

Review

Ion uptake and acid secretion in zebrafish (*Danio rerio*)

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Accepted 21 January 2009

Summary

Transepithelial transport is one of the major processes involved in the mechanism of homeostasis of body fluids in vertebrates including fish. The current models of ion regulation in fish gill ionocytes have been proposed mainly based on studies in traditional model species like salmon, trout, tilapia, eel and killifish, but the mechanisms are still being debated due to the lack of convincing molecular physiological evidence. Taking advantage of plentiful genetic databases for zebrafish, we studied the molecular/cellular mechanisms of ion regulation in fish skin/gills. In our recently proposed model, there are at least three subtypes of ionocytes in zebrafish skin/gills: Na⁺-K⁺-ATPase-rich (NaR), Na⁺-Cl⁻ cotransporter (NCC) and H⁺-ATPase-rich (HR) cells. Specific isoforms of transporters and enzymes have been identified as being expressed by these ionocytes: zECaC, zPMCA2 and zNCX1b by NaR cells; zNCC gill form by NCC cells; and zH⁺-ATPase, zNHE3b, zCA2-like a and zCA15a by HR cells. Serial molecular physiological experiments demonstrated the distinct roles of these ionocytes in the transport of various ions: HR, NaR and NCC cells are respectively responsible for acid secretion/Na⁺ uptake, Ca²⁺ uptake and Cl⁻ uptake. The expression, regulation and function of transporters in HR and NaR cells are much better understood than those in NCC cells. The basolateral transport pathways in HR and NCC cells are still unclear, and the driving forces for the operations of apical NHE and NCC are another unresolved issue. Studies on zebrafish skin/gill ionocytes are providing new insights into fish ion-regulatory mechanisms, but the zebrafish model cannot simply be applied to other species because of species differences and a lack of sufficient molecular physiological evidence in other species.

Key words: mitochondria-rich cells, embryo, ion regulation, acid-base regulation.

Introduction

Homeostasis of body fluids is vital for vertebrates including fish. Compared with terrestrial vertebrates, fish face a more challenging task to achieve internal homeostasis because aquatic environments present ionic and/or osmotic gradients which are mostly hostile to body fluids of fish. How fish operate and regulate their ionic and osmotic regulation mechanisms to achieve internal homeostasis has long been of scientific curiosity as well as being one of the most important issues in fish physiology. Transepithelial ion-transport mechanisms in fish gills have been studied most extensively, probably because gills, a unique organ in aquatic animals, show dramatic changes in their function in response to fluctuations in the aquatic environment. In gills, mitochondria-rich (MR) cells (formerly called chloride cells), which are specialized ionocytes, are the major cell type responsible for transepithelial ion-transport functions. Based on numerous studies with conventional model species (e.g. salmon, trout, eel, tilapia, killifish, etc.), many models for the ion-transport mechanisms in gill MR cells have been proposed. However, there is still much debate about these models probably because of differences in species, acclimation situations, and/or experimental design; the lack of specific probes or antibodies to identify related transporters; and deficiencies of direct evidence with molecular physiological approaches. Genomic sequencing has been explored in human and many model species including zebrafish. Compared with traditional model species of fish physiology, zebrafish have several advantages including plenty of genetic databases and mutants, as well as the applicability of many molecular/cellular physiological approaches, and these

advantages are being augmented because of continuous updating of genetic information and the development of new methodologies. With these advantages, recent studies have explored ion-regulatory mechanisms in the skin and gills of zebrafish, and a platform for molecular physiological approaches to understand fish ion regulation and its controlling pathways has accordingly been established (Fig. 1). The present review focuses on introducing progress in our understanding of ion uptake and acid base regulation in different ionocyte types in zebrafish and attempts to clarify some issues under debate by comparing previous studies on traditional model species.

H⁺-ATPase rich (HR) cells

In vivo evidence for Na⁺-uptake/acid-secretion functions

In zebrafish skin and gills, HR cells have been demonstrated to be responsible for acid-secretion and Na⁺-uptake mechanisms (Lin et al., 2006; Esaki et al., 2007; Horng et al., 2007; Hwang and Lee, 2007). So far, at least two pathways have been proposed for the apical transport of Na⁺ in fish gill cells (Hirose et al., 2003; Perry et al., 2003; Evans et al., 2005): (1) an apical V-type H⁺-ATPase electrically linked to Na⁺ absorption *via* the epithelial Na⁺ channel (ENaC) and (2) an electroneutral exchange of Na⁺ and H⁺ *via* an apical Na⁺/H⁺ exchanger (NHE). As the uptake of Na⁺ *via* passive exchange with H⁺ has been questioned on thermodynamic grounds (Kirschner, 1983; Avella and Bornancin, 1989), most of the latter studies favoured the major role of H⁺-ATPase in acid-secretion and Na⁺-uptake mechanisms. Accordingly, immunocytochemistry (ICC) with heterologous or homologous antibodies and *in situ*

