

# Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms

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**Hwang P, Lee T, Lin L.** Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am J Physiol Regul Integr Comp Physiol* 301: R28–R47, 2011. First published March 30, 2011; doi:10.1152/ajpregu.00047.2011.—Fish encounter harsh ionic/osmotic gradients on their aquatic environments, and the mechanisms through which they maintain internal homeostasis are more challenging compared with those of terrestrial vertebrates. Gills are one of the major organs conducting the internal ionic and acid-base regulation, with specialized ionocytes as the major cells carrying out active transport of ions. Exploring the iono/osmoregulatory mechanisms in fish gills, extensive literature proposed several models, with many conflicting or unsolved issues. Recent studies emerged, shedding light on these issues with new opened windows on other aspects, on account of available advanced molecular/cellular physiological approaches and animal models. Respective types of ionocytes and ion transporters, and the relevant regulators for the mechanisms of NaCl secretion, Na<sup>+</sup> uptake/acid secretion/NH<sub>4</sub><sup>+</sup> excretion, Ca<sup>2+</sup> uptake, and Cl<sup>−</sup> uptake/base secretion, were identified and functionally characterized. These new ideas broadened our understanding of the molecular/cellular mechanisms behind the functional modification/regulation of fish gill ion transport during acute and long-term acclimation to environmental challenges. Moreover, a model for the systematic and local carbohydrate energy supply to gill ionocytes during these acclimation processes was also proposed. These provide powerful platforms to precisely study transport pathways and functional regulation of specific ions, transporters, and ionocytes; however, very few model species were established so far, whereas more efforts are needed in other species.

NaCl secretion; ion uptake; acid-base regulation; ammonia excretion; functional regulation; ionocytes; transporters; energy supply

TELEOST FISHES, LIKE terrestrial vertebrates, maintain ionic/osmotic homeostasis in body fluids to allow normal operations of cell functions and activities. Fish encounter harsh ionic/osmotic gradients derived from their aquatic environments, and the mechanisms for maintaining internal homeostasis are more challenging for fish compared with terrestrial vertebrates. To maintain an internal hydromineral balance, teleosts have evolved sophisticated iono/osmoregulatory mechanisms, which are mainly achieved by the gills, kidneys, and intestine. Among these osmoregulatory organs, the gills are the unique one for aquatic animals, and specific ionocytes, mitochondrion-rich (MR) cells (formerly called chloride cells), are the major cells in fish gills that actively transport ions. These ionocytes secrete and absorb ions in seawater (SW) and freshwater (FW) environments, respectively, in addition to carrying out acid-base regulation and ammonia excretion functions (39, 40, 49, 54, 64, 66, 67, 108, 129, 130). Fish iono/osmoregulatory mechanisms have long been an important topic for fish physiology

itself and also for comparative studies from an evolutionary point of view. Much literature has accumulated that explore the iono/osmoregulatory mechanisms of fish gills; however, many issues remain debatable, conflicting, or unsolved, due to differences in species and experimental designs, as well as limitations of methodologies. Presently, highly advanced approaches employing cell biology/molecular physiology and model animals are available, new answers and knowledge of these debated, unresolved issues are emerging, and new windows on other aspects of fish iono/osmoregulation were recently opened. Therefore, instead of making a detailed overview, the intent of the present review is to summarize recent progress in related issues, particularly emphasizing details behind cellular and molecular pathways.

## *Ion Regulation in SW*

**NaCl secretion.** The current ion-transporting system depicted in the working model for NaCl secretion of gill epithelial ionocytes in SW teleosts includes (Fig. 1) the ouabain-inhibited Na<sup>+</sup>-K<sup>+</sup>-ATPase (NKA) and bumetanide-sensitive Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> cotransporter 1 (NKCC1) located in the basolateral membrane; the anion channel cystic fibrosis transmem-

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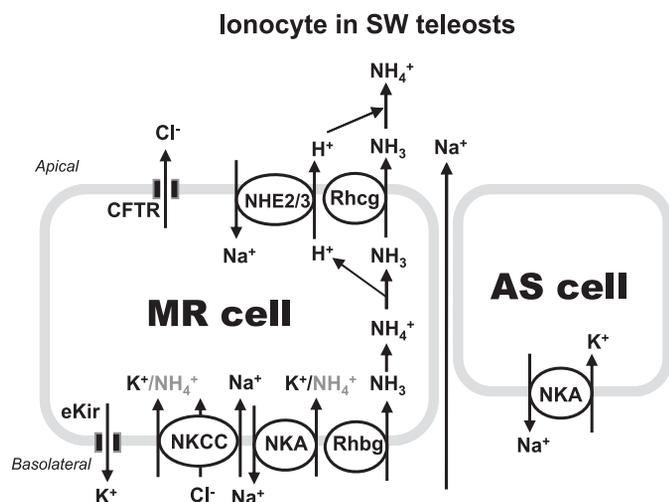


Fig. 1. Model of ionocytes [mitochondrion-rich (MR) cells] in seawater (SW) teleosts. For details, refer to the text (sections *Na uptake/acid secretion* and *Ca<sup>2+</sup> uptake*). AS cell, accessory cell; CFTR, cystic fibrosis transmembrane conductance regulator; eKir, inwardly rectifying K<sup>+</sup> channel; NHE, Na<sup>+</sup>/H<sup>+</sup> exchanger; NKA, Na<sup>+</sup>-K<sup>+</sup>-ATPase; NKCC1, Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter; Rhcg and Rhbg, Rhesus glycoproteins. Ion in dark gray, an unidentified transport pathway.

brane conductance regulator (CFTR) in the apical membrane; and the tight-junction proteins, claudins, and occludins, located between adjacent epithelial cells. The basolateral NKCC1 mediates the entry of Na<sup>+</sup> and Cl<sup>-</sup> into the cellular compartment down the electrochemical gradient provided by NKA, followed by passive exit of Cl<sup>-</sup> and Na<sup>+</sup>, respectively, through the apical channel CFTR and paracellular tight-junction pathway (see *Permeability and tight junctions* below) (40, 54, 66, 108). In addition, basolateral recycling of K<sup>+</sup> is achieved by an inwardly rectifying K<sup>+</sup> channel, which increases the expression in gills of SW-acclimated eels (157, 175). This generally accepted model was formed in the 1970s, and the remaining related ion transport pathways were identified and clarified by molecular and protein evidences in the past decades.

Functional NKA consists of two protein subunits, the catalytic  $\alpha$ -subunit and glycoprotein  $\beta$ -subunit, assembled in a 1:1 ratio to create an  $\alpha\beta$ -heterodimer. NKA provides an electrochemical gradient to drive operations of other transport pathways and exhibits four  $\alpha$ - ( $\alpha_1 \sim 4$ ) and three  $\beta$ -isoforms ( $\beta_1 \sim 3$ ) in mammals (139, 158). Some teleosts have more NKA subunit isoforms; for example, in zebrafish, nine distinct NKA  $\alpha$ -subunit genes (seven  $\alpha_1$ , one  $\alpha_2$ , and one  $\alpha_3$ ) have been identified by analyzing the expressed sequence tag and genomic databases (90). Changes in branchial NKA responses (i.e., mRNA abundances and protein amounts, specific activities, and immunoreactive cell numbers) of euryhaline teleosts were thought to be necessary for acclimation to environmental salinity challenge (66). Different affinities of gill NKA for Na<sup>+</sup> and K<sup>+</sup> were reported in FW- and SW-acclimated milkfish (*Chanos chanos*), tilapia (*Oreochromis mossambicus*), and pufferfish (*Tetraodon nigroviridis*) (92). In addition, NKA proteins isolated from FW- and SW-acclimated rainbow trout (*Oncorhynchus mykiss*) gills were found to have different biochemical properties (123). It is thought that different NKA isoforms might correlate with the kinetic or biochemical properties of NKA to match physiological demands for adaptation.

Because the  $\alpha$ -subunit is the catalytic subunit of NKA, alteration of protein expressions of NKA  $\alpha$ -isoforms was thought to be a crucial adaptation mechanism for euryhaline teleosts in environments with various salinities. NKA  $\alpha$ -isoforms were reported in gills of several euryhaline species [see review by Hwang and Lee (66)], and different increasing levels of  $\alpha_1$ - and  $\alpha_3$ -mRNA, as well as protein (with heterologous antibodies) were found in SW tilapia but not in FW fish (42, 87). It was also reported that the protein abundance of  $\alpha_1$ - and  $\alpha_3$ -isoforms, but not the  $\alpha_2$ -isoform, of NKA in gills of milkfish increased with environmental salinities (159). Isoform switches were supposed to fulfill the requirements of altering NKA activity with various Na<sup>+</sup> and K<sup>+</sup> affinities in the euryhaline tilapia and milkfish. A similar notion was also supported by recent studies on salmonids. Those studies focused on reciprocal mRNA expression of two NKA  $\alpha_1$ -subtypes,  $\alpha_{1a}$  and  $\alpha_{1b}$ , in response to salinity changes, and led to the idea that each NKA  $\alpha_{1a}$ - and  $\alpha_{1b}$ -subtype in salmonid gills has respective functions in fish residing in SW and FW (16, 102, 121, 145). McCormick et al. (112) further used specific antibodies against these two NKA  $\alpha_1$ -subtypes to reveal salinity-dependent combined patterns of protein abundance and immunolocalization in gills of Atlantic salmon (*Salmo salar*). Likewise, double immunocytochemical experiments indicated the respective existence of two NKA isoforms in two distinct groups of ionocytes in gills of FW salmon ( $\alpha_{1a}$  and  $\alpha_{1b}$ ) (112) and tilapia ( $\alpha_1$  and  $\alpha_3$ ) (87). Interestingly, in stenohaline FW zebrafish (*Danio rerio*), three subtypes of NKA  $\alpha_1$ , ATP1a1.1, -2, and -5, were respectively colocalized by double in situ hybridization in different types of ionocytes (90) (also refer to sections *Na uptake/acid secretion*, *Ca<sup>2+</sup> uptake*, and *Cl<sup>-</sup> uptake/base secretion* below). On the other hand, two NKA  $\alpha_{1a}$ - (at a much lower expression level) and  $\alpha_{1b}$ -subtypes were only coexpressed in a group of ionocytes in SW salmon gills (112). It will be challenging and interesting to determine how the two NKA  $\alpha_1$ -subtypes functions in the same ionocyte to achieve the NaCl secretion mechanism in SW gills. Since NKA consists of an ( $\alpha\beta$ )<sub>2</sub>-protein complex, the  $\beta$ -subunit is required for proper functioning and potentially influences the catalytic activity of the enzyme (48). Functional properties of NKA isoforms were more clearly characterized in mammals than in fishes. In rat, Na<sup>+</sup> and K<sup>+</sup> affinities varied among NKA isoform combinations with the respective rank orders of  $\alpha_2\beta_1 > \alpha_1\beta_1 > \alpha_3\beta_1$ , and  $\alpha_1\beta_1 > \alpha_2\beta_1 > \alpha_3\beta_1$  (8). Future studies on the expressions of branchial NKA  $\beta$ -isoforms will certainly extend our knowledge of the roles of NKA in NaCl secretion of marine teleosts.

NKCC1 is thought to be the secretory isoform of NKCC, a member of the SLC12A family. Two isoforms of NKCC1, 1a and 1b, were cloned from European eel (*Anguilla anguilla*), Mozambique tilapia, and brackish-water medaka (*Oryzias latipes*) (33, 53, 76). In gills of these species, the main expressed isoform is the NKCC1a gene. Furthermore, using whole-mount in situ hybridization, the NKCC1a gene was demonstrated to be localized in ionocytes of medaka gills (76), which supports immunocytochemical data from extensive previous studies (66). When euryhaline teleosts were exposed to SW, increased mRNA levels of branchial NKCC1a (33, 53, 76, 99, 150, 169, 175) and elevated protein levels of branchial NKCC1 (43, 76, 148) were reported. The observations strongly suggest a role of

NKCC1a in the NaCl secretion function in gill ionocytes of euryhaline teleosts during acclimation to SW.

