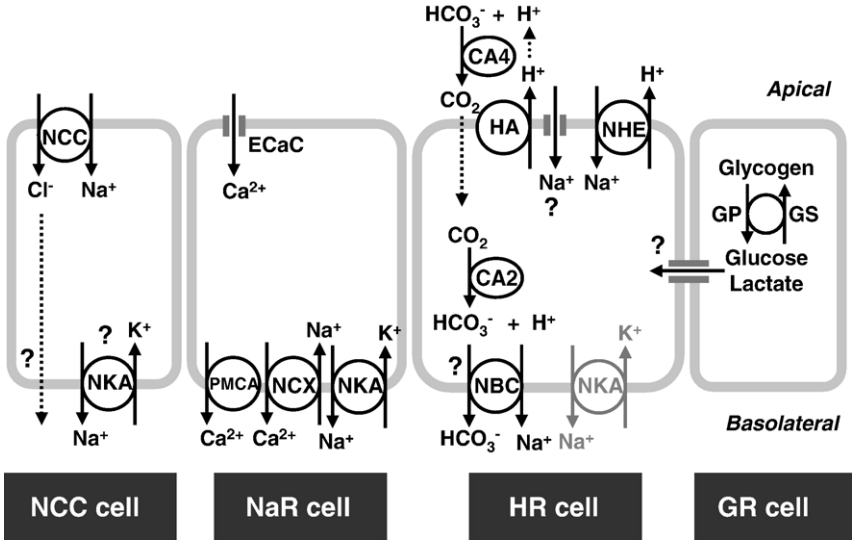

Fig. 7. A proposed model of ion regulatory mechanisms in zebrafish gill/skin ionocytes. In this model, there are at least three subtypes of ionocytes, NaR (Na^+–K^+-ATPase-rich) cells, HR (H^+-ATPase-rich) cells, and NCC (thiazide-sensitive Na^+/Cl^- cotransporter expressing) cells and another type of gill cell, GR (glycogen-rich) cells (data from tilapia, Tseng et al., 2007). NaR cells, HR cells, and NCC cells express different sets of ion transporters and enzymes to carry out Ca^{2+} uptake, Na^+ uptake/acid secretion, and Cl^- uptake, respectively. CA2, CAII; CA4, CAIV-like; ECaC, epithelial Ca^{2+} channel; GP, glycogen phosphorylase; GS, glycogen synthase; HA, V-H^+-ATPase; NBC, Na^+/HCO_3^- cotransporter; NCX, NCX1b; NHE, NHE3b; NKA, Na^+–K^+-ATPase. A question mark indicates no molecular or physiological evidence for the pathway. NKA in the gray color indicates lower NKA expression.

signaling protein inactivating Cl^- secretion via CFTR) (Kohn et al., 2003), and the mechanisms through which these transporters are coordinated to achieve the secretion process. Functional and physiological significances of the different responses of gill NKA α-subunit isoforms to environmental salinities deserve further investigation. The increased NKA activity in hyposmotic environments in some species like killifish, striped bass, and milkfish, is another puzzling story which remains to be explored.

Studies on FW fish gill Na^+/Cl^- uptake mechanisms have been accelerating, but the mechanisms are still being debated compared with those for NaCl secretion. The current models of ion regulation in FW gill cells were proposed mainly based on studies in traditional model species like salmon, trout, tilapia, eel, and killifish (Marshall 2002; Hirose et al., 2003; Perry et al., 2003; Evans et al., 2005). Many inconsistencies have been reported so far, which were claimed to be due to differences among species, various experimental designs, or acclimation conditions as discussed above. However, some possible reasons should be taken into consideration. One is the specificities of the heterologous antibodies, which have been used for immunocytochemistry or Western blotting and which have not been appropriately characterized before use. Another is the transporter isoforms. Several isoforms are generally known to exist in a transporter family since the genomes of human and several fish species have been sequenced (Cheng et al., 2003). Data mining of the genetic databases, and cloning and expression localization with specific probes are necessary to determine if specific isoforms of a transporter exist in fish gill ionocytes, and then further functional studies of these transporter isoforms will be convincing and of physiological significance. Compared with salmon, trout, tilapia, etc., zebrafish (and/or fugu) is a new and more powerful model species because of its plentiful genetic database and mutants, as well as the various established molecular physiological approaches (e.g., gain-of-function and loss-of-function). Based on recent studies discussed above, a model of ion regulatory mechanisms in zebrafish gill/skin ionocytes is proposed (Fig. 7). In this new model, there are at least three subtypes of ionocytes (NaR cells, HR cells, and NCC-expressing cells) in zebrafish gill/skin, and relevant transporters and enzymes are thought to achieve the transport of different ions in the ionocyte subtypes, respectively, although many unknown points deserve more exploration in the future: (1) molecular evidence for the existence of ENaC or other equivalent transporters and their expressions in ionocytes, (2) coordination of the NHE and ENaC/H^+-ATPase pathways in the functions of Na^+ uptake and acid secretion, (3) molecular physiological evidence for whether CAIV or other isoforms provide the driving force for Na^+ and/or Cl^- uptake, (4) molecular physiological evidence for the involvement of NCC, SLC4 (like AE1), and/or SLC26 (like pendrin) in Cl^- uptake mechanisms, and (5) molecular physiological evidence for the driving force to operate NCC, AE1, and/or pendrin, (6) transcription factors to control the differentiations of different subtypes of ionocytes.

A sufficient energy supply is a prerequisite for the operation of ion regulation mechanisms in fish gill MR cells. The energy requirements (oxygen consumption) for gills appear to be relatively small compared to that for the whole fish. During acclimation to different environmental salinities, carbohydrate metabolism plays a major role in the energy supply for ion regulation in gills, and the liver is definitely the major source for supplying carbohydrate metabolites to gills. A new window has been opened for examining the spatial and temporal relations between the gills and liver in partitioning of the energy supply for ion regulation mechanisms during salinity or other challenges. Further questions are how metabolites are transported between GR cells (the energy supplier) and ionocytes (the energy consumer), and how energy metabolism and ion regulation are coordinated during environmental challenges.