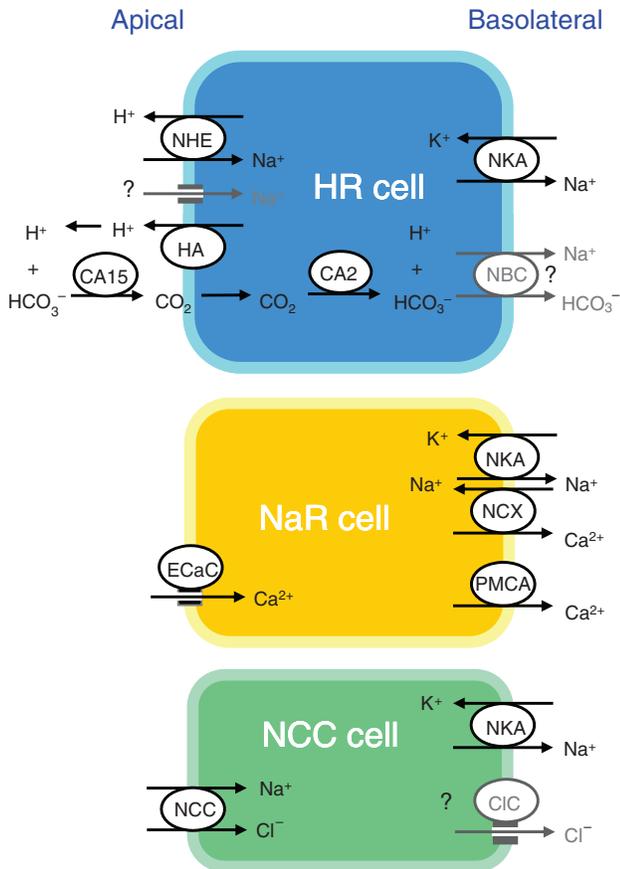


Fig. 1. Model of the ion-regulatory mechanism in zebrafish skin/gill ionocytes. Three types of ionocytes, H⁺-ATPase-rich (HR), Na⁺-K⁺-ATPase-rich (NaR) and Na⁺-Cl⁻ cotransporter (NCC) cells, express different sets of ion transporters and/or enzymes, and are respectively responsible for acid secretion/Na⁺ uptake, Ca²⁺ uptake and Cl⁻ uptake. A question mark indicates an unidentified pathway. CA2, carbonic anhydrase 2-like a; CA15, carbonic anhydrase 15a; CIC, Cl⁻ channel; ECaC, epithelial Ca²⁺ channel; HA, H⁺-ATPase; NBC, Na⁺-HCO₃⁻ cotransporter; NCC, Na⁺-Cl⁻ cotransporter; NCX, Na⁺/Ca²⁺ exchanger 1b; NHE, Na⁺/H⁺ exchanger 3b; NKA, Na⁺-K⁺-ATPase; PMCA, plasma membrane Ca²⁺-ATPase 2.

hybridization (ISH) with RNA probes were used to identify the existence of V-type H⁺-ATPase in fish gills. The distribution of the enzyme in ionocytes and/or pavement cells has been debated, and this has been ascribed to antibody specificities, and/or differences in species, populations or acclimation conditions [see detailed discussion by Evans and colleagues (Evans et al., 2005)]. Under such controversy, we raised the question of whether there is any *in vivo* evidence to demonstrate the acid-secretion function in cells expressing V-type H⁺-ATPase. An *in vivo* approach, the scanning ion-selective electrode technique (SIET), was developed to enable non-invasive measurement of ion fluxes from single cells and epithelial structures (Smith et al., 1999; Smith and Trimarchi, 2001), and it was initially used to detect Cl⁻ excretion from MR cells in isolated opercular epithelium of seawater (SW) fish in a landmark study on fish MR cells (Foskett and Scheffey, 1982). After SIET measurements, the intact fish can be directly subjected to subsequent analysis to identify cells measured by SIET. In zebrafish embryonic skin, a group of cells was demonstrated to secrete substantial bafilomycin-dependent H⁺ currents compared