The CFTR, a cAMP-activated Cl<sup>-</sup> channel (CIC), mainly appears in branchial ionocytes of SW fish, indicating its role in NaCl secretion upon salinity challenge (9, 66). Increased levels of CFTR genes were found in different teleost species upon exposure to hyperosmotic environments (9, 121, 150, 175). Higher amount of CFTR protein were also detected in SW-adapted teleosts (153, 161, 175). In addition, Tse et al. (175) reported that higher CFTR gene abundances occurred in ionocytes than in pavement cells. Glucocorticoid- and osmotic-responsive elements were identified in upstream regions of the killifish CFTR gene (*Fundulus heteroclitus*) (156). This accumulated evidence supports the crucial role of CFTR, similar to that of NKCC, in NaCl secretion function in SW fish gill ionocytes (9, 121, 150, 175).

*Acid-base regulation in SW fish.* Acid-base regulation in fish is primarily accomplished by branchial transfer of relevant ions (H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>). Due to their low plasma Pco<sub>2</sub> and bicarbonate concentration (indicating a low buffering capacity), fish cannot adjust the plasma pH by modulating ventilation or CO<sub>2</sub> excretion as effectively as air breathers (40). The plasma pH in fish must be adjusted by the differential excretion of H<sup>+</sup> or HCO<sub>3</sub><sup>-</sup> to the ambient water by gills (40, 67, 129). In hypertonic SW environments, fish excrete metabolic acids through the apical Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE), which is generally believed to play a critical role in SW ionocytes (29). In contrast to hypotonic FW, high NaCl concentrations in SW provide the driving force of Na<sup>+</sup>/H<sup>+</sup> exchange across apical membranes of ionocytes. Another acid transporter, H-ATPase (HA), which is generally thought to be critical for acid secretion in FW fish (refer to section *Na uptake/acid secretion* below), may be involved in base secretion instead of acid secretion in SW fish, according to the recent studies (see below).

In recent years, the molecular identity and cellular localization of NHE isoforms and HA in marine and euryhaline fishes were reported. It should be noted that elasmobranchs excrete salt by the rectal gland instead of gills; therefore, ionocytes in their gills are probably specific for acid-base regulation in SW. Interestingly, the euryhaline stingray (*Dasyatis sabina*) was also acclimated to FW to investigate the mechanisms of acid-base regulation and ion uptake. Double immunohistochemistry and/or in situ hybridization studies revealed two distinct ionocytes in stingray gills (22, 135, 136): one expressing apical NHE3/basolateral NKA for H<sup>+</sup> secretion and Na<sup>+</sup> uptake, and the other using apical pendrin-like Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers/basolateral HA to drive apical HCO<sub>3</sub><sup>-</sup> secretion and Cl<sup>-</sup> uptake. Studies in the stingray acclimated to either FW (with a higher transporter expression) or SW (with a lower transporter expression) suggested that the two types of ionocytes, respectively, contribute to acid secretion/Na<sup>+</sup> uptake and base secretion/Cl<sup>-</sup> uptake (22, 136).

In gills of the marine elasmobranch, the dogfish (*Squalus acanthias*), two distinct types of ionocytes were also identified that, respectively, express basolateral HA and NKA; HA expression increased in animals exposed to metabolic alkalosis, suggesting that the basolateral HA is involved in base secretion (171, 173, 174), as proposed in the euryhaline stingray (22, 136). NKA-rich ionocytes were reported to coexpress apical NHE3 (with an anti-stingray NHE3 antibody) (23) or NHE2 mRNA and protein (with an anti-dogfish NHE2 antibody) (28).

Earlier work using heterologous antibodies (against mammalian NHE2) demonstrated the colocalization of NHE2 and NKA in gills of several elasmobranch species (36) and increased protein levels following acid infusion (171). Similarly, the marine teleost, *Myoxocephalus octodecemspinosus*, was also reported to develop two types of ionocytes in its gills: one expressing apical NHE2 (with a homologous antibody) with basolateral NKA, and the other basolateral HA (183). These two cell types were proposed to conduct, respectively, acid secretion and base secretion, similar to that of elasmobranch gill ionocytes (18). Taken together, the role of NHEs in acid secretion in SW fish is well accepted; however, it is still unclear whether both NHE2 and NHE3 are coexpressed in the same ionocytes; if so, what are the functional differences between the two?

#### *Ion Regulation in FW*

*Na uptake/acid secretion.* Two working models were proposed for apical Na<sup>+</sup> uptake/acid secretion functions in FW fish gills (Fig. 2) (39, 40, 64, 66, 67): 1) HA electrically linking with the epithelial Na<sup>+</sup> channel (ENaC), and 2) the electro-neutral NHE. Molecular evidence for the existence of the ENaC in fish is not available so far; however, many studies still support the model of HA/ENaC. Most of the data supporting the role of ENaC were derived from pharmacological experiments using ENaC inhibitors in intact fish (37, 138) or isolated cells (126, 142). Parks and colleagues (127) addressed how the electrochemical gradient between FW and the intracellular milieu of gill ionocytes favors the operation of linking HA/ENaC, but never to NHE. However, no homolog of mammalian ENaC or its equivalent was found in teleost genomes (187). On the other hand, evidence for the role of HA in FW fish gill Na<sup>+</sup> uptake/acid secretion mechanisms is abundant and mostly consistent and was obtained from physiological experiments (acid or hypercapnic effect on enzyme expression/activity), pharmacological approaches with HA inhibitors, and cellular localization of the enzyme (11, 37, 58, 93). In rainbow trout gills, only the group of peanut lectin agglutinin-negative (PNA<sup>-</sup>) cells showed much higher HA expression and bafilomycin-sensitive, acid-activated <sup>22</sup>Na<sup>+</sup> uptake function, compared with PNA<sup>+</sup> cells (45, 142). Recent studies on zebrafish provide convincing in vivo molecular physiological evidence on this issue. A group of ionocytes, HA-rich (HR) cells was demonstrated to secrete H<sup>+</sup> using a scanning ion-selective electrode technique (SIET) in the skin of intact embryos (93) and absorb Na<sup>+</sup> using a Na<sup>+</sup> green fluorescence reagent (37). Further loss-of-function studies reinforced the functional role of HA in HR cells. Knockdown of HA by specific morpholinos decreased the surface H<sup>+</sup> activity in embryonic skin and simultaneously resulted in a decline in the whole body Na<sup>+</sup> content in embryos acclimated to low-Na<sup>+</sup> FW (58). That study was supported by pharmacological evidence (37), in which bafilomycin, an HA inhibitor, was found to impair Na<sup>+</sup> green accumulation in zebrafish HR cells.

The thermodynamic operation of apical Na<sup>+</sup>/H<sup>+</sup> exchange through the NHE has been debated for a long time (3, 127). Much of the emerging literature provides the cellular localization and expression data of various NHE isoforms in FW gill ionocytes [refer to details in a review by Evans (39)]. Recently, NHE3b was found to be the only one (of 8 isoforms) specifi-

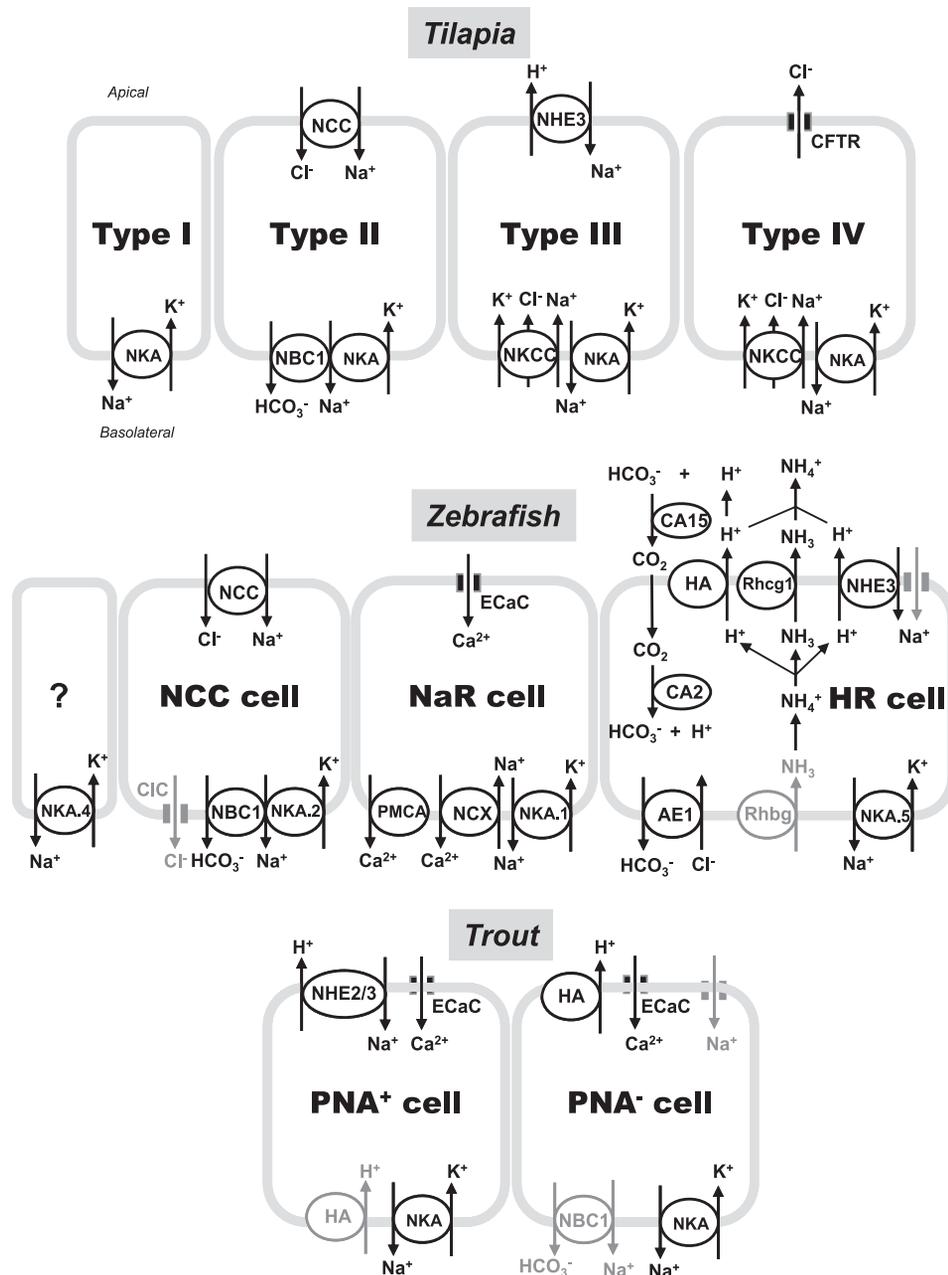


Fig. 2. Models of ionocytes in different freshwater species. Details refer to the text (sections *Ion Regulation in FW*, *Ammonia Excretion*, and *Identification and Comparison of Ionocyte Subtypes in Different Species*). Tilapia: type I, II, III, and IV (i.e., the SW; refer to Fig. 1) cells. Zebrafish: unidentified (?),  $\text{Na}^+\text{-Cl}^-$  cotransporter (NCC),  $\text{H}^+$ -ATPase (HA)-rich (HR), and NKA-rich (NaR) cells. Rainbow trout: peanut lectin agglutinin (PNA)<sup>+</sup> and PNA<sup>-</sup> cells. AE1, anion exchanger 1b; CA, carbonic anhydrase 2-like a; CA2 (CA15), carbonic anhydrase 2-like a (15a); CIC,  $\text{Cl}^-$  channel; ECaC, epithelial  $\text{Ca}^{2+}$  channel; NBC1,  $\text{Na}^+\text{-HCO}_3^-$  cotransporter (NBCe1b in zebrafish); NCX,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger 1b; NHE (NHE3b in zebrafish); NAK.1–5, NKA  $\alpha_1$ -subunit subtypes; PMCA, plasma membrane  $\text{Ca}^{2+}$ -ATPase 2. Transporters indicated in dark gray had no mRNA or protein localization data.

cally expressed in the apical membrane of HR-type ionocytes in zebrafish (201). Similarly, NHE3 is in a group of gill cells in Osorezan dace (*Tribolodon hakonensis*) (51) and type III ionocytes of tilapia (53, 68), and NHE2/3 are in PNA<sup>+</sup> ionocytes of rainbow trout (72). Upregulation of both  $\text{Na}^+$  uptake function and NHE expression was reported in several species in low- $\text{Na}^+$  FW or other experimental situations (68, 96, 200, 201). Moreover, a specific NHE inhibitor, EIPA, was found to decrease the  $\text{Na}^+$  green accumulation in zebrafish HR cells (37) and the  $\text{Na}^+$  uptake activity (by SIET) in medaka embryonic skin ionocytes (200). All of these results reinforce the role of NHEs in the  $\text{Na}^+$  uptake mechanism in specific types of ionocytes in FW fish. On the other hand, the roles of NHEs in acid-base regulation mechanisms in FW gills appear to be species specific (also refer to section *Acid-base regulation in SW fish* above). For instance, NHE2 expression in rainbow

trout gill PNA<sup>+</sup> cells was upregulated during hypercapnic acidosis (72). Similarly, NHE3 expression in dace gills (51) was stimulated by low-pH FW. In contrast, low-pH FW was reported to suppress NHE3b expression, but enhance HA expression and function of HR cells in zebrafish gills and embryonic skin (21, 59).