Acknowledgments

Our original research was financially supported by grants from the National Science Council and Academia Sinica of Taiwan, ROC. We thank Dr. T. Kaneko (University of Tokyo) for his discussion and encouragement, and also extend our thanks to Y. C. Tung for her technical and secretarial assistance.

References

Avella, M., Bornancin, M., 1989. A new analysis of ammonia and sodium-transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 142, 155–175.

Bakkers, J., Hild, M., Kramer, C., Furutani-Seiki, M., Hammerschmidt, M., 2002. Zebrafish delta Np63 is a direct target of bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. Dev. Cell Biol. 2, 617–627.

Blasiole, B., Canfield, V., Degrave, A., Thisse, C., Thisse, B., Rajarao, J., Levenson, R., 2002. Cloning, mapping, and developmental expression of a sixth zebrafish Na, K-ATPase alpha1 subunit gene (atp1a1a5). Gene Expr. Patterns 2, 243–246.

Blomqvist, S.R., Vidarsson, H., Fitzgerald, S., Johansson, B.R., Ollerstam, A., Brown, R., Persson, A.E., Bergstrom, G.G., Enerback, S., 2004. Distal renal tubular acidosis in mice that lack the forkhead transcription factor *Foxi1*. J. Clin. Invest. 113, 1560–1570.

Boeuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. C 130, 411–423.

Boisen, A.M., Amstrup, J., Novak, I., Grosell, M., 2003. Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). Biochim. Biophys. Acta 1618, 207–218.

Brauer, P.R., Sanmann, J.N., Petzel, D.H., 2005. Effects of warm acclimation on Na^+,K^+-ATPase alpha-subunit expression in chloride cells of Antarctic fish. Anat. Rec. 285A, 600–609.

Brown, P., 1992. Gill chloride cell surface-area is greater in fresh-water-adapted adult sea trout (*Salmo trutta* L) than those adapted to seawater. J. Fish Biol. 40, 481–484.

Brown, A.M., Tekkok, S.B., Ransom, B.R., 2003. Glycogen regulation and functional role in mouse white matter. J. Physiol. 549, 501–512.

Canfield, V.A., Loppin, B., Thisse, B., Thisse, C., Postlethwait, J.H., Mohideen, M.A., Rajarao, S.J., Levenson, R., 2002. Na, K-ATPase a and b subunit genes exhibit unique expression patterns during zebrafish embryogenesis. Mech. Dev. 116, 51–59.

Chang, I.C., Hwang, P.P., 2004. Cl^- uptake mechanism in freshwater-adapted tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. 77, 406–414.

Chang, I.C., Lee, T.H., Yang, C.H., Wei, Y.W., Chou, F.I., Hwang, P.P., 2001. Morphology and function of gill mitochondria-rich cells in fish acclimated to different environments. Physiol. Biochem. Zool. 74, 111–119.

Chang, I.C., Wei, Y.Y., Chou, F.I., Hwang, P.P., 2003. Stimulation of Cl^- uptake and morphological changes in gill mitochondria-rich cells in freshwater tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. 76, 544–552.

Chang, J.C., Wu, S.M., Tseng, Y.C., Lee, Y.C., Baba, O., Hwang, P.P., in press. Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus*) during acclimation to seawater. J Exp Biol.

Chen, C.N., Lin, L.Y., Lee, T.H., 2004. Ionocyte distribution in gills of the Euryhaline milkfish, *Chanos chanos* (Forsskal, 1775). Zool. Stud. 43, 772–777.

Cheng, K.C., Levenson, R., Robishaw, J.D., 2003. Functional genomic dissection of multimeric protein families in zebrafish. Dev. Dyn. 228, 555–567.

Choi, J.Y., Shah, M., Lee, M.G., Schultheis, P.J., Shull, G.E., Muallem, S., Baum, M., 2000. Novel amiloride-sensitive sodium-dependent proton secretion in the mouse proximal convoluted tubule. J. Clin. Invest. 105, 1141–1146.

Chretien, M., Pisam, M., 1986. Cell Renewal and differentiation in the gill epithelium of freshwater-adapted or saltwater-adapted euryhaline fish as revealed by [H^3] thymidine autoradiography. Biol. Cell 56, 137–150.

Conte, F.P., Lin, D.H.Y., 1967. Kinetics of cellular morphogenesis in gill epithelium during sea water adaptation of *Oncorhynchus* (Walbaum). Comp. Biochem. Physiol. 23, 945–957.

Crombie, H.J., Bell, M.V., Tytler, P., 1996. Inhibition of sodium-plus-potassium-stimulated adenosine triphosphatase (Na^+–K^+-ATPase) by protein kinase C activators in the gills of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B 113, 765–772.

Cutler, C.P., Cramb, G., 2002. Two isoforms of the $Na^+/K^+/2Cl^-$ cotransporter are expressed in the European eel (*Anguilla anguilla*). Biochim. Biophys. Acta, Biomembr. 1566, 92–103.

Cutler, C.P., Sanders, I.L., Hazon, N., Cramb, G., 1995. Primary sequence, tissue-specificity and expression of the Na^+,K^+-ATPase a-1 subunit in the European eel (*Anguilla anguilla*). Comp. Biochem. Physiol. B 111, 567–573.

D'Cotta, H., Valotaire, C., Le Gac, F., Prunet, P., 2000. Synthesis of gill Na^+–K^+-ATPase in Atlantic salmon smolts: differences in a-mRNA and a-protein levels. Am. J. Physiol. 278, R101–R110.

Daborn, K., Cozzi, R.R.F., Marshall, W.S., 2001. Dynamics of pavement cell-chloride cell interactions during abrupt salinity change in *Fundulus heteroclitus*. J. Exp. Biol. 204, 1889–1899.