with other cells by scanning across the apical openings of cells with the SIET probe, and this group of cells was identified as HR cells based on the specific localizations of V-type H⁺-ATPase mRNA and protein (Lin et al., 2006) (Fig. 2A,B). This was the first *in vivo* evidence for the acid-secretion function of an ionocyte, HR cells, in fish. Similar acid-secreting ionocytes have been reported in mammalian kidney, epididymis and vas deferens, amphibian urinary bladder and skin, and insect midgut, etc. (Brown and Breton, 1996). Subsequent studies explored the functions of HR cells in ion-regulatory mechanisms in zebrafish. Translational knockdown of H⁺-ATPase in HR cells with specific A subunit (zATP6V1a) morpholinos was found to decrease the surface H⁺ concentration in skin and whole-body Na⁺ content in morphants, and to impair the survival of morphants in an acidic environment (Hornig et al., 2007), indicating the role of HR cells in zebrafish Na⁺-uptake/acid-secretion mechanisms. At the same time, another molecular physiological study by Esaki and colleagues (Esaki et al., 2007) also supported this notion: Sodium Green, a fluorescence probe for Na⁺, was used to signal Na⁺ uptake to demonstrate that HR cells in zebrafish embryonic skin are the exact site for absorbing Na⁺ based on the accumulation of fluorescence signals only by HR cells.

Apical Na⁺-uptake pathway

The next question concerns the apical Na⁺-uptake pathways in zebrafish HR cells. According to the current models described above, ENaC or NHE was the first candidate to be considered. In a study by Boisen and colleagues (Boisen et al., 2003), 10⁻⁵ to 10⁻⁴ mol l⁻¹ of amiloride (a Na⁺-uptake inhibitor) did not affect the Na⁺ uptake in zebrafish acclimated to soft fresh water (FW) ([Na⁺]=0.035 mmol l⁻¹, [Ca²⁺]=0.004 mmol l⁻¹, pH 6.0), while 5 × 10⁻⁵ mol l⁻¹ EIPA (an NHE-specific inhibitor) enhanced its uptake; thus it was concluded that the pathway for Na⁺ uptake may be distinct from ENaC. In a study by Esaki and colleagues (Esaki et al., 2007), Na⁺ accumulation (monitored by Sodium Green) in HR cells in the skin of zebrafish embryo was inhibited by both 10⁻⁴ mol l⁻¹ amiloride and 10⁻⁵ mol l⁻¹ EIPA, but not by a lower concentration (10⁻⁵ mol l⁻¹) of amiloride, and NHE was therefore suggested to be the major player in the Na⁺-uptake pathway. Subsequently, Yan and colleagues (Yan et al., 2007) provided convincing molecular physiological evidence to support the argument proposed by Boisen and colleagues (Boisen et al., 2003) and Esaki and coworkers (Esaki et al., 2007). In the zebrafish SL9 family, eight members, zNHE1, -2, -3a, -3b, -5, -6, -7 and -8, were identified, and all of them, except zNHE3a, are expressed in gills. However, triple ISH and ICC experiments demonstrated that only zNHE3b mRNA is specifically and predominantly expressed in HR cells (Yan et al., 2007). Acclimation to a low-Na⁺ artificial FW that stimulated fish Na⁺ uptake (Chang et al., 2001) also caused upregulation of zNHE3b mRNA expression in zebrafish gills (Yan et al., 2007), indicating the major role of NHE3b in the apical Na⁺-uptake pathway of zebrafish HR cells.

So far, no orthologues of the mammalian ENaC have been found in databases of the genomes of zebrafish, fugu and other fish species. However, several studies on other species including rainbow trout favoured the operation of ENaC-like channels in the apical Na⁺-uptake pathway. Goss and colleagues (Reid et al., 2003; Parks et al., 2007) used peanut lectin agglutinin (PNA) to isolate two types of ionocytes, PNA⁻ and PNA⁺ cells, from rainbow trout (*Oncorhynchus mykiss*) gills and demonstrated phenamil-sensitive ²²Na⁺ uptake and intracellular acidification in the isolated PNA⁻ MR cells, providing *in vitro* pharmacological and physiological