Based on the expression and function of HA and NHEs, FW fish gill ionocytes, trout PNA<sup>-</sup> cells, and zebrafish HR cells were proposed to be analogous to mammalian A-type intercalated cells or proximal tubular cells, respectively (39, 66, 67). Membrane-bound and cytosolic carbonic anhydrases (CA4 and CA2) play important roles in epithelial acid secretion/ $\text{Na}^+$  uptake mechanisms in both mammalian A-type intercalated cells and proximal tubular cells (140). In FW fish gill ionocytes, functional characterization of the cytosolic CA in ion uptake and acid-base regulation pathways is well documented

(40, 49, 66). However, there were no available data on the expression and function of a membrane-bound CA until a recent study on zebrafish (96). CA2-like a (a cytosolic form) and CA15a (a membrane-bound CA4-like isoform) were identified to be coexpressed in zebrafish HR ionocytes (96). Knock-down of either isoform caused impacts on  $\text{Na}^+$  uptake and  $\text{H}^+$  secretion functions in zebrafish skin HR cells (96). A CA inhibitor, ethoxzolamide, was found to obviously decrease the accumulation of  $\text{Na}^+$  green fluorescence in zebrafish skin HR cells (37). Zebrafish gill CA15a mRNA expression was up-regulated after acclimation to low- $\text{Na}^+$  or low-pH FW (96). Taken together, both membrane-bound and cytosolic CAs are essential for achieving the epithelial acid secretion/ $\text{Na}^+$  uptake mechanisms in zebrafish HR-type ionocytes as in mammalian A-type intercalated cells or proximal tubular cells.

The anion exchanger (AE1/SLC4A1) and electrogenic  $\text{Na}^+$ - $\text{HCO}_3^-$  cotransporter (NBCe1/SLC4A4) are basolateral transporters in mammalian A-type intercalated cells and proximal tubular cells, respectively (1, 146). In the current models of fish gill  $\text{Na}^+$  uptake/acid secretion mechanisms, NBC was repeatedly proposed as a major player mainly based on the gene expression data after acid or hypercapnic treatment (40, 66, 67, 127). There are few data available on localization of NBC in fish gill ionocytes. For instance, NBCe1 was localized in a certain group of gill ionocytes in the Osorezan dace (51) and rainbow trout (126). So far, there are no data on the colocalization of NBC with either NHE or HA in the same gill ionocytes, which is physiologically important for achieving  $\text{Na}^+$  uptake/acid secretion functions by ionocytes. Surprisingly, a recent study on zebrafish with a double in situ hybridization approach indicated that NBCe1b (SLC4A4b) mRNA was not detected in HR cells (64), but was coexpressed with the  $\text{Na}^+$ - $\text{Cl}^-$  cotransporter (NCC) (SLC12A10.2) (88), a marker of another type of ionocytes in zebrafish (191) (also refer to section *Cl<sup>-</sup> uptake/base secretion* below). Similar approaches were used to identify that AE1b (SLC4A1b) was the only member of the SLC4A family that is specifically expressed by HR cells (88). A functional analysis with loss of function or treatment with low pH/low  $\text{Na}^+$  provided molecular physiological evidence for the role of AE1b in  $\text{Na}^+$  uptake/acid secretion, the known functions of HR-type ionocytes in zebrafish (88). In support of these data, basolateral localization of AE1 with an anti-tilapia AE1 antibody in gill ionocytes (marked with NKA) was also reported in the pufferfish (162). On the other hand, an NKA  $\alpha_1$ -subunit subtype, ATP1a1a.5, was localized in zebrafish HR cells (90). After acclimation to low  $\text{Na}^+$ , ATP1a1a.5 expression in zebrafish gills was upregulated, as was that of other HR cell genes (NHE3b, CA15a, and AE1b), suggesting a role in the epithelial  $\text{Na}^+$  uptake mechanism in HR cells (90).

Recent molecular physiological studies accumulated more convincing data in zebrafish than in other species, for the identification and functional analysis of transporters and enzymes involved in  $\text{Na}^+$  uptake/acid secretion (64, 67). Accordingly, zebrafish HR cells could provide a more comprehensive model for these epithelial transport pathways. In apical membranes of HR cells, NHE3b and HA transport  $\text{H}^+$  out of the cells, and this  $\text{H}^+$  combines with environmental  $\text{HCO}_3^-$  to generate  $\text{H}_2\text{O}$  and  $\text{CO}_2$  by carbonic CA15a. Subsequently,  $\text{CO}_2$  enters HR cells and is hydrated by cytosolic CA2-like a to form  $\text{H}^+$  and  $\text{HCO}_3^-$ . Basolateral AE1b extrudes cytosolic  $\text{HCO}_3^-$

out of the cells to fulfill the epithelial acid secretion function, and this also provides an intracellular pH gradient favorable for the operation of apical NHE3b to achieve the apical  $\text{Na}^+$  uptake mechanism. On the other hand, basolateral NKA is responsible for the excretion of  $\text{Na}^+$  and also provides an intracellular negative potential.

*Ca<sup>2+</sup> uptake.* In FW fish, besides dietary intake, the gills are the main site (>97% of the whole body) of  $\text{Ca}^{2+}$  uptake from the aquatic environment to maintain the  $\text{Ca}^{2+}$  balance (44). According to the current mammalian model for transcellular  $\text{Ca}^{2+}$  transport,  $\text{Ca}^{2+}$  is transported across the apical membrane through apical epithelial  $\text{Ca}^{2+}$  channels (ECaC, transient receptor potential vanilloid 5, and/or transient receptor potential vanilloid 6), while intracellular  $\text{Ca}^{2+}$  is bound to calbindins that facilitate diffusion to the basolateral membrane and is extruded via the basolateral plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and/or  $\text{Na}^+$ / $\text{Ca}^{2+}$  exchanger (NCX) (55). A similar model (Fig. 2) was also proposed for ionocytes of FW fish gills (44). It took time for molecular evidence from recent studies to become available for the existence and functional roles of relevant  $\text{Ca}^{2+}$  transporters in fish. Initially, ECaC was cloned from fugu, zebrafish, and trout (124, 141, 151). Epithelial  $\text{Ca}^{2+}$  channel, two basolateral transporter isoforms (PMCA2 and NCX1b), and an NKA  $\alpha_1$ -subunit subtype (ATP1a1a.2) were identified as being coexpressed in a subset of ionocytes,  $\text{Na}^+$ - $\text{K}^+$ -ATPase-rich (NaR) cells, in zebrafish (90, 91, 124). In rainbow trout, however, MR cells and pavement cells were proposed as sites of  $\text{Ca}^{2+}$  uptake on the basis of ECaC expression in both cell types (151). ECaC was demonstrated to be the major player for the epithelial  $\text{Ca}^{2+}$  uptake pathway in zebrafish skin and gills, based on ECaC loss-of-function experiments (176) and expression/function upregulation of ECaC (but not PMCA2 or NCX1b) by low- $\text{Ca}^+$  FW (91, 124, 176). Supporting the zebrafish data, acclimation to soft water or an infusion of  $\text{CaCl}_2$  upregulated ECaC mRNA and/or protein expressions in trout gills (152). Notably, the  $\text{Ca}^+$  uptake capacity of isolated rainbow trout gill PNA<sup>+</sup> cells was about threefold higher than that of PNA<sup>-</sup> cells (46), while ECaC mRNA levels in PNA<sup>-</sup> cells were higher, although nonsignificantly, compared with those of PNA<sup>+</sup> cells (151) (also refer to section *Identification and Comparison of Ionocyte Subtypes in Different Species* below).

In mammalian kidney cells, calbindin-D<sub>9k</sub> and -D<sub>28k</sub> are known to play important roles in facilitating cytosolic  $\text{Ca}^{2+}$  diffusion to achieve transcellular  $\text{Ca}^{2+}$  transport (55). Other calcium-binding proteins, parvalbumin and S100, are also involved in  $\text{Mg}^{2+}$  reabsorption or  $\text{Ca}^{2+}$  homeostasis in mammals (34, 97). Interestingly, pvalb3a and S100A11 suggest a salt-and-pepper expression pattern in zebrafish embryonic skin (60, 82), similar to that of skin ionocytes (61). Further studies are needed to characterize whether these calcium-binding proteins play a role in cytosolic  $\text{Ca}^{2+}$  diffusion in fish gill/skin  $\text{Ca}^{2+}$  uptake mechanisms.

*Cl<sup>-</sup> uptake/base secretion.* It was long proposed that apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange is the major pathway of  $\text{Cl}^-$  uptake linked to base secretion in FW fish gills, and that an anion exchanger (AE1) was one of the candidates proposed (40, 66, 67). Early pharmacological studies supported this notion, but convincing molecular evidence for AE1 expression by gill cells was scarce and debatable (64, 67, 128, 172). Moreover, low- $\text{Cl}^-$  FW, which can stimulate the  $\text{Cl}^-$  uptake function, did not

cause a significant change in the mRNA expression of AE1 in zebrafish gills (64), raising a question about the role of AE1 in  $\text{Cl}^-$  uptake mechanisms. In a  $\text{Cl}^-$ /base secretion model of mammalian B-type intercalated cells, pendrin is the major transporter responsible for apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange (188, 189). In a group of gill cells of a euryhaline elasmobranch, the Atlantic stingray, Piermarini et al. (136) first identified the colocalization of apical pendrin and basolateral HA (by double immunocytochemistry with heterogeneous antibodies), similar to mammalian kidney B-type intercalated cells (also refer to section *Acid-base regulation in SW fish* above). The gill protein expression of pendrin was stimulated by FW acclimation compared with the SW control (136). In a recent pharmacological study on trout gills, a subset of isolated ionocytes (probably  $\text{PNA}^+$  cells) showed DIDS-sensitive,  $\text{Cl}^-$ -free induced alkalization, suggesting the involvement of  $\text{Cl}^-/\text{HCO}_3^-$  in the apical  $\text{Cl}^-$  uptake by cells (128). However, it was not until recently that studies provided molecular physiological evidence, which shed some light on the role of apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in the FW fish  $\text{Cl}^-$  uptake mechanism. Pendrin (SLC26A4) and two paralog (SLC26A3 and -6) mRNAs were localized in certain groups of gill cells in both zebrafish embryos and adults, and only a small portion (<10%) of SLC26A3-expressing cells coexpressed basolateral NKA (7, 131). Our laboratory's recent preliminary experiments indicated the colocalization of SLC26A4 in most NaR cells (basolaterally labeled with an NKA antibody) (Fig. 3, A and B; R. D. Chen, Y. C. Tseng, and P. P. Hwang, unpublished data). Bayaa and colleagues demonstrated the role of these transporters in the  $\text{Cl}^-$  uptake mechanism by discovering  $\text{Cl}^-$  uptake defects in zebrafish morphants injected with specific morpholinos of SLC26A3, -4, and -6 (7). These data were further supported by data of low- $\text{Cl}^-$  stimulation of SLC 26 members' mRNA expression in both embryos and adult gills (7, 131). On the other hand, the driving mechanism for apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in FW fish gill cells is still being debated. Chemical gradients ( $\text{Cl}^-$  or  $\text{HCO}_3^-$ ) across apical membranes of FW gill cells appear to be unfavorable for  $\text{Cl}^-/\text{HCO}_3^-$  exchange (172). Similar to mammalian kidney B-type intercalated cells, cytosolic CA2 and basolateral HA were proposed to create microenvironments enriched with  $\text{HCO}_3^-$  to drive the apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (11, 19, 172). However, there are no data so far available to support the coexpression of SLC26 members, cytosolic CA2, and basolateral HA in the same types of ionocytes in a fish species. On the other hand, stoichiometry of SLC26 members may be another possibility to explain the driving mechanism. Mammalian SLC26A3, -4, and -6 show different respective stoichiometries for transporting  $2\text{Cl}^-/1\text{HCO}_3^-$ ,  $1\text{Cl}^-/1\text{HCO}_3^-$ , and  $1\text{Cl}^-/2\text{HCO}_3^-$  (122). Recent functional characterization studies also identified some electrogenic SLC26 members in teleosts, SLC26a6A and -6B in mefugu (*Takifugu obscurus*) (85) and SLC26a6 in toadfish (50). This suggests a possibility that the intracellular negative potential of FW gill cells may facilitate apical  $\text{Cl}^-/\text{HCO}_3^-$  transport via electrogenic SLC26 members to achieve epithelial  $\text{Cl}^-$  uptake/base secretion functions.