Dang, Z.C., Lock, R.A.C., Flik, G., Bonga, S.E.W., 1999. Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. Am. J. Physiol. 277, R320–R331.

Dang, Z.C., Balm, P.H.M., Flik, G., Bonga, S.E.W., Lock, R.A.C., 2000. Cortisol increases Na^+/K^+-ATPase density in plasma membranes of gill chloride cells in the freshwater tilapia *Oreochromis mossambicus*. J. Exp. Biol. 203, 2349–2355.

De Boeck, G., Vlaeminck, A., Van der Linden, A., Blust, R., 2000. The energy metabolism of common carp (*Cyprinus carpio*) when exposed to salt stress: an increase in energy expenditure or effects of starvation? Physiol. Biochem. Zool. 73, 102–111.

De Robertis, E.M., Kuroda, H., 2004. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu. Rev. Cell Dev. Biol. 20, 285–308.

Deane, E.E., Woo, N.Y.S., 2004. Differential gene expression associated with euryhalinity in sea bream (*Sparus sarba*). Am. J. Physiol. 287, R1054–R1063.

Deane, E.E., Kelly, S.P., Luk, J.C.Y., Woo, N.Y.S., 2002. Chronic salinity adaptation modulates hepatic heat shock protein and insulin-like growth factor I expression in black sea bream. Mar. Biotechnol. 4, 193–205.

Eddy, F.B., 1982. Osmotic and ionic regulation in captive fish with particular reference to Salmonids. Comp. Biochem. Physiol. B 73, 125–141.

Edwards, S.L., Tse, C.M., Toop, T., 1999. Immunolocalisation of NHE3-like immunoreactivity in the gills of the rainbow trout (*Oncorhynchus mykiss*) and the blue-throated wrasse (*Pseudolabrus tetrious*). J. Anat. 195, 465–469.

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., Hirose, S., 2007. Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on *foxi3a*. Am. J. Physiol. 292, R470–R480.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Febry, R., Lutz, P., 1987. Energy partitioning in fish — the activity-related cost of osmoregulation in a euryhaline cichlid. J. Exp. Biol. 128, 63–85.

Feng, S.H., Leu, J.H., Yang, C.H., Fang, M.J., Huang, C.J., Hwang, P.P., 2002. Gene expression of Na^+–K^+-ATPase a1 and a3 subunits in gills of the teleost *Oreochromis mossambicus*, adapted to different environmental salinities. Mar. Biotechnol. 4, 379–391.

Fenwick, J.C., Wendelaar Bonga, S.E., Flik, G., 1999. In vivo bafilomycin-sensitive Na^+ uptake in young freshwater fish. J. Exp. Biol. 202, 3659–3666.

Flik, G., Verbost, P.M., Wendelaar Bonga, S.E., 1995. Calcium transport process in fishes. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and molecular approaches to fish ionic regulation. Academic, San Diego, pp. 317–342.

Flik, G., Kaneko, T., Greco, A.M., Li, J., Fenwick, J.C., 1997. Sodium dependent ion transporters in trout gills. Fish Physiol. Biochem. 17, 385–396.

Galvez, F., Reid, S.D., Hawkings, G., Goss, G.G., 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. Am. J. Physiol. 282, R658–R668.

Galvez, F., Wong, D., Wood, C.M., 2006. Cadmium and calcium uptake in isolated mitochondria-rich cell populations from the gills of the freshwater rainbow trout. Am. J. Physiol. 291, R170–R176.

Gamba, G., 2005. Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. Physiol. Rev. 85, 423–493.

Geering, K., 2006. FXYD proteins: new regulators of Na-K-ATPase. Am. J. Physiol. 290, F241–F250.

Georgalis, T., Gilmour, K.M., Yorston, J., Perry, S.F., 2006. Roles of cytosolic and membrane-bound carbonic anhydrase in renal control of acid-base balance in rainbow trout, *Oncorhynchus mykiss*. Am. J. Physiol. 291, F407–F421.

Gilmour, K.M., Bayaa, M., Kenney, L., McNeill, B., Perry, S.F., 2007. Type IV carbonic anhydrase is present in the gills of spiny dogfish (*Squalus acanthias*). Am. J. Physiol. 292, R556–R567.

Goss, G.G., Adamia, S., Galvez, F., 2001. Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. Am. J. Physiol. 281, R1718–R1725.

Gracia-Lopez, V., Rosas-Vazquez, C., Brito-Perez, R., 2006. Effects of salinity on physiological conditions in juvenile common snook *Centropomus undecimalis*. Comp. Biochem. Physiol. A 145, 340–345.

Gross, E., Pushkin, A., Abuladze, N., Fedotoff, O., Kurtz, I., 2002. Regulation of the sodium bicarbonate cotransporter kNBC1 function: role of Asp(986), Asp(988) and kNBC1-carbonic anhydrase II binding. J. Physiol. 544, 679–685.

Guynn, S.R., Scofield, M.A., Petzel, D.H., 2002. Identification of mRNA and protein expression of the Na/K-ATPase a1-, a2- and a3-subunit isoforms in Antarctic and New Zealand nototheniid fishes. J. Exp. Mar. Biol. Ecol. 273, 15–32.

Haney, D.C., Nordlie, F.G., 1997. Influence of environmental salinity on routine metabolic rate and critical oxygen tension of *Cyprinodon variegatus*. Physiol. Zool. 70, 511–518.

Hirai, N., Tagawa, M., Kaneko, T., Seikai, T., Tanaka, M., 1999. Distributional changes in branchial chloride cells during freshwater adaptation in Japanese sea bass *Lateolabrax japonicus*. Zool. Scr. 16, 43–49.

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M.H., Romero, M.F., Hirose, S., 2003. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. Am. J. Physiol. 284, R1199–R1212.

Hiroi, J., McCormick, S.D., 2007. Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. J. Exp. Biol. 210, 1015–1024.