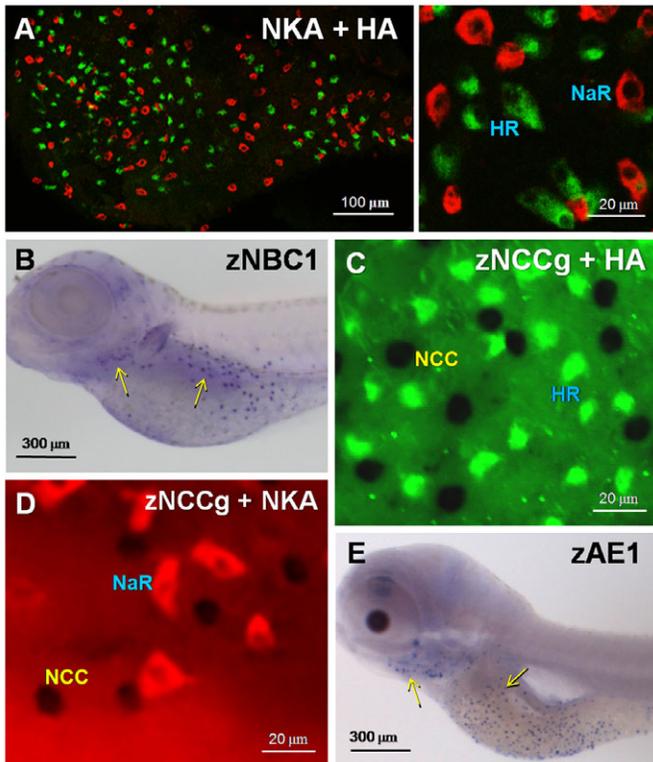


Fig. 2. Double immunocytochemistry and/or *in situ* hybridization of ionocytes in zebrafish embryos at 5 days post-fertilization. (A) NaR cells (red) were labelled with NKA while HR cells (green) were labelled with H⁺-ATPase (HA). Labelled cells are shown on the right at higher magnification. (B) zNBC1 mRNA was expressed in a specific group of ionocytes (arrows). (C,D) NCC cells (black) labelled with zNCCg mRNA were different from HR cells (green) and NaR cells (red). (E) zAE1 mRNA was expressed in a specific group of ionocytes (arrows). Antibodies for immunocytochemistry: an anti-avian NKA α -subunit monoclonal antibody and an anti-killifish HA subunit polyclonal antibody. RNA probes for *in situ* hybridization: zNCCg, nt639–2162 (EF591989); zNBC1, nt1363–3217 (EF634453); zAE1, nt1363–3217 (FJ211592).

evidence for the involvement of ENaC-like channels in the Na⁺-uptake mechanism of fish gills. In a recent review, Parks and colleagues (Parks et al., 2008) emphasized the thermodynamic constraints that prevent electroneutral apical NHE from functioning in most FW environments, thus favouring the applicability of ENaC-like channels to fish Na⁺-uptake mechanisms. Interestingly, Perry and colleagues (Ivanis et al., 2008) recently used double ISH and/or ICC to colocalize the mRNA and protein of both NHE2 and -3 in PNA⁺ MR cells in gill sections of rainbow trout. This evidence appears to be against the argument of Goss's group, as described above, of the unfavourable operation of NHE in isolated trout gill cells (Parks et al., 2007; Parks et al., 2008). These differences between zebrafish and rainbow trout indicate that apical Na⁺-uptake mechanisms may be species specific. However, several urgent issues remain to be resolved to comprehensively clarify these diverse pathways; convincing molecular evidence for the existence of ENaC or equivalent channels and the mechanism to drive NHE's electroneutral operation. In mammals, amiloride-sensitive cation channels (ASICs) are members of the same gene family of ENaC, but ISH data indicated that none of the six zebrafish ASIC paralogues was specifically expressed in zebrafish gills (Paukert et al., 2004). On the other hand, the NHE family and another Na⁺/H⁺

antiporter (NHA) family belong to the same monovalent cation proton antiporter (CPA) superfamily (Brett et al., 2005). Bacterial NHA was proven to show electrogenic stoichiometry with 2 H⁺ for every Na⁺ (Taglicht et al., 1993). In zebrafish genetic databases, we found an orthologue of bacterial NHA. It will be challenging and interesting to study whether zebrafish NHA is also electrogenic so that it can transport Na⁺ and H⁺ in situations that might not favour the operation of zebrafish NHE3 (S. F. Perry, personal communication).