Another candidate for the apical transport pathway in the FW  $\text{Cl}^-$  uptake mechanism is the NCC (Fig. 2). The NCC, a thiazide-sensitive membrane protein, was initially identified in the flounder urinary bladder (144) and is specifically expressed in apical membranes of mammalian distal convoluted tubules,

which reabsorb 5 ~ 10% of the total filtered  $\text{Na}^+/\text{Cl}^-$  by the kidneys (143). It was not until recent studies that a new window was opened on exploring the roles of the NCC in FW fish  $\text{Cl}^-$  and/or  $\text{Na}^+$  uptake mechanisms. Hiroi and coworkers (53, 69) first identified an ortholog of the NCC from tilapia and colocalized the protein (with a homologous antibody) to the apical membrane of type II (mainly with convex apical membrane) ionocytes, but not to other cell types. Acclimation to deionized FW or low- $\text{Cl}^-$  artificial FW caused an increase in the density of NCC-expressing type II ionocytes and a concomitant upregulation of NCC mRNA in tilapia adult gills and embryonic yolk sac membranes, compared with those of the FW controls (68, 69). Those studies on tilapia proposed a role of the NCC in  $\text{Cl}^-$  and  $\text{Na}^+$  uptake mechanisms. In addition, a recent in vivo electrophysiological study (by SIET) of tilapia embryos further demonstrated NCC's function in  $\text{Cl}^-$  uptake. An inward metolazone-sensitive  $\text{Cl}^-$  current occurred over convex ionocytes that express NCC immunoreactivity in embryonic skin (57). In support of tilapia studies, the NCC was also identified in a specific group of zebrafish ionocytes; NCC cells and NCC mRNA expression and  $\text{Cl}^-$  uptake function in embryos were stimulated in low- $\text{Cl}^-$  artificial FW (191). Translational knockdown of the NCC impaired both the  $\text{Cl}^-$  influx and content in zebrafish morphant embryos, but stimulated  $\text{Na}^+$  influx and content, as well as the expression of NHE3b mRNA (191). That study provided molecular physiological evidence for the role of the NCC in  $\text{Cl}^-$  uptake, but with a minor effect on  $\text{Na}^+$  uptake (191). Even with those studies, the driving mechanism for the NCC in FW gill/skin ionocytes is still an unresolved issue (64, 66). Both the  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations in FW would appear to be too low to permit operation of the NCC (64, 66, 191), which is an electroneutral transporter, and needs a chemical gradient to drive the cotransport of  $\text{Cl}^-$  and  $\text{Na}^+$  (144). Recently, according to electrophysiological measurements by Hornig et al. (57),  $\text{Cl}^-/\text{Na}^+$  concentrations in the microenvironment were higher than those in ambient FW due to outward  $\text{Cl}^-/\text{Na}^+$  fluxes from neighboring pavement cells, and these ionic gradients may be high enough to drive the NCC.

As discussed above, the  $\text{Na}^+$  uptake function monitored with fluorescent  $\text{Na}^+$  green is confined to HR-type cells, but not to other types of ionocytes in zebrafish embryonic skin (37). Incubation with metolazone (a thiazide-like, NCC-specific inhibitor) reduced both  $\text{Cl}^-$  and  $\text{Na}^+$  influxes in zebrafish embryos (191). NBCe1b, a basolateral transporter that eliminates intracellular  $\text{Na}^+$  from cells, was colocalized in NCC cells of zebrafish (88) and also in NCC-expressing type II ionocytes of tilapia (F. Furukawa, T. Kaneko, personal communication). Our preliminary experiments on zebrafish demonstrated that knockdown of GCM2, a transcriptional factor specifically targeting differentiation of HR cells, resulted in the disappearance of HR cells (the ionocytes mainly responsible for  $\text{Na}^+$  uptake; refer to section *Na uptake/acid secretion* above) (21, 38), but also caused an increase in both the number of NCC cells and  $\text{Na}^+$  uptake function in zebrafish morphants (Fig. 3, C and D; W. J. Chang, Y. F. Wang, P. P. Hwang, unpublished data). This implies a redundant or supplementary role of NCC-expressing cells in  $\text{Na}^+$  uptake in FW fish gills, and the exact role of the NCC in the  $\text{Na}^+$  uptake function deserves further exploration.

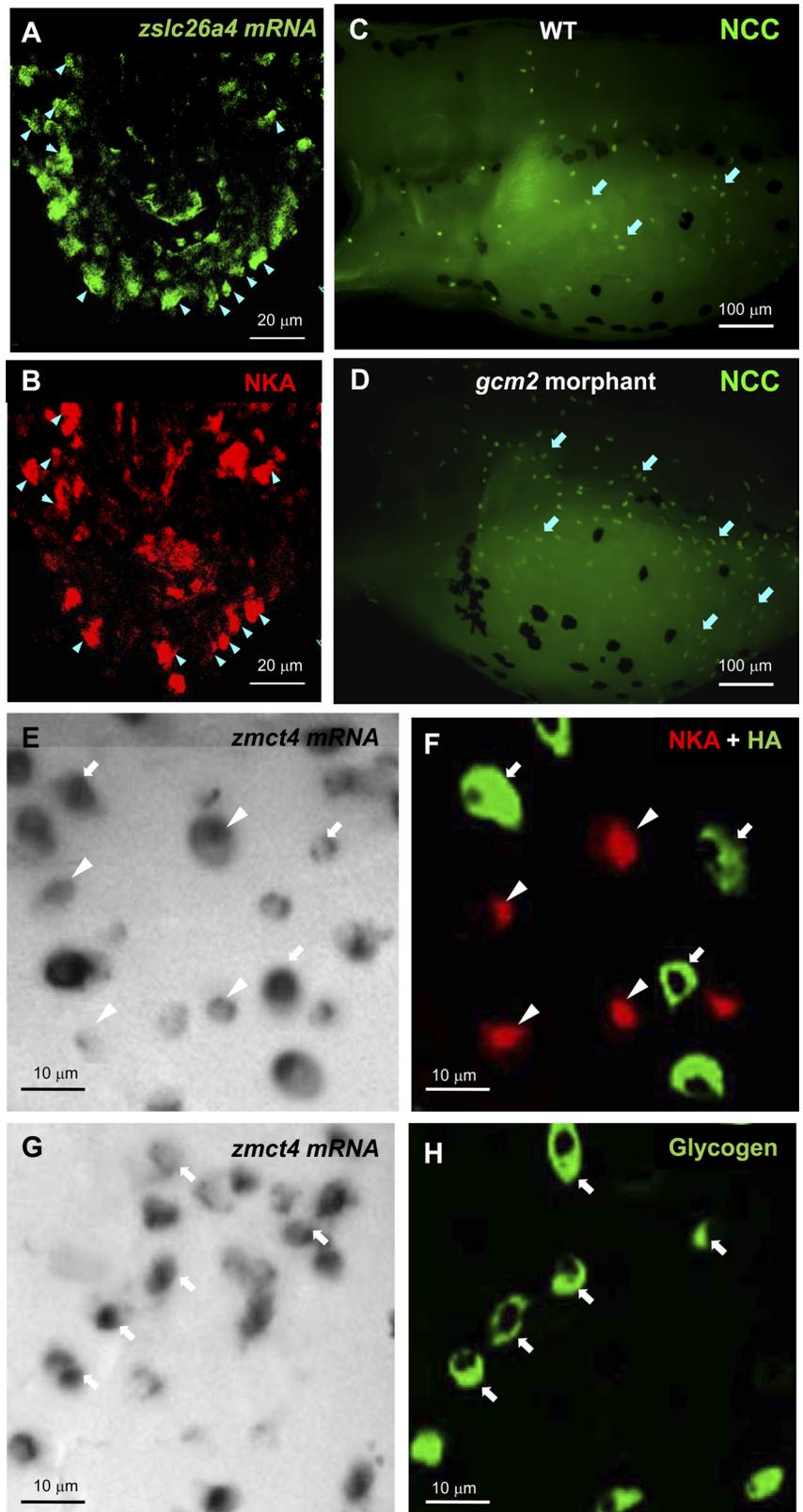


Fig. 3. In situ hybridizational and immunocytochemical images of ionocytes in zebrafish gills and embryonic skin. *A* and *B*: colocalization (arrowhead) of *zslc26a4* (pendrin) mRNA and NKA in a frozen section of zebrafish adult gills. *C* and *D*: comparison of skin NCC cells (arrow) between *gcm2* morphant and wild-type (WT) embryos at 3 days postfertilization. *E* and *F*: colocalization of monocarboxylate transporter (*zmct4*) mRNA in NaR-type (labeled with NKA, arrowhead) and HR-type (labeled with HA, arrow) ionocytes, respectively, in the skin of 3-day postfertilization zebrafish embryos. *G* and *H*: colocalization of *zmct4* mRNA in glycogen-rich (GR) cells (labeled with glycogen, arrow). RNA probes and antibodies were the *zslc26a4* RNA probe, a 1,196-bp fragment of NM\_001165915; *zmct4* RNA probe, a 364-bp fragment of NM\_212708; NKA,  $\alpha_5$  anti-chicken NKA  $\alpha$ -subunit monoclonal antibody; NCC, anti-zebrafish NCC polyclonal antibody; HA, anti-zebrafish HA A subunit polyclonal antibody; and glycogen, anti-glycogen monoclonal antibody [refer to Tseng et al. (178)]. The morpholinos used were directed against zebrafish *gcm2* [5'-AAACT-GATCTGAGGATTTGGACATG-3', refer to Change et al. (21)].

In models of mammalian distal convoluted tubule cells and B-type intercalated cells, CICs are the major basolateral transporter in collaboration with apical pendrin and the NCC, respectively, which carry out the epithelial  $\text{Cl}^-$  uptake mech-

anism (143, 188). In mammalian cells, CIC3 and -5 mainly reside in the endosomal/lysosomal system and in synaptic vesicles and were recently suggested to act as a  $\text{Cl}^-/\text{H}^+$  exchanger associated with luminal acidification (74). Fish

orthologs of mammalian CIC3 and -5 were first cloned from tilapia (113). A recent study on the euryhaline pufferfish demonstrated colocalization of CIC3 (with a heterologous antibody) in the basolateral membrane of gill NKA-rich ionocytes (160). Furthermore, acclimation to low  $\text{Cl}^-$  FW stimulated CIC3 protein levels in pufferfish gills, but did not affect mRNA expression (160). Differences in the cellular localization and physiological function of CIC3 between fish gill cells and mammalian cells are of evolutionary significance; however, whether other CIC orthologs are involved in basolateral  $\text{Cl}^-$  transport in FW fish gill ionocytes is so far an open issue. On the other hand, basolateral NKA in NCC-expressing ionocytes may establish a negative intracellular gradient to drive the electrogenic CIC (and/or NBCe1, see above) to eliminate  $\text{Cl}^-$  (and/or  $\text{Na}^+$ ) from cells. Immunocytochemical localization of NKA in tilapia type II ionocytes (53, 69) and the presence of ATP1a.2 (a subtype of the NKA  $\alpha_1$ -subunit) mRNA by in situ hybridization in zebrafish NCC-type ionocytes (90) support this notion.