Hiroi, J., Kaneko, T., Tanaka, M., 1999. In vivo sequential changes in chloride cell morphology in the yolk-sac membrane of mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. J. Exp. Biol. 202, 3485–3495.

Hiroi, J., McCormick, S.D., Ohtani-Kaneko, R., Kaneko, T., 2005. Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. J. Exp. Biol. 208, 2023–2036.

Hirose, S., Kaneko, T., Naito, N., Takei, Y., 2003. Molecular biology of major components of chloride cells. Comp. Biochem. Physiol. B 136, 593–620.

Hoenderop, J.G., Nilius, B., Bindels, R.J., 2005. Calcium absorption across epithelia. Physiol. Rev. 85, 373–422.

Horng, J.L., Lin, L.Y., Huang, C.J., Katoh, F., Kaneko, T., Hwang, P.P., 2007. Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). Am. J. Physiol. 292, R2068–R2076.

Hossler, F.E., Ruby, J.R., Mcilwain, T.D., 1979. Gill Arch of the mullet, *Mugil cephalus*.2. modification in surface ultrastructure and Na,K-ATPase content during adaptation to various salinities. J. Exp. Zool. 208, 399–405.

Hossler, F.E., Musil, G., Karnaky, K.J., Epstein, F.H., 1985. Surface ultrastructure of the gill arch of the killifish, *Fundulus heteroclitus*, from seawater and fresh water, with special reference to the morphology of apical crypts of chloride cells. J. Morphol. 185, 377–386.

Hsiao, C.D., You, M.S., Guh, Y.J., Ma, M., Jiang, Y.J., Hwang, P.P., 2007. A positive regulatory loop between *foxi3a* and *foxi3b* is essential for specification and differentiation of zebrafish epidermal ionocytes. PLoS ONE 2, e302.

Hulander, M., Kiernan, A.E., Blomqvist, S.R., Carlsson, P., Samuelsson, E.J., Johansson, B.R., Steel, K.P., Enerback, S., 2003. Lack of pendrin expression leads to deafness and expansion of the endolymphatic compartment in inner ears of *Foxi1* null mutant mice. Development 130, 2013–2025.

Hwang, P.P., 1988. Multicellular complex of chloride cells in the gills of freshwater teleosts. J. Morphol. 196, 15–22.

Hwang, P.P., Hirano, R., 1985. Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. J. Exp. Zool. 236, 115–126.

Hwang, P.P., Fang, M.J., Tsai, J.C., Huang, C.J., Chen, S.T., 1998. Expression of mRNA and protein of Na^+–K^+-ATPase alpha subunit in gills of tilapia (*Oreochromis mossambicus*). Fish Physiol. Biochem. 18, 363–373.

Iwama, G.K., Thomas, P.T., Forsyth, R.H.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fish. 8, 35–56.

Johansen, K., Pettersson, K., 1981. Gill O_2 consumption in a teleost fish, *Gadus morhua*. Respir. Physiol. 44, 277–284.

Katoh, F., Kaneko, T., 2003. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining technique. J. Exp. Biol. 206, 4113–4123.

Katoh, F., Hasegawa, S., Kita, J., Takagi, Y., Kaneko, T., 2001. Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. Can. J. Zool. 79, 822–829.

Katoh, F., Hyodo, S., Kaneko, T., 2003. Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. J. Exp. Biol. 206, 793–803.

Kelly, S.P., Woo, N.Y.S., 1999. The response of sea bream following abrupt hyposmotic exposure. J. Fish Biol. 55, 732–750.

Keys, A., Willmer, E.N., 1932. "Chloride secreting cells" in the gills of fishes, with special reference to the common eel. J. Physiol. (Lond.) 76, 368–378.

King, J.A.C., Hossler, F.E., 1991. The gill arch of the striped bass (*Morone saxatilis*). 4. Alterations in the ultrastructure of chloride cell apical crypts and chloride efflux following exposure to seawater. J. Morphol. 209, 165–176.

Kirschner, L.B., 1983. Sodium-chloride absorption across the body-surface-frog skins and other epithelia. Am. J. Physiol. 244, R429–R443.

Kirschner, L.B., 1995. Energetics of osmoregulation in freshwater vertebrates. J. Exp. Zool. 271, 243–252.

Kirschner, L.B., 2004. The mechanism of sodium chloride uptake in hyperregulating aquatic animals. J. Exp. Biol. 207, 1439–1452.

Kültz, D., Jürss, K., Jonas, L., 1995. Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*). Cell Tissue Res. 279, 65–73.

Kohn, A., Chakravarty, D., Kültz, D., 2003. Teleost Fh14-3-3a protein protects *Xenopus* oocytes from hyperosmolality. J. Exp. Zool. 299A, 103–109.

Krogh, A., 1937. Osmotic regulation in freshwater fishes by active absorption of chloride ions. Z. Vgl. Physiol. 24, 656–666.

Lang, F., Capasso, G., Schwab, M., Waldegger, S., 2005. Renal tubular transport and the genetic basis of hypertensive disease. Clin. Exp. Nephrol. 9, 91–99.

Laurent, P., Dunelerb, S., Chevalier, C., Lignon, J., 1994. Gill epithelial cells kinetics in a freshwater teleost, *Oncorhynchus mykiss* during adaptation to ion-poor water and hormonal treatments. Fish Physiol. Biochem. 13, 353–370.

Lee, T.H., Hwang, P.P., Feng, S.H., 1996a. Morphological studies of gill and mitochondria-rich cells in the stenohaline cyprinid teleosts, *Cyprinus carpio* and *Carassius auratus*, adapted to various hypotonic environments. Zool. Stud. 35, 272–278.

Lee, T.H., Hwang, P.P., Feng, S.H., Huang, F.L., 1996b. The gill structure and branchial mitochondria-rich cells of the medaka, *Oryzias latipes*. Acta Zool. Taiwanica 7, 43–50.

Lee, T.H., Hwang, P.P., Lin, H.C., Huang, F.L., 1996c. Mitochondria-rich cells in the branchial epithelium of the teleost, *Oreochromis mossambicus*, acclimated to various hypotonic environments. Fish Physiol. Biochem. 15, 513–523.

Lee, T.H., Tsai, J.C., Fang, M.J., Yu, M.J., Hwang, P.P., 1998. Isoform expression of Na^+–K^+-ATPase a-subunit in gills of the teleost *Oreochromis mossambicus*. Am. J. Physiol. 44, R926–R932.

Lee, T.H., Hwang, P.P., Shieh, Y.E., Lin, C.H., 2000. The relationship between 'deep-hole' mitochondria-rich cells and salinity adaptation in the euryhaline teleost, *Oreochromis mossambicus*. Fish Physiol. Biochem. 23, 133–140.

Lee, T.H., Feng, S.H., Lin, C.H., Hwang, Y.H., Huang, C.L., Hwang, P.P., 2003. Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. Zool. Sci. 20, 29–36.

Liao, B.K., Pan, T.C., Deng, A.N., Shiao, J.C., Chen, S.C., Chou, M.Y., Hwang, P.P., 2006. Expression and regulation of Ca^{2+} transporters and Ca^{2+} uptake in zebrafish. VII International Congress on the Biology of Fish, St. John's, p. 144.

Lin, L.Y., Hwang, P.P., 2004. Mitochondria-rich cell activity in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae acclimatized to different ambient chloride levels. J. Exp. Biol. 207, 1335–1344.

Lin, C.H., Lee, T.H., 2005. Sodium or potassium ions activate different kinetics of gill Na, K-ATPase in three seawater- and freshwater-acclimated euryhaline teleosts. J. Exp. Zool. 303A, 57–65.

Lin, Y.M., Chen, C.N., Lee, T.H., 2003. The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and fresh water. Comp. Biochem. Physiol. A 135, 489–497.

Lin, C.H., Huang, C.L., Yang, C.H., Lee, T.H., Hwang, P.P., 2004a. Time-course changes in the expression of Na, K-ATPase and the morphometry of mitochondrion-rich cells in gills of euryhaline tilapia (*Oreochromis mossambicus*) during freshwater acclimation. J. Exp. Zool. 301A, 85–96.

Lin, C.H., Tsai, R.S., Lee, T.H., 2004b. Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol. A 138, 287–295.

Lin, L.Y., Horng, J.L., Kunkel, J.G., Hwang, P.P., 2006a. Proton pump-rich cell secretes acid in skin of zebrafish larvae. Am. J. Physiol. 290, C371–C378.

Lin, Y.M., Chen, C.N., Yoshinaga, T., Tsai, S.C., Shen, I.D., Lee, T.H., 2006b. Short-term effects of hyposmotic shock on Na^+/K^+-ATPase expression in gills of the euryhaline milkfish, *Chanos chanos*. Comp. Biochem. Physiol. A 143, 406–415.

Lyndon, A.R., 1994. A method for measuring oxygen-consumption in isolated–perfused gills. J. Fish Biol. 44, 707–715.

Lytle, C., Xu, J.C., Biemesderfer, D., Forbush III, B., 1995. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. Am. J. Physiol. 269, C1496–C1505.

Lytton, J., Lin, J.C., Guidotti, G., 1985. Identification of two molecular forms of (Na^+,K^+)-ATPase in rat adipocytes. Relation to insulin stimulation of the enzyme. J. Biol. Chem. 260, 1177–1184.

Mackinnon, M., Enesco, H.E., 1980. Cell renewal in the gills of the fish *Barbus conchonius*. Can J. Zool. 58, 650–653.

Madsen, S.S., Jensen, M.K., Nhr, J., Kristiansen, K., 1995. Expression of Na^+–K^+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. Am. J. Physiol. 269, R1339–R1345.

Maetz, J., Garciaromeu, F., 1964. The mechanism of sodium and chloride uptake by the gills of a freshwater fish, *Carassius auratus*. II. Evidence for NH_4^+/Na^+ and HCO_3^-/$C1^-$ ion exchanges. J. Gen. Physiol. 47, 1209–1227.

Maina, J.N., 1990. A study of the morphology of the gills of an extreme alkalinity and hyperosmotic adapted teleost *Oreochromis alcalicus grahami*

(Boulenger) with particular emphasis on the ultrasturcture of the chloride cells and their modifications with water dilution. A SEM and TEM study. Anat. Embryol. 181, 83–89.

Marshall, W.S., 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J. Exp. Zool. 293, 264–283.

Marshall, W.S., Bryson, S.E., 1998. Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp. Biochem. Physiol. A 119, 97–106.

Marshall, W.S., Bryson, S.E., Midelfart, A., Hamilton, W.F., 1995. Low conductance anion channel activated by camp in teleost Cl^- secreting cells. Am. J. Physiol. 37, R963–R969.

Marshall, W.S., Lynch, E.M., Cozzi, R.R., 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to seawater. J. Exp. Biol. 205, 1265–1273.

Marshall, W.S., Ossum, C.G., Hoffmann, E.K., 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J. Exp. Biol. 208, 1063–1077.

McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781–794.

McCormick, S.D., Sundell, K., Bjornsson, B.T., Brown, C.L., Hiroi, J., 2003. Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). J. Exp. Biol. 206, 4575–4583.

Miguel Mancera, J., Laiz Carrion, R., del Pilar Martin del Rio, M., 2002. Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata* L). Gen. Comp. Endocrinol. 129, 95–103.

Miyazaki, H., Kaneko, T., Uchida, S., Sasaki, S., Takei, Y., 2002. Kidney-specific chloride channel, OmClC-K, predominantly expressed in the diluting segment of freshwater-adapted tilapia kidney. Proc. Natl. Acad. Sci. U. S. A. 99, 15782–15787.

Moreau, M., Leclerc, C., 2004. The choice between epidermal and neural fate: a matter of calcium. Int. J. Dev. Biol. 48, 75–84.