### Ammonia Excretion

Ammonia excretion in fish is largely accomplished by the gills. Krogh (84) initially proposed the linked ammonia excretion and  $\text{Na}^+$  uptake in FW fish. The mechanisms underlying the excretion of ammonia across fish gills were reviewed recently (70, 192, 195, 198). Herein, we emphasize recent progress on Rhesus (Rh) glycoproteins, which are involved in branchial ammonia excretion (Fig. 2). As ammonia can exist as a dissolved ammonia gas ( $\text{NH}_3$ ) and ammonium ions ( $\text{NH}_4^+$ ), the present review used the conventional term "ammonia" when the chemical was not specified, and chemical symbols ( $\text{NH}_3$  or  $\text{NH}_4^+$ ) when specified.

The human Rh blood type antigen was long linked to destructive antibody production; however, the role of Rh glycoproteins in ammonia transport of erythrocytes and nonerythrocytes was only recently discovered. In 1997, Marini et al. (105) first identified sequence similarities between Rh glycoproteins and the Mep/Amt family found in microorganisms and plants that transport ammonia by Mep/Amt proteins. Soon afterwards, the human erythroid RhAG and its nonerythroid homologs, RhBG and RhCG, were demonstrated to indeed behave as ammonia transporters (104, 194). Nakada et al. (117) were the first to identify four Rh glycoproteins (Rhag, Rbhg, Rhcg1, and Rhcg2) in pufferfish (*Takifugu rubripes*) and their distributions in gills. They found that Rhag, Rbhg, and Rhcg2 are distributed on gill lamellae, which were long thought to be the location of  $\text{O}_2$  and  $\text{CO}_2$  exchange and probably  $\text{NH}_3$  gas excretion. On the contrary, Rhcg1 exhibited a somewhat unexpected localization in ionocytes, which are relatively minor among the population of gill epithelial cells and are specialized to actively absorb or secrete  $\text{NaCl}$  to maintain body fluid homeostasis. With isoform-specific antibodies, Rbhg and Rhcg2 were, respectively, localized to the basolateral and apical membrane of pavement cells, whereas Rhcg1 was localized to the apical membrane of ionocytes. Interestingly, the blood form (Rhag) was localized to pillar cells, which line blood channels in gill lamellae. In zebrafish, Nakada et al. (116) also identified four isoforms in gills and found that Rhcg1 was not expressed in all ionocytes, but only in HR cells, which are acid-secreting cells (93). This specific expression

was further demonstrated to be critical for ammonia excretion by zebrafish embryos (155). In addition to Rhcg1, the distribution of Rbhg in zebrafish somewhat differs from that in the pufferfish (12). Rbhg was localized to the basolateral membrane of an unidentified cell (which was unlike ionocytes in our opinion) in gills and skin of zebrafish embryos (12). Although the cellular distribution is not as clear as that in pufferfish and zebrafish, Rh proteins were identified in gills of several species, including mangrove killifish (*Kryptolebias marmoratus*) (62), rainbow trout (120), longhorn sculpin (*Myoxocephalus octodecemspinosus*) (30), toadfish (192), weatherloach (*Misgurnus anguillicaudatus*) (114), and medaka (200).

In addition to the in vitro characterization of Rh proteins with an oocyte expression system (116, 119), the expression level of Rh proteins was shown to be correlated with ammonia excretion in vivo. In rainbow trout, Nawata et al. (120) found that Rhcg2 and Rbhg mRNA expressions were induced by 12 ~ 48 h of high environmental ammonia treatment, and these increases were specific to pavement cells using density gradient-based separation of branchial cells. A similar response in various Rh proteins was also found in gills of mangrove killifish (62) and pufferfish (119). The upregulation of Rh proteins in response to high environmental ammonia suggests that Rh proteins are involved in ammonia excretion. Because there is no known inhibitor of Rh proteins, loss-of-function evidence demonstrating their function in vivo depends on genetic approaches. In a zebrafish study, Shih et al. (155) used a morpholino knockdown technique to block the translation of Rhcg1 in embryos and demonstrated that Rhcg1 is involved in ammonia excretion by HR cells. Braun et al. (12) further demonstrated that knockdown of other Rh proteins also partially blocked ammonia excretion in zebrafish.

Those in vitro and in vivo studies support the hypothesis that Rh proteins are involved in ammonia transport. However, which form of ammonia, the ionic  $\text{NH}_4^+$  or  $\text{NH}_3$  gas, is transported by Rh proteins is still being debated. The functional properties of Rh proteins were recently reviewed (193, 198). Some in vitro studies provided evidence to support a model that Rh proteins are  $\text{NH}_3$  gas channels (103), whereas others suggested the dual transport of  $\text{NH}_3$  and  $\text{NH}_4^+$  (6) or electrogenic  $\text{NH}_4^+$  transport (118). An X-ray crystallography study (81) analyzed the molecular structure of AmtB, a bacterial homolog of Rh glycoproteins from *Escherichia coli*. Based on molecular analysis of this structure, a model was proposed whereby a 20-Å-long hydrophobic pore serves as a channel that has four  $\text{NH}_3$ - or  $\text{NH}_4^+$ -binding sites. The first site facing the extracellular medium serves as a vestibule that recruits  $\text{NH}_3/\text{NH}_4^+$  binding predominantly as charged  $\text{NH}_4^+$ . Once  $\text{NH}_4^+$  is recruited,  $\text{H}^+$  is released extracellularly, and  $\text{NH}_3$  is conducted through the hydrophobic pore. On the intracellular side,  $\text{NH}_3$  recruits an intracellular  $\text{H}^+$  and is released as  $\text{NH}_4^+$ . Given the known relatedness, the structure of Amt proteins was used as a template for homologous modeling of human Rh proteins to gain insights into their functions (15, 17). Those studies suggested that the three-dimensional structures of Rh proteins were similar to those of Amt proteins. Recently, X-ray crystallography of a bacterial Rh protein (NeRh) strengthened our understanding of Rh functions (89, 101). Those studies showed that the structure of NeRh is similar to that of Amt, an  $\text{NH}_3$  gas channel.

In vivo studies on zebrafish and medaka embryos suggested that ammonia is mainly excreted in the form of  $\text{NH}_3$  gas (155, 200). By probing  $\text{NH}_4^+$  and  $\text{H}^+$  concentrations at the surface of fish embryos, those studies showed a reverse correlation between surface  $\text{NH}_4^+$  and  $\text{H}^+$  concentrations, suggesting that  $\text{NH}_3$  gas is excreted and combined with  $\text{H}^+$  to form ionic  $\text{NH}_4^+$  at the surface. The apical Rhcg was suggested to be the  $\text{NH}_3$  channel involved in the excretion of "acid trapping" ammonia. As proposed by Wright et al. (199) in an earlier study, the acid-trapping hypothesis of ammonia excretion suggests that the acidification of the external boundary layer adjacent to the gill epithelium facilitates the diffusive movement of  $\text{NH}_3$ , since an acidic environment increases the conversion of  $\text{NH}_3$  to  $\text{NH}_4^+$ . The conversion of  $\text{NH}_3$  to  $\text{NH}_4^+$  serves to maintain a favorable blood-to-water  $\text{NH}_3$  gradient. A study by Wright et al. (199) on rainbow trout suggested that  $\text{CO}_2$  excretion by gills is the major source of the acidic layer. In zebrafish embryos, Shih et al. (155) used morpholino knockdown and a bafilomycin inhibitor to demonstrate that  $\text{H}^+$  secretion by HA in HR cells provides an alternate source of surface acidification to promote  $\text{NH}_3$  diffusion via Rhcg1. In cultured rainbow trout gill epithelial cells, bafilomycin was shown to inhibit ammonia efflux (182). In an air-breathing fish, the weatherloach, HA and Rhcg1 were colocalized to a group of ionocytes that are not rich in NKA. Treatment with bafilomycin decreased ammonia excretion, which also supports HA being involved in the acid-trapping mechanism (114). However, this HA-associated mechanism is not universal to all fish species. In some species, such as killifish (78) and medaka (200), HA was not found in the apical domain of ionocytes or pavement cells. Furthermore, a recent study on medaka embryos showed that NHE3 and Rhcg1 mRNAs were colocalized in a group of ionocytes, and ammonia excretion was blocked by EIPA (an analog of amiloride, specific for NHE but not ENaC), but not bafilomycin, suggesting that NHE is critical for ammonia excretion (200).

Recent studies on Rh proteins also provide new insights into the mechanism of  $\text{Na}^+$  uptake in FW fish (Fig. 2). In zebrafish, Rhcg1 mRNA expression was found to be induced by ion-poor FW, suggesting that Rhcg1 may be involved in adaptation mechanisms for the environment of low ionic strength (116). Similarly, medaka branchial Rhcg1 mRNA expression was induced by low- $\text{Na}^+$  FW acclimation (200). In a similar manner, Rhcg2 mRNA in rainbow trout was also upregulated in cultured branchial cells pretreated with low- $\text{Na}^+$  medium (182). Interestingly, a linkage between ammonia excretion and  $\text{Na}^+$  uptake was revealed. Tsui and colleagues (182) examined the kinetics of  $\text{NH}_4^+/\text{Na}^+$  transport in cultured trout gill cells and suggested that branchial  $\text{Na}^+/\text{NH}_4^+$  exchange is mediated by a putative protein complex, including the Rh proteins, NHE, HA, and ENaC. This "protein complex model" was further elaborated by Wright and Wood (198). However, it should be noted that the cellular distributions of those suggested proteins in trout gills are yet unclear, and whether they are located in the same type of ionocytes is uncertain. In an in vivo study, Wu and colleagues (200) provided additional solid evidence for an ammonium-dependent sodium uptake mechanism in ionocytes of medaka. They used SIET to probe  $\text{Na}^+$ ,  $\text{NH}_4^+$ , and  $\text{H}^+$  gradients at ionocytes in the embryonic skin of medaka and demonstrated that  $\text{Na}^+$  uptake and ammonia excretion by ionocytes are tightly associated. Both  $\text{Na}^+$  uptake and am-

monia excretion were blocked by an NHE inhibitor (EIPA), but not by an HA inhibitor (bafilomycin). In situ hybridization labeled NHE3, Rhcg1, and Rhbg in the same ionocytes, and their mRNA expression levels were induced by low- $\text{Na}^+$  FW acclimation. This evidence suggests the involvement of Rh proteins and NHE3 in the ammonium-dependent sodium uptake model. In this model (Fig. 2), Rhcg1 and NHE3 are functionally and probably physically associated in apical membranes of ionocytes. Rhcg1 facilitates ammonia diffusion and thus generates an  $\text{H}^+$  gradient across apical membranes of ionocytes. This  $\text{H}^+$  gradient may drive  $\text{Na}^+$  uptake via NHE3. Meanwhile, the excreted  $\text{H}^+$  combines with external  $\text{NH}_3$  to form  $\text{NH}_4^+$  and thus maintains the  $\text{NH}_3$  gradient across the apical membrane (the acid-trapping mechanism). As mentioned in section *Na uptake/acid secretion* above, the driving force of NHE in gills of FW fish has been questioned for several decades. This model may be a solution to this long-debated question.

Evidence of a role for Rh proteins in branchial ammonia excretion is greater in FW fish than in SW fish. In the mangrove killifish acclimated to 15 parts per thousand SW, Rhcg and Rhbg mRNA expressions in the gills and skin were induced by high-ammonia exposure (62). In SW pufferfish exposed to high external ammonia, their branchial Rhcg1, HA, NHE3, NKA, and NKCC1 mRNAs were found to be upregulated, suggesting that ammonia excretion is mainly mediated by ionocytes (119). A recent study suggested that the coupling role of NHE3 and Rhcg1 in apical membranes of ionocytes in SW fish is similar to that in FW medaka (200). In SW-acclimated medaka embryos, unpublished data by SIET probing of the  $\text{H}^+$  gradient at ionocytes showed that the apical surface of cells was alkalinized after ammonia loading (S. T. Liu, L. Tsung, L. Y. Lin, unpublished data). This phenomenon was also found in zebrafish and FW-acclimated medaka, suggesting nonionic  $\text{NH}_3$  excretion by ionocytes (155, 200). However, further studies on SW fish are required to extend our understanding of Rh proteins and ammonia excretion in SW.

#### *Identification and Comparison of Ionocyte Subtypes in Different Species*

As discussed above, NaCl secretion mechanisms in SW fish gills are relatively better understood than the ion uptake mechanism in FW fish. It is generally accepted that SW fish gills have only one type of ionocyte expressing a similar set of ion transporters (refer to section *NaCl secretion* above) among most teleosts (Fig. 1). But then, two distinct types of ionocyte were previously proposed in SW gills (refer to section *Acid-base regulation in SW fish* above); however, this was reported in only one teleost species (18) and needs to be confirmed in more species in the future. In the case of FW teleosts, the transport and regulation of various ions are achieved by respective sets of ion transporters (and/or enzymes) through a variety of pathways in the gills. Accordingly, exploring whether fish gills develop pleomorphic ionocytes (or MR cells) to conduct these different transport functions, as do the mammalian kidneys, has long been an important issue for fish physiologists. In early studies, different subtypes of ionocytes were identified in several species, mainly based on the ultrastructures (by electron microscopic observations) and NKA immunoreactivities, and their functions were accordingly de-

rived from correlated data of cell morphologies, NKA expression/activity, and/or related ion fluxes (54, 64, 66, 67).

Given a variety of cell biological (localization of ion transporters and isolation of cells), molecular physiological (loss-of-function), electrophysiological, and pharmacological approaches, recent studies identified ionocyte subtypes according to criteria with greater physiological significance. Hence, these resources provide powerful platforms for further studies on related ion-transport mechanisms. In tilapia embryonic skin (Fig. 2), four subtypes (types I, II, III, and IV) of ionocytes were classified by multiple labeling for different transporters (53, 68): for type I ionocytes, only basolateral NKA staining was seen; for type II, basolateral NKA and apical NCC staining was evident; for type III, basolateral NKA/NKCC and apical NHE3 staining was seen; and for type IV (i.e., the SW type), basolateral NKA/NKCC and apical CFTR staining was documented (53, 68). Subsequent acclimation experiments with different salinities or ionic compositions further suggested that these ionocytes are associated with their respective functions: type II with NaCl uptake; type III with Na<sup>+</sup> uptake; and type IV with Cl<sup>-</sup>/Na<sup>+</sup> secretion (53, 68, 69). On the other hand, in zebrafish embryonic skin and adult gills (Fig. 2), HR cells, NaR cells, NCC cells, and a group of unidentified cells were identified by double/triple in situ hybridization and immunocytochemistry. These ionocytes were demonstrated to be responsible for the transportation of different ions by a loss-of-function analysis of related ion transporters (or enzymes) that are coexpressed in each type of ionocyte. HR cells showed apical HA/NHE3b/Rhcg1, basolateral AE1b/NKA (ATP1a1a.5), and two CAs (membrane CA15a and cytosolic CA2-like a), which are responsible for Na<sup>+</sup> uptake/acid secretion/NH<sub>4</sub><sup>+</sup> excretion (refer to section *Na uptake/acid secretion* above). NaR cells coexpressed ECaC/PMCA2/NCX1b/NKA (ATP1a1a.1) mRNAs and carried out Ca<sup>2+</sup> uptake (refer to section *Ca<sup>2+</sup> uptake* above). NCC cells coexpressed NCC/NBCe1b/NKA (ATP1a1a.2) mRNAs and absorbed Cl<sup>-</sup> and/or Na<sup>+</sup> (refer to section *Cl<sup>-</sup> uptake/base secretion* above). Surprisingly, the case of rainbow trout differs from those of tilapia and zebrafish (Fig. 2). PNA was used to classify two subtypes of ionocytes, PNA<sup>+</sup> and PNA<sup>-</sup>, from isolated rainbow trout gill cells (45). Subsequent in vitro pharmacological and physiological experiments suggested the distinct ion-transport functions of these two ionocyte subtypes: PNA<sup>-</sup> cells are involved in bafilomycin-sensitive, acid-activated Na<sup>+</sup> uptake (refer to section *Na uptake/acid secretion* above), and PNA<sup>+</sup> cells are involved in Cl<sup>-</sup> uptake/base secretion and Ca<sup>2+</sup> uptake (refer to sections *Cl<sup>-</sup> uptake/base secretion* and *Ca<sup>2+</sup> uptake* above). Most recently, a pharmacological study further indicated the intracellular pH regulation-related NHE activity of both PNA<sup>+</sup> and PNA<sup>-</sup> cells (125). Obviously, isolation approaches might not be efficient enough to identify all (or more) subtypes of ionocytes from isolated trout gills (64, 67). Moreover, colocalization of the related transporters in each subtype of cells largely remains unclear. This is probably one of the reasons why some subsequent studies did not support the proposed transport functions by PNA<sup>-</sup>/PNA<sup>+</sup> cells. Immunocytochemistry and in situ hybridization studies revealed that PNA<sup>-</sup> cells expressed both ECaC mRNA and proteins, while PNA<sup>+</sup> cells expressed NHE2 mRNA, NHE3 mRNA/proteins, and ECaC mRNA (72, 151). Indeed, Shahsavarani et al. (151) further reported four populations of cells in rainbow trout gills, ECaC-

positive, NKA-positive, ECaC/NKA-positive, and ECaC/NKA-negative cells, confirming the broad distribution of ECaC among PNA<sup>-</sup>, PNA<sup>+</sup>, and other types of gill cells.

Identification of ionocyte subtypes is important for comparisons among species and the generalization of a comprehensive working model of fish ion regulation mechanisms, but also to provide a platform for the precise study of the transport pathway and regulation of each ion or each ionocyte subtype. For example, recent studies clearly indicated that acute salinity challenge caused the disappearance of type II (for Cl<sup>-</sup>/Na<sup>+</sup> uptake) and a transition of type III (for Na<sup>+</sup> uptake) to type IV (for Cl<sup>-</sup>/Na<sup>+</sup> secretion) ionocytes in tilapia gills (24). In a hypophysectomy study, NCC expression and the type II cell (expressing NCC) number in tilapia gills were found to have decreased, and these impairments could only be rescued by prolactin replacement therapy (13). In zebrafish, both stannocalcin 1 and calcitonin were found to control the Ca<sup>2+</sup> uptake function through specifically downregulating ECaC expression in NaR cells, but they were not associated with other Ca<sup>2+</sup> transporters (PMCA or NCX) (86, 176). Taken together, the models for tilapia and zebrafish allow us to explore the more detailed and precise mechanisms of ion regulation in the two species, but also knowledge gained from these is helpful to accelerate studies of other species by analyzing the correct targets (e.g., ionocyte types and ion transporters).

#### *Functional Modification and Regulation*

Fish must regulate the transport functions and capacities in gill/skin ionocytes to maintain their internal homeostasis to cope with aquatic environments, which usually fluctuate in temperature, pH, ion levels, and salinity. Upon acute environmental challenge, fish have to modify or enhance the functions of preexisting ionocytes through complicated signaling pathways in the short term, of minutes to hours. For long-term (days to weeks) acclimation to harsh environments, fish have to increase the ionocyte number, transporter expression, and thus the entire functional capacity. Recent studies using molecular, cellular, physiological, and electrophysiological approaches have made some progress in our knowledge related to these issues.

*Regulation of activity, signaling, and expression of transporters.* Alterations in NKA activity/kinetics to match the demands of fish gill epithelial cells are essential for an enzyme to execute its physiological functions. In addition to expressing NKA  $\alpha$ - and  $\beta$ -subunits (refer to section *NaCl secretion* above), assisting FXYD protein is seen to be a crucial regulatory component. FXYD proteins are a family of small single-membrane-spanning proteins that are known to interact with NKA and modulate its kinetic properties (47). Wang et al. (190) first found a pufferfish FXYD (FXYP9) and its interaction with NKA in gills and demonstrated that FXYD expression is salinity dependent. Pufferfish FXYD protein and mRNA levels were reduced, which elevated NKA activity through their interactions in gill ionocytes (190). Meanwhile, Tipsmark (164) identified eight FXYD protein isoforms (i.e., FXYP2, -5, -6, -7, -8, -9, -11, and -12) in various tissues of Atlantic salmon. Expression of FXYD isoforms in gills and kidneys differed during FW-to-SW acclimation, suggesting their physiological significance during salinity acclimation (164). Among these isoforms, FXYD11 was expressed almost

exclusively in gills, and parallel increases in total NKA  $\alpha$ -subunits and FXD11 protein expression were induced upon transfer from FW to SW, providing more support for the role of FXD11 in modulating NKA kinetic properties (170). Other FXD isoforms were also found to be expressed in zebrafish, and only FXD11 mRNA and protein were expressed in a specific type of ionocyte, NaR cells, in adult gills and/or in embryonic skin (147). Diluted FW was found to stimulate zebrafish FXD11 mRNA expression, and an in situ proximity ligation assay demonstrated the close association between NKA and FXD11 (147). In addition, knockdown of zebrafish FXD11 caused functional impairment of NKA and a subsequent decrease in the ion-transporting ability of ionocytes, which might lead to feedback upregulation of NKA expression (147). Taken together, regulation of NKA activity by FXD appears to play some roles in fish gill ion uptake and secretion mechanisms in FW and SW.

Many studies addressed the importance of NKCC1 expression for long-term (in terms of days) salinity acclimation, as described above (refer to section *NaCl secretion* above); however, much remains unknown about the regulation of NKCC1 activity and expression during acute (within hours) salinity acclimation. It was hypothesized that regulation of NKCC1 was under rapid control, being, respectively, turned on and off via phosphorylation and dephosphorylation in response to cell shrinkage (hypertonic challenge) and cell swelling (hypotonic challenge) (56, 109). When SW-acclimated teleosts were exposed to FW, branchial NKCC1 protein amounts remained constant (SW level) for 7 days posttransfer (76, 99, 169). Therefore, upon hypotonic challenge, gill NKCC1 of SW teleosts might be inhibited to become the inactive form by dephosphorylation with Ser/Thr protein phosphatases, including protein phosphatase 1 until 7 days (76, 99, 107). Kang et al. (76) suggested that NKCC1 expressions (inactive form) maintained in FW-acclimated teleosts were involved in the endurance of the hypoosmoregulatory ability. Recently, Flemmer et al. (43) demonstrated that, within 1 h of transfer of killifish to water of a higher or lower salinity, the fractional level of NKCC1 phosphorylation, respectively, increased or decreased. They also reported that phosphorylation in excised killifish gills increased in response to forskolin stimulation of the cAMP-PKA pathway. Potential phosphorylation sites and interaction sites for Sterile 20 protein-related proline alanine-rich kinase (SPAK) and oxidative stress response 1 kinase (ORS1) in the mammalian NKCC were reported in the deduced protein sequence of NKCC1a (76, 99, 107). In addition, elevated SPAK gene levels were recently found in killifish gills (43). Thus gill NKCC1 was thought to be activated by phosphorylation with SPAK and ORS1, leading to the rapid excretion of  $\text{Cl}^-$  when euryhaline fish were transferred to hyperosmotic media.

Regulation of CFTR expression in gills of fish upon acute salinity challenge is not clearly understood yet. Marshall (106) indicated the protein trafficking of CFTR in gill ionocytes. Previous studies showed that killifish CFTR responded to hyperosmotic challenge, and gill CFTR proteins and ion currents were upregulated within 1 h (153). On the other hand, when SW killifish (*Fundulus heteroclitus*) were exposed to FW, the CFTR signal localized to the apical side of gill ionocytes disappeared within 1 day (79). The case of SW seabass transferred directly to FW was similar, in that bran-

chial CFTR mRNA was downregulated within 1 day, followed by decreases in the CFTR protein over 7 days (9). Hence, regulation of CFTR was thought to be transcriptional (9). Using different approaches, Marshall et al. (109) reported that CFTR in the inner opercular epithelia of euryhaline killifish was activated by a mechanism composed of an osmotically mediated, cAMP-independent, and hormone-mediated, cAMP-dependent pathway to secrete  $\text{Cl}^-$ , possibly through tyrosine phosphorylation of CFTR by a focal adhesion kinase. The above data suggest that modulating protein function via differential phosphorylation may contribute to the tolerance of various teleost species to osmotic stresses.