Morgan, J.D., Iwama, G.K., 1991. Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish Aquat. Sci. 48, 2083–2094.

Morgan, J.D., Iwama, G.K., 1998. Salinity effects on oxygen consumption, gill Na^+,K^+-ATPase and ion regulation in juvenile coho salmon. J. Fish Biol. 53, 1110–1119.

Morgan, J.D., Iwama, G.K., 1999. Energy cost of NaCl transport in isolated gills of cutthroat trout. Am. J. Physiol. 277, R631–R639.

Morgan, J.D., Sakamoto, T., Grau, E.G., Iwama, G.K., 1997. Physiological and respiratory responses of the Mozambique Tilapia (*Oreochromis mossambicus*) to salinity acclimation. Comp. Biochem. Physiol. A 117, 391–398.

Moser, M.L., Hettler, W.F., 1989. Routine metabolism of juvenile spot, *Leiostomus xanthurus* (Lacepede), as a function of temperature, salinity and weight. J. Fish Biol. 35, 703–707.

Nakano, K., Tagawa, M., Takemura, A., Hirano, T., 1998. Temporal changes in liver carbohydrate metabolism associated with seawater transfer in *Oreochromis mossambicus*. Comp. Biochem. Physiol. B 119, 721–728.

Pan, T.C., Liao, B.K., Huang, C.J., Lin, L.Y., Hwang, P.P., 2005. Epithelial Ca^{2+} channel expression and Ca^{2+} uptake in developing zebrafish. Am. J. Physiol. 289, R1202–R1211.

Parks, S.K., Tresguerres, M., Goss, G.G., 2007. Interactions between Na^+ channels and Na^+-HCO_3^- cotransporters in the freshwater fish gill MR cell: a model for transepithelial Na^+ uptake. Am. J. Physiol. 292, C935–C944.

Paukert, M., Sidi, S., Russell, C., Siba, M., Wilson, S.W., Nicolson, T., Grunder, S., 2004. A family of acid-sensing ion channels from the zebrafish — widespread expression in the central nervous system suggests a conserved role in neuronal communication. J. Biol. Chem. 279, 18783–18791.

Pech, V., Kim, Y.H., Weinstein, A.M., Everett, L.A., Pham, T.D., Wall, S.M., 2007. Angiotensin II increases chloride absorption in the cortical collecting duct in mice through a pendrin-dependent mechanism. Am. J. Physiol. 292, F914–F920.

Perry, S.F., Flik, G., 1988. Characterization of branchial transepithelial calcium fluxes in freshwater trout, *Salmo gairdneri*. Am. J. Physiol. 254, R491–R498.

Perry, S.F., Randall, D.J., 1981. Effects of amiloride and SITS on branchial ion fluxes in rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 215, 225–228.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. J. Exp. Zool. 300A, 53–62.

Pfeiffer-Guglielmi, B., Fleckenstein, B., Jung, G., Hamprecht, B., 2003. Immunocytochemical localization of glycogen phosphorylase isozymes in rat nervous tissues by using isozyme-specific antibodies. J. Neurochem. 85, 73–81.

Philpott, C.W., Copeland, D.E., 1963. Fine structure of chloride cells from three species of *Fundulus*. J. Cell Biol. 18, 389–404.

Piermarini, P.M., Verlander, J.W., Royaux, I.E., Evans, D.H., 2002. Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch. Am. J. Physiol. 283, R983–R992.

Pisam, M., Rambourg, A., 1991. Mitochondria-rich cells in the gill epithelium of teleost fishes — an ultrastructural approach. Int. Rev. Cytol. 130, 191–232.

Pisam, M., Caroff, A., Rambourg, A., 1987. Two types of chloride cells in the gill epithelium of a freshwater-adapted euryhaline fish: Lebistes reticulatus; their modifications during adaptation to saltwater. Am. J. Anat. 179, 40–50.

Pisam, M., Prunet, P., Boeuf, G., Rambourg, A., 1988. Ultrastructural features of chloride cells in the gill epithelium of the atlantic salmon, Salmo salar, and their modifications during smoltification. Am. J. Anat. 183, 235–244.

Pisam, M., Boeuf, G., Prunet, P., Rambourg, A., 1990. Ultrastructural features of mitochondria-rich cells in stenohaline freshwater and seawater fishes. Am. J. Anat. 187, 21–31.

Pisam, M., Le Moal, C., Auperin, B., Prunet, P., Rambourg, A., 1995. Apical structures of "mitochondria-rich" α and β cells in euryhaline fish gill: their behaviour in various living conditions. Anat. Rec. 241, 13–24.

Planelles, G., 2004. Chloride transport in the renal proximal tubule. Pflugers Arch. 448, 561–570.

Plaut, I., 2000. Resting metabolic rate, critical swimming speed, and routine activity of the euryhaline cyprinodontid, *Aphanius dispar*, acclimated to a wide range of salinities. Physiol. Biochem. Zool. 73, 590–596.

Polakof, S., Arjona, F.J., Sangiao-Alvarellos, S., Martin del Rio, M.P., Mancera, J.M., Soengas, J.L., 2006. Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream *Sparus auratus*. J. Comp. Physiol. B 176, 441–452.

Purkerson, J.M., Schwartz, G.J., 2007. The role of carbonic anhydrases in renal physiology. Kidney Int. 71, 103–115.

Qiu, A., Hogstrand, C., 2004. Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, *Fugu rubripes*. Gene 342, 113–123.

Quentin, F., Chambrey, R., Trinh-Trang-Tan, M.M., Fysekidis, M., Cambillau, M., Paillard, M., Aronson, P.S., Eladari, D., 2004. The Cl^-/HCO_3^- exchanger pendrin in the rat kidney is regulated in response to chronic alterations in chloride balance. Am. J. Physiol., Renal Fluid Electrolyte Physiol. 287, F1179–F1188.