Recently, several functional genomic studies examined the effects of environmental stresses, including salinities and ion levels, on gill transcriptomic and proteomic profiles in several euryhaline species (41, 75, 77, 100, 137). In addition to ion transporter genes, genomewide transcriptomic/proteomic analyses of fish gills provided a valuable diversity of novel or functionally unknown genes that are possible candidates that can be tested. These analyses further broadened our understanding of fish ion transport mechanisms and their functional regulation during acclimation to environmental challenges. Notably, some recent studies emphasized short-term ( $\sim 12$  h) profiles of the gill transcriptome during acute exposure to different salinities, as in the euryhaline goby (*Gillichthys mirabilis*), or  $\text{Ca}^{2+}$  levels, as in the green-spotted puffer (41, 137). Differentially regulated genes were dominantly related to cytoskeletal structure/organization, energy metabolism, proteolysis/molecular degradation, or the cell cycle, and these were proposed to be associated with osmotic stress sensing, and the signaling and regulation of ion transporters, as well as the functional/morphological regulation of ionocytes (41, 137). Further studies, e.g., precise localization (mRNA and protein) in ionocytes or other gill cells and functional analyses of these genes, should be conducted to explore related short-term mechanisms involved in gill-acclimation responses to environmental changes.

*Functional changes, activation, proliferation, and differentiation of ionocytes.* In early studies, the morphology (apical structure and cellular ultrastructure) and NKA immunoreactivity of gill ionocytes (or MR cells) were well compared between SW- and FW-acclimated fish. Accordingly, SW and FW types of ionocytes were proposed to be associated with salt secretion and absorption functions, respectively (40, 54, 66, 108). Triggering the salt secretion function by SW-type ionocytes and simultaneously suppressing the salt absorption function by FW-type cells during acute salinity challenge in a timely and sufficient manner are critical events for euryhaline teleosts. These functional and morphological modifications from FW- to SW-type ionocytes are known to occur on the order of hours. A recent study using SIET provided convincing in vivo data to demonstrate rapid functional changes in ionocytes during acute transfer to different salinities (154). Ionocytes in embryonic skin showed inward (i.e., uptake) and outward (i.e., secretion)  $\text{Na}^+/\text{Cl}^-$  currents from apical openings in medaka acclimated to FW and SW, respectively (154). Sequential probing of the same ionocytes showed that functional changes from inward currents to outward ones were completed in 2  $\sim$  5 h immediately after transfer from FW to SW, and the reverse change during transfer from SW to FW was also accomplished in 5 h (154). A Ussing chamber study of killifish also reported that

the isolated opercular epithelium, which contains abundant ionocytes like the gills, initially showed an increased CFTR  $\text{Cl}^-$  current (i.e.,  $\text{Cl}^-$  secretion function) as early as 1 h immediately after transfer from FW to SW (153) (refer to section *Regulation of activity, signaling, and expression of transporters* above). These rapid functional modifications on the basis of electrophysiological profiles concur with morphological changes in ionocytes, as described below, and reflect the functional plasticity of ionocytes during acute transfer to different salinities.

Determining whether the rapid appearance of SW-type ionocytes originates from stem cells (or undifferentiated cells) or directly from the transformation of the preexisting FW-type cells has been a challenging and important issue for fish physiologists. This issue remained a puzzle until some elegant and precise studies were carried out on tilapia. Following sequential *in vivo* observations by confocal microscopy [fluorescence 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DEASPEI), a marker for mitochondria], 75% of skin ionocytes in tilapia embryos survived for 96 h after transfer from FW to SW (52). This suggests that the acute functional (see the above paragraph on ionocyte electrophysiology) and morphological changes (see below) upon salinity challenge mainly occur in preexisting ionocytes, and they are mostly not associated with the recruitment of new ionocytes. This notion was supported by the appearance of changes in the cell size (52) and apical morphology (from convex/concave to pit types) (24) within 1 day of salinity challenge. The transformation from type III ionocytes (FW type, with NHE3 used as the marker transporter) to type IV (SW type, with NKCC used as the marker transporter) (24) reflects the functional change from salt uptake to salt secretion (154) during acute salinity challenge. These cellular events also occurred in the case of tilapia acclimation to ion-deficient FW. Upon transfer from high- $\text{Cl}^-$  to low- $\text{Cl}^-$  FW, >90% of ionocytes in tilapia embryonic skin survived for 24 h based on the sequential *in vivo* observations of DEASPEI fluorescence (94), and a group of ionocytes [the NCC type later identified by Horng et al. (57)] changed their apical opening (concave to convex or unopened to opened) within 4 ~ 24 h (94, 95). This indicates that stimulation of  $\text{Cl}^-$  uptake function on acute exposure to low- $\text{Cl}^-$  FW was mainly associated with activation of preexisting ionocytes (57, 94, 95). Similarly, type II and III ionocytes in tilapia gills increased their apical opening sizes and transporter expressions (NCC and NHE3, respectively) within 1 day after transfer from FW to deionized FW (24).

It was long documented that, through cell renewal and proliferation, the number of ionocytes increases to enhance the overall ionoregulatory functions after long-term (in terms of days or weeks) acclimation to a changed environment or treatment with exogenous hormones (40, 66, 110). Studies using cell biological and molecular approaches explored the detailed mechanisms behind this. For instance, higher mitotic activity or a higher turnover rate was reported in gill epithelium after transfer from FW to SW in early studies (31, 40, 183). Using flow cytometry on isolated eel gill cells, Wong and Chan (196) found a higher turnover rate of undifferentiated cells in SW fish than in FW ones, supporting a high ionocyte number after SW challenge. However, changes in gill cell turnover rates appear to be species dependent or based on the environment encountered by the fish. In tilapia embryonic skin traced

by sequential *in vivo* observations, ionocytes showed similar turnover rates for 3 days of acclimation in control FW and SW (52), or for 1 ~ 2 days of acclimation in low- $\text{Cl}^-$  and high- $\text{Cl}^-$  FW (94). In zebrafish embryos, acclimation to acidic FW (pH 4) for 4 days stimulated the proliferation of epidermal stem cells [with the transcription factor, p63, used as the marker (60)] without an effect on cell apoptosis (monitored by a terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay), which resulted in increased HR cell numbers and their acid-secretion function (59).

A recent series of studies on zebrafish dissected the molecular pathways for the differentiation and specification of ionocytes. NaR and HR ionocytes were found to differentiate from the same stem cells by differential determinations of two duplicated forkhead transcription factors, FOXI3a and -3b, and another transcription factor, GCM2, which is the specific factor for the terminal differentiation of HR cells (21, 38, 61, 67, 73). Those studies opened up a new window to further explore molecular and cellular mechanisms of the functional regulation of ionocytes and neuroendocrine control. To cope with acidic FW, zebrafish embryos enhance their acid-secretion ability by stimulating acid-secreting functions ( $\text{H}^+$  activity as measured by the SIET) of single HR cells and also by increasing HR cell numbers (59). Increased HR cell numbers originate from stimulation of stem cell proliferation, as well as the terminal differentiation of HR cells by GCM2 (21). On the other hand, to cope with low temperatures (12°C), zebrafish gill ionocytes enhance ionoregulatory functions to compensate for cold-induced ionic imbalances (25). This functional recovery is the result of an extended lifespan (due to delayed apoptosis) and sustained ionocyte functions (due to FOXI3a-mediated stimulation of preexisting progenitor cells into ionocytes) (25). In addition, these regulatory functions in the proliferation of epidermal stem cells and differentiation of ionocytes during acclimation to harsh environments were further demonstrated to be controlled by neuroendocrines, such as isotocin, in zebrafish (26). Moreover, a recent study demonstrated that FOXI3 is involved in the differentiation of ionocytes in medaka (*Oryzias latipes*), a euryhaline species (163). Henceforth, it will be a challenging issue to determine whether these transcription factors are also involved in functional changes and regulation of ionocytes during acclimation to salinity changes.

*Permeability and tight junctions.* Tight junctions regulate paracellular transport and play an important role for osmoregulation by balancing transcellular pathways, the properties of which were identified in various epithelia (40). Tight junctions are located on the apical lateral sides of numerous epithelia, including teleost gills (2). Previous transmission electron microscopic studies of fish adult gills and embryonic skin showed that the ultrastructure of tight junctions between epithelial MR cells and accessory cells changed to being “leaky” after transfer from FW to SW (63, 65). The presence of leaky tight junctions between these two cell types is crucial to providing a paracellular pathway for  $\text{Na}^+$  secretion by fish gill epithelium (149), and thus is associated with higher osmotic permeability in SW gills than in FW ones (71). The claudin transmembrane protein family is believed to be the main structural and functional component of tight junctions (83). Although the ultrastructure and electrophysiology of tight junctions were well examined in teleosts (63, 65, 80, 149, 197), teleost claudins

were only recently identified and shown to be expressed in various tissues of fish (98). In mammals, ~24 claudin members were identified (185). However, in fish, ~57 members were identified because of tandem gene duplication and/or whole-genome duplication events, which were probably induced by the diversity of aquatic environments (98). Moreover, in tiger fugu, gene-duplication events were suggested to produce 17 genes, including genes 27, 28, and 30, which are similar to mammalian claudins 3 and 4 (98).

SW induced expression of claudin isoform 10e in Atlantic salmon gills, while FW induced expression of isoforms 27a and 30 in gills of Atlantic salmon and isoforms 28a and 30 in gills of tilapia (165, 167). SW-induced (10e) and FW-induced (27a and 30) claudins were all stimulated by cortisol, while other osmoregulatory hormones, i.e., growth hormone and prolactin, did not affect these claudin isoforms (166). In addition, the salinity-dependent differential mRNA expression of gill claudin 27 isoforms was found between two pufferfish species, *T. nigroviridis* and *T. biocellatus* (5, 35). On the other hand, transfer of euryhaline flounder, tilapia, and pufferfish from FW to SW decreased the mRNA or protein abundances of claudins 3 and 4, and the opposite reactions were found with SW-to-FW transfer (4, 35, 165, 168). Since the overexpression of claudins 3 and 4 reduced paracellular permeability in mammalian epithelial cells (32, 186), it was assumed that increased abundances of claudins 3 and 4 contributed to the reduction in epithelial permeability in fish gills. Furthermore, cortisol treatment of cultured epithelia of pufferfish gills dose-dependently increased or decreased mRNA abundance of selected claudin isoforms (14). All of these salinity-dependent alterations indicate that claudins might be vital to permeability changes associated with salinity adaptation and possibly the formation of deeper tight junctions in FW gills of euryhaline teleosts.

#### Energy Supply for Ionocytes

The energy supply for the iono/osmoregulatory mechanisms in fish gills is another important issue, since activation and synthesis of the related ion transporters are highly energy consuming [refer to detailed reviews by Boef and Payan (10), Hwang and Lee (66), and Tseng and Hwang (179)]. Effects of different environmental salinities on whole fish oxygen consumption have long been used as a way to estimate the energy required to cope with salinity challenges. However, the whole fish oxygen consumption cannot delineate the partitioning of energy costs for gills only, the most important organ responsible for fish iono/osmoregulatory mechanisms. Efforts extended using isolated gills showed that oxygen consumption of gills constituted <4 and <20%, respectively, of that of the whole fish in cutthroat trout and Atlantic salmon (111, 115). On the other hand, energy requirements for all transport processes could not be estimated using isolated/perfused gill arch preparations (10). Despite the limitations in methodologies, previous studies provided important information about the energy requirements of iono/osmoregulation on salinity changes. Identification and functional analysis of ion transporters can be used to examine specific ion transport functions in each type of ionocyte (refer to section *Identification and Comparison of Ionocyte Subtypes in Different Species* above), and also to look at the energy supply at the level of ionocytes during acclimation to changed environments, as discussed below.