Ransom, B.R., Fern, R., 1997. Does astrocytic glycogen benefit axon function and survival in CNS white matter during glucose deprivation? Glia 21, 134–141.

Reid, S.D., Hawkings, G.S., Galvez, F., Goss, G.G., 2003. Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. J. Exp. Biol. 206, 551–559.

Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na^+/K^+-ATPase a-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol. 206, 4475–4486.

Ron, B., Shimoda, S.K., Iwama, G.K., Grau, E.G., 1995. Relationships among ration, salinity, 17a-methyltestosterone and growth in the euryhaline tilapia, *Oreochromis mossambicus*. Aquaculture 135, 185–193.

Sakamoto, T., Ando, M., 2002. Calcium ion triggers rapid morphological oscillation of chloride cells in the mudskipper, *Periophthalmus modestus*. J. Comp. Physiol. B 172, 435–439.

Sakamoto, T., Uchida, K., Yokota, S., 2001. Regulation of the ion-transporting mitochondrion-rich cell during adaptation of teleost fishes to different salinities. Zool. Sci. 18, 1163–1174.

Sangiao-Alvarellos, S., Arjona, F.J., Martin del Rio, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and

metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291–4304.

Sardella, B.A., Cooper, J., Gonzalez, R.J., Brauner, C.J., 2004. The effect of temperature on juvenile Mozambique tilapia hybrids (*Oreochromis mossambicus*×*O. urolepis hornorum*) exposed to full-strength and hypersaline seawater. Comp. Biochem. Physiol. A 137, 621–629.

Sasai, S., Kaneko, T., Hasegawa, S., Tsukamoto, K., 1998. Morphological alteration in two types of gill chloride cells in Japanese eel (*Anguilla japonica*) during catadromous migration. Can. J. Zool. 76, 1480–1487.

Scheiner-Bobis, G., 2002. The sodium pump — its molecular properties and mechanics of ion transport. Eur. J. Biochem. 269, 2424–2433.

Schoenmakers, T.J., Flik, G., 1992. Sodium-extruding and calcium-extruding sodium/calcium exchangers display similar calcium affinities. J. Exp. Biol. 168, 151–159.

Schultheis, P.J., Clarke, L.L., Meneton, P., Miller, M.L., Soleimani, M., Gawenis, L.R., Riddle, T.M., Duffy, J.J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P.S., Lorenz, J.N., Shull, G.E., 1998. Renal and intestinal absorptive defects in mice lacking the NHE_3 Na^+/H^+ exchanger. Nat. Genet. 19, 282–285.

Scott, G.R., Richards, J.G., Forbush, B., Isenring, P., Schulte, P.M., 2004. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol. 287, C300–C309.

Scott, G.R., Claiborne, J.B., Edwards, S.L., Schulte, P.M., Wood, C.M., 2005. Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J. Exp. Biol. 208, 2719–2729.

Seidelin, M., Madsen, S.S., Blenstrup, H., Tipsmark, C.K., 2000. Time-course changes in the expression of Na^+, K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. Physiol. Biochem. Zool. 73, 446–453.

Seidelin, M., Madsen, S.S., Cutler, C.P., Cramb, G., 2001. Expression of gill vacuolar-type H+-ATPase B subunit, and Na^+, K^+-ATPase a1 and b1 subunit messenger RNAs in smolting *Salmo salar*. Zool. Sci. 18, 315–324.

Semple, J.W., Green, H.J., Schulte, P.M., 2002. Molecular cloning and characterization of two Na/K-ATPase isoforms in *Fundulus heteroclitus*. Mar. Biotechnol. 4, 512–519.

Shahsavarani, A., Perry, S.F., 2006. Hormonal and environmental regulation of epithelial calcium channel in gill of rainbow trout (*Oncorhynchus mykiss*). Am. J. Physiol. 291, R1490–R1498.

Shahsavarani, A., McNeill, B., Galvez, F., Wood, C.M., Goss, G.G., Hwang, P.P., Perry, S.F., 2006. Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 209, 1928–1943.

Shen, I.D., Chiu, Y.H., Lee, T.H., Hwang, P.P., in press. Localization of chloride transporters in gill epithelium of the grass pufferfish, *Takifugu nephobles*. J Fish Soc Taiwan.

Shirai, N., Utida, S., 1970. Development and degeneration of chloride cell during seawater and freshwater adaptation of Japanese eel, *Anguilla japonica*. Z. Zellforsch. Mikrosk. Anat. 103, 247–264.

Shiraishi, K., Hiroi, J., Kaneko, T., Matsuda, M., Hirano, T., Mori, T., 2001. In vitro effects of environmental salinity and cortisol on chloride cell differentiation in embryos of Mozambique tilapia, *Oreochromis mossambicus*, measured using a newly developed 'yolk-ball' incubation system. J. Exp. Biol. 204, 1883–1888.

Singer, T.D., Tucker, S.J., Marshall, W.S., Higgins, C.F., 1998. A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. Am. J. Physiol. 43, C715–C723.

Singer, T.D., Clements, K.M., Semple, J.W., Schulte, P.M., Bystriansky, J.S., Finstad, B., Fleming, I.A., McKinley, R.S., 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Can. J. Fish Aquat. Sci. 59, 125–135.

Smith, H.W., 1930. The absorption and excretion of water and salts by marine teleosts. Am. J. Physiol. 93, 480–505.

Sullivan, G.V., Fryer, J.N., Perry, S.F., 1996. Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. Can. J. Zool. 74, 2095–2103.

Suzuki, Y., Itakura, M., Kashiwagi, M., Nakamura, N., Matsuki, T., Sakuta, H., Naito, N., Takano, K., Fujita, T., Hirose, S., 1999. Identification by differential display of a hypertonicity-inducible inward rectifier potassium channel highly expressed in chloride cells. J. Biol. Chem. 274, 11376–11382.

Swanson, C., 1998. Interactive effects of salinity on metabolic rate, activity, growth and osmoregulation in the euryhaline milkfish (*Chanos chanos*). J. Exp. Biol. 201, 3355–3366.

Tang, C.H., Lee, T.H., 2007. The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*. Comp. Biochem. Physiol. A 147, 521–528.

Tang, C.H., Lee, T.H., in press. The novel correlation of carbonic anhydrase II and anion exchanger 1 in gills of the spotted green pufferfish, *Tetraodon nigrovirids*. J. Exp. Zool. 307A. doi:10.1002/jez.391.

Tipsmark, C.K., Madsen, S.S., Seidelin, M., Christensen, A.S., Cutler, C.P., Cramb, G., 2002. Dynamics of Na^+,K^+,$2Cl^-$ cotransporter and Na^+,K^+-ATPase expression in the branchial epithelium of brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*). J. Exp. Zool. 293, 106–118.

Tipsmark, C.K., Madsen, S.S., Borski, R.J., 2004. Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J. Exp. Zool. 301A, 979–991.

Tresguerres, M., Katoh, F., Orr, E., Parks, S.K., Goss, G.G., 2006. Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? Physiol. Biochem. Zool. 79, 981–996.

Tsai, J.C., Hwang, P.P., 1998. Effects of wheat germ agglutinin and colchicine on microtubules of the mitochondria-rich cells and Ca^{2+} uptake in tilapia (*Oreochromis mossambicus*) larvae. J. Exp. Biol. 201, 2263–2271.

Tse, W.K.F., Au, D.W.T., Wong, C.K.C., 2006. Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. Biochem. Biophys. Res. Commun. 346, 1181–1190.

Tseng, Y.C., Huang, C.J., Chang, J.C., Teng, W.Y., Baba, O., Fann, M.J., Hwang, PP., 2007. Glycogen phosphorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia. Am. J. Physiol. 293, R482–R491.

Uchida, K., Kaneko, T., 1996. Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. Zool. Sci. 13, 655–660.

Uchida, S., Sasaki, S., 2005. Function of chloride channels in the kidney. Annu. Rev. Physiol. 67, 759–778.

Uchida, K., Kaneko, T., Yamauchi, K., Hirano, T., 1996. Morphometrical analysis of chloride cell activity in the gill filaments and lamellae and changes in Na^+, K^+-ATPase activity during seawater adaptation in chum salmon fry. J. Exp. Zool. 276, 193–200.

Uchida, K., Kaneko, T., Miyazaki, H., Hasegawa, S., Hirano, T., 2000. Excellent salinity tolerance of Mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater. Zool. Sci. 17, 149–160.

Urayama, O., Nakao, M., 1979. Organ specificity of rat sodium-activated and potassium-activated adenosine-triphosphatase. J. Biochem. 86, 1371–1381.

van der Heijden, A.J., Verbost, P.M., Bijvelds, M.J., Atsma, W., Wendelaar Bonga, S.E., Flik, G., 1999. Effects of sea water and stanniectomy on branchial Ca^{2+} handling and drinking rate in eel (*Anguilla anguilla* L.). J. Exp. Biol. 202, 2505–2511.

Varsamos, S., Diaz, J.P., Charmantier, G., Flik, G., Blasco, C., Connes, R., 2002. Branchial chloride cells in sea bass (*Dicentrarchus labrax*) adapted to fresh water, seawater, and doubly concentrated seawater. J. Exp. Zool. 293, 12–26.

Verlander, J.W., Kim, Y.H., Shin, W., Pham, T.D., Hassell, K.A., Beierwaltes, W.H., Green, E.D., Everett, L., Matthews, S.W., Wall, S.M., 2006. Dietary Cl^- restriction upregulates pendrin expression within the apical plasma membrane of type B intercalated cells. Am. J. Physiol. 291, F833–F839.

Wagner, C.A., 2007. The emerging role of pendrin in renal chloride reabsorption. Am. J. Physiol. 292, F912–F913.

Wagner, C.A., Finberg, K.E., Breton, S., Marshansky, V., Brown, D., Geibel, J.P., 2004. Renal vacuolar H^+-ATPase. Physiol. Rev. 84, 1263–1314.

Wilson, J.M., Laurent, P., Tufts, B.L., Benos, D.J., Donowitz, M., Vogl, A.W., Randall, D.J., 2000. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. J. Exp. Biol. 203, 2279–2296.

Wilson, J.M., Whiteley, N.M., Randall, D.J., 2002. Ionoregulatory changes in the gill epithelia of coho salmon during seawater acclimation. Physiol. Biochem. Zool. 75, 237–249.

Wong, C.K.C., Chan, D.K.O., 1999a. Chloride cell subtypes in the gill epithelium of Japanese eel *Anguilla japonica*. Am. J. Physiol. 277, R517–R522.

Wong, C.K.C., Chan, D.K.O., 1999b. Isolation of viable cell types from the gill epithelium of Japanese eel *Anguilla japonica*. Am. J. Physiol. 276, R363–R372.

Woo, N.Y.S., Fung, A.C.Y., 1981. Studies on the biology of the red sea bream *Chrysophrys major*. 4. Metabolic effects of starvation at low-temperature. Comp. Biochem. Physiol. A 69, 461–465.

Woo, N.Y.S., Kelly, S.P., 1995. Effects of salinity and nutritional status on growth and metabolism of *Sparus sarba* in a closed seawater system. Aquaculture 135, 229–238.

Woo, A.L., Noonan, W.T., Schultheis, P.J., Neumann, J.C., Manning, P.A., Lorenz, J.N., Shull, G.E., 2003. Renal function in NHE_3-deficient mice with transgenic rescue of small intestinal absorptive defect. Am. J. Physiol. 284, F1190–F1198.

Wu, Y.C., Lin, L.Y., Lee, T.H., 2003. Na^+,K^+,$2Cl^-$cotransporter: a novel marker for identifying freshwater- and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. Zool. Stud. 42, 186–192.