Previous studies reported the utilization of different metabolic substrates as energy supplies during salinity acclimation. In such cases, carbohydrate metabolism appears to play a major role in the energy supply for fish iono/osmoregulation, and the liver is definitely the major source supplying carbohydrate metabolites to iono/osmoregulatory organs [refer to the detailed review by Tseng and Hwang (179)]. Indeed, an early study on tilapia indicated that isolated gill cells oxidized more glucose and lactate than other metabolites (alanine or oleate) (132). Some recent molecular physiological studies on tilapia and zebrafish provided new insights into the carbohydrate metabolite supply and translocation in ionocytes during acclimation to environmental challenges. In tilapia, a novel type of gill cell, glycogen-rich (GR) cells, was identified as the cells that serve as an energy depository and supply emergent energy (66, 179). GR cells, which reside next to ionocytes, coexpress glycogen phosphorylase (GP) (a gill-specific form, tGPGG) and glycogen synthase (GS), which initialize, respectively, glycogenolysis and glycogenesis (20, 178). In vivo and in vitro pharmacological experiments with caffeine (a GP inhibitor) demonstrated the transport of glucose (or other energy metabolites) from GR cells to neighboring ionocytes for operation of ion regulation (66). This spatial relationship in energy translocation between gill GR cells and ionocytes (Fig. 4) is analogous to that between astrocytes and neurons in mammal brains (133). In the rat brain, GP and glycogen mainly exist in astrocytes and astroglial cells, but never in neurons. During energy deprivation, glycogen is degraded to lactate and is

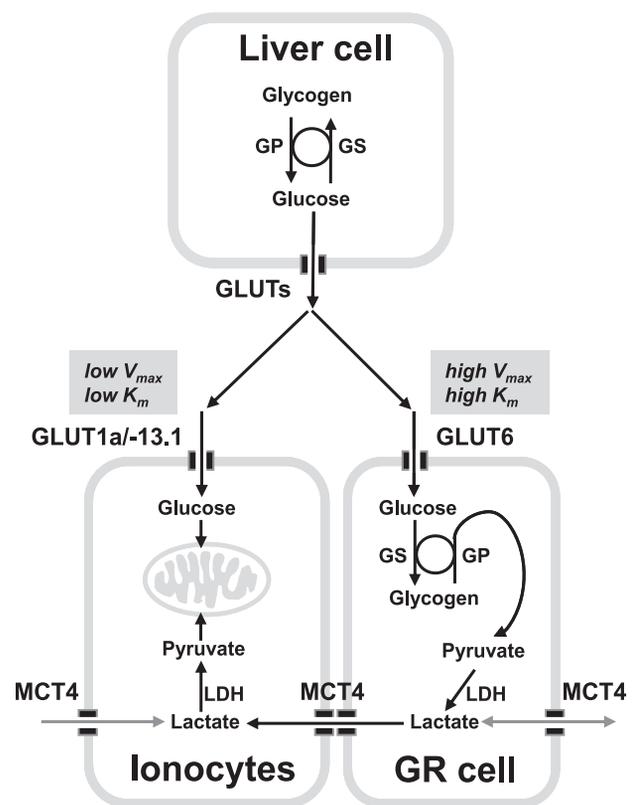


Fig. 4. Model of the carbohydrate energy supply of ionocytes. For details, refer to the text (section *Energy Supply for Ionocytes*). GLUT, glucose transporter; GP, glycogen phosphorylase; GS, glycogen synthase; LDH, lactate dehydrogenase. Dark gray line, unidentified transport pathway.

shuttled from astrocytes to high-energy-requiring neurons (133, 134). Indeed, our preliminary double in situ hybridization/immunocytochemistry results demonstrated the colocalization of a monocarboxylate transporter isoform (MCT4) in both GR cells and ionocytes in zebrafish (Fig. 3, *E–H*; Y. C. Tseng, Z. J. Kao, S. T. Liu, and P. P. Hwang, unpublished data), as well as the localization of lactate dehydrogenase 1/5 (enzymes that convert lactate to pyruvate or the reverse) in both cell types (180). These events suggest the translocation of lactate or pyruvate between these types of cells. By examining the time course changes in NKA activity, glycogen content, and the protein and/or activity of GP/GS in gills and liver, Chang et al. (20) further explored the spatial and temporal partitioning of the energy supply for iono/osmoregulation by gill ionocytes during salinity acclimation. In tilapia, GR cells accumulate glycogen as local carbohydrate reserves in the gills. Glycogenolysis in gill GR cells is initially (1 ~ 3 h after SW challenge) activated to provide prompt energy for neighboring ionocytes to trigger ion secretion mechanisms, and several (6 ~ 12) h later, liver cells begin to degrade their glycogen stores for a subsequent energy supply (20). As Tseng and Hwang (179) proposed, the liver is the central carbohydrate reserve for the entire system, but the role of gills as a local energy supply must be considered for emergency requirements during acute environmental challenge.

The proposed model of energy translocation between gill ionocytes and GR cells (179) was further supported by some recent molecular physiological studies on zebrafish glucose transporters (zGLUTs) (177, 181). Among 18 members of the zebrafish SLC2 family, zGLUT-1a and -13.1 are coexpressed by, respectively, NaR and NCC/HR types of ionocytes, while zGLUT-6 is expressed by GR cells (177). These zGLUTs were demonstrated to transport glucose according to a functional analysis of overexpressed transporters in *Xenopus* oocytes (181). The roles of these transporters in supplying energy to ionocytes were identified by both acclimation experiments and loss-of-function approaches. Acclimation to ion-deficient artificial FW caused parallel stimulation of mRNA expression of the respective ion transporter and zGLUT in each type of ionocyte (177). In addition, knockdown of zGLUTs impaired the ion uptake functions in the respective types of ionocyte (181). Further transport kinetics analyses indicated higher D-glucose affinity in ionocytes expressing zGLUT-1a (3.4 mM) and -13.1 (1.5 mM) than in GR cell's zGLUT-6 (17.5 mM) (181), and these differences appeared to be of physiological significance. zGLUT-1a and -13.1 enable ionocytes to effectively compete for hexose absorption to carry out ionic regulation (a function of ionocytes) under a situation of low blood glucose due to extensive energy consumption (181). On the contrary, in a postprandial situation of high blood glucose, zGLUT-6 (with a much lower affinity and higher  $V_{\max}$ ) allows GR cells to absorb the excess glucose from the blood for energy deposition (a function of GR cells) (181). Similar physiological significance was also found in GLUTs expressed in the mammalian brain. A low  $K_m$  (~1.4 mM) for mammalian GLUT-3 (a major isoform in neurons) has a higher affinity to compete for glucose than GLUT-1 (3~7 mM) and GLUT-2 (17 mM) expressed by astrocytes and oligodendrocytes (184, 202). Taken together, a timely and sufficient supply of energy to ionocytes to carry out ionoregulatory functions is definitely a more important event than storing energy in GR cells. This

strategy is particularly critical when acute environmental change has created the need for functional regulation and emergent energy demands in fish gills (Fig. 4). More fish species should be studied in the future to support this notion.

### Conclusions and Perspectives

Recently, much more works regarding gill iono/osmoregulatory mechanisms have been done in FW than in SW fish, probably because the mechanisms in FW gills are more complicated. In SW teleosts, NaCl secretion and the related ion transporters (CFTR, NKCC, NKA, and inwardly rectifying  $K^+$  channel) have been clearly identified and functionally characterized. Acid-base regulation mechanisms have been mostly studied in marine elasmobranchs, for example, the involvement of NHE/NKA (apical/basolateral) and pendrin/HA in acid and base secretion, respectively. Teleosts studies are important to gain a better understanding of acid-base regulation iono/osmoregulatory mechanisms in SW.

With the availability of advanced cellular/molecular physiological approaches and model animals, research on gill ionic uptake and acid-base regulation mechanisms in FW teleosts is accelerating, and recent progress has boosted our knowledge in this field. Functions of  $Na^+$  uptake/acid secretion/ $NH_4^+$  are linked together and are achieved by well-identified sets of ion transporters and enzymes including NHE3 (and/or NHE2), HA, Rh proteins, and membrane and cytosolic forms of CA, AE1, and NKA. Based on new molecular/electrophysiological evidence, Rhcg has been shown to transport  $NH_3$  in a gaseous form through an acid-trapping mechanism with an acidic boundary layer provided by apical NHE and/or HA. Notably, linking NHE3/Rhcg1 in the newly proposed “ammonium-dependent sodium uptake” model raises the possibility that the  $H^+$  gradient created by Rhcg1 may drive the electroneutral NHE3. To achieve the  $Ca^{2+}$ -uptake function, ECaC and the three basolateral components, NCX, PMCA, and NKA, work together, for which ECaC is the major regulatory transporter during functional regulation. Apical NCC and basolateral NBCe1/CIC, recently identified transporters, are involved in  $Cl^-$  and/or  $Na^+$  uptake functions; however, the functional analysis of NBCe1 remains to be done in the future. On the other hand, pendrin and other SLC26 members are also involved in  $Cl^-$  uptake/base secretion mechanisms in teleost gills, as proposed in elasmobranchs. Further studies, however, are needed to identify and colocalize the relevant transporters that are necessary to achieve these epithelial transport functions. Furthermore, the driving mechanisms for apical NCC and pendrin are yet unresolved. Based on the expression and functional characterization of specific sets of transporters, classification of subtypes of ionocytes is well established in several model species (zebrafish, tilapia, and rainbow trout), and these provide a powerful platform to precisely elucidate detailed mechanisms and functional regulation of specific ion transport pathways. Identification and colocalization of relevant transporters in a specific group of ionocytes deserve more efforts in other species and would be helpful to establish a more general and comprehensive model of fish gill ionoregulatory mechanisms.

Upon acute environmental challenge, fish have to modify or enhance the functions of gill ionocytes, and the molecular/cellular mechanisms behind these are recently being dissected.

FXD, a regulatory component of NKA activity, appears to play significant roles in both ion secretion and uptake mechanisms in teleost gills. Initial studies on signaling pathways in fish gills during acute salinity challenges focused on NKCC and CFTR, and it is suggested that further studies extend those efforts to possible candidate molecules that were proposed by recent genome-wide functional genomics analyses. Functional (e.g., from NaCl infuses to outfluxes) and morphological (from one subtype to another subtype of ionocytes) transformation or modifications of preexisting ionocytes occur in a matter of hours (as rapidly as 1 h) immediately after an acute environmental challenge, and these molecular and cellular events are supported by a series of recent *in vivo* electrophysiological and cell biological experiments. For long-term (on the order of days or weeks) acclimation, enhancement of the entire gill capacity for ion transport functions is achieved by the augmented expression of transporters in specific groups of ionocytes. This could be the outcome of stimulation of transporters in preexisting ionocytes, and/or it may originate from increased numbers of ionocytes due to recruitment of new cells. Recent studies on the molecular pathways of ionocyte development further extend our understanding of these cellular events. For instance, one strategy for the functional regulation of ionocytes during acclimation to environmental changes is to stimulate the proliferation of epidermal stem cells and/or FOXI3-mediated differentiation of ionocytes. Accumulating molecular physiological data on specific claudin isoforms further reinforced an earlier notion that permeability regulation is a vital component in ion transport mechanisms of fish gills during environmental salinity challenge.

A sufficient and timely energy supply is a prerequisite for the operation of iono/osmoregulatory mechanisms in fish gills to cope with a changing environment. Carbohydrate metabolite translocation has been found between fish gill ionocytes and neighboring GR cells. GR cells deposit glycogen as an energy source and can provide a local energy supply to ionocytes. From spatial and temporal points of view, the liver is the central carbohydrate reserve for the entire system, while the gills (GR cells) play an important role in the local energy supply for emergency requirements during acute environmental challenge. This model requires more support from future data in other species.

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#### DISCLOSURES

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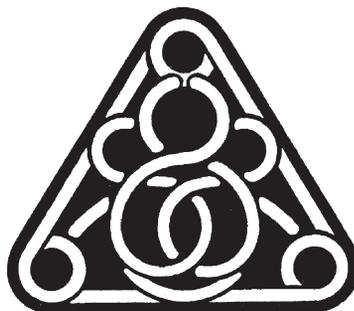
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# Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms

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