Membrane-associated and cytosolic carbonic anhydrases
Zebrafish HR cells specifically express apical H⁺-ATPase and NHE as do mammalian renal proximal tubular cells (Yan et al., 2007), which are responsible for about 70% of sodium and 80% of bicarbonate reabsorption in mammalian nephrons (Wagner et al., 2004). As HR cells were demonstrated to have similar functions of acid–base regulation and Na⁺ uptake, they should also express carbonic anhydrases (CAs) and the basolateral H⁺-HCO₃⁻ cotransporter (NBC) to fulfill the transepithelial transport mechanisms, as do proximal tubular cells. Recently, Lin and colleagues (Lin et al., 2008) identified 20 CA isoforms and cloned 10 of them. Triple ISH and ICC results showed that only two isoforms, zCA2-like a and zCA15a, were specifically expressed in HR cells but not in other cells in zebrafish skin/gills. Subsequent zCA2-like a or zCA15a knockdown experiments provided molecular physiological evidence to support the roles of the two CA isoforms in the acid–base regulation and Na⁺-uptake functions of zebrafish HR cells. Morphants of zCA15a had increased H⁺ activity at the apical surface of HR cells at 24h post-fertilization, while those of zCA2-like a showed no change. Later, at 96h post-fertilization, both zCA15a and zCA2-like a showed decreased H⁺ activity and increased Na⁺ uptake, with concomitant upregulation of zNHE3b and downregulation of zATP6V1a (H⁺-ATPase A-subunit) expression (Lin et al., 2008), demonstrating the roles of the 2 CA isoforms in the functions of HR cells. A study by Hirose's group (Esaki et al., 2007) also supported this notion; zCA2-like mRNA was colocalized in Sodium Green-accumulating HR cells, and 10 $\mu\text{mol l}^{-1}$ ethoxzolamide (a CA inhibitor) was found to abolish 65% of the Sodium Green accumulation in zebrafish HR cells. On the other hand, acclimation to both acidic and low-Na⁺ FW caused upregulation of zCA15a expression but did not change the zCA2-like a mRNA level in zebrafish gills (Lin et al., 2008). Interestingly, knockdown of zCA2-like a caused upregulation of zCA15a expression, while knockdown of zCA15a did not affect the expression of zCA2-like a in zebrafish morphants. It is probable that the intact zCA2-like a is sufficient to overcome the physiological defects caused by zCA15a knockdown, and it may provide sufficient CA activity to fulfill the physiological needs in zebrafish coping with different environments. In mammalian kidneys, CA2 accounts for 95% of CA activity and shows the highest catalytic rate, compared with other membrane-associated CA paralogues (Purkerson and Schwartz, 2007). Taken together, zebrafish HR cells appear to be similar to mammal renal proximal tubular cells (Purkerson and Schwartz, 2007), in which apical CA4 (an orthologue of zCA15a) and cytosolic CA2 (an orthologue of zCA2-like a) are involved in driving NHE3. In structural terms, zCA15a has a glycosylphosphatidylinositol lipid anchor, through which the enzyme may be tethered to the outer leaflet of the plasma membrane, as are trout and human CA4 in apical and basolateral membranes of kidney cells (Georgalis et al., 2006; Purkerson and Schwartz, 2007). Impairing the function of zCA15a by injecting specific morpholinos caused a direct decline in the H⁺

concentration at the apical surface of HR cells within 24h, supporting the apical localization of the enzyme. This, however, needs to be verified using a specific antibody in the future. On the other hand, it is interesting and important to study whether there are other CA isoforms, like CA12 and CA14, which are also expressed and function in zebrafish HR cells as in mammalian kidney cells (Purkerson and Schwartz, 2007).

Basolateral transport pathways

NBC1 has been immunocytochemically identified in gill ionocytes of Osorezan dace (*Tribolodon hakonensis*) (Hirata et al., 2003) and rainbow trout (Parks et al., 2007). In a study on isolated rainbow trout gill PNA⁻ cells (Parks et al., 2007), electrogenic NBC activity was supported by DIDS (an NBC inhibitor)-sensitive, Na⁺-induced membrane potential depolarization as observed *via* imaging of the voltage-sensitive dye bis-oxonol. The identification and functional analysis of NBC in zebrafish HR cells remain to be done. Our preliminary experiments showed that an NBC1 isoform was expressed in a specific group of cells in zebrafish skin/gills cells (Fig. 2B), and further experiments are needed to identify the cell type that expresses NBC1. In mammalian proximal tubular cells, Na⁺-K⁺-ATPase (NKA) pumps cytosolic Na⁺ across the basolateral membrane and also provides an intracellular negative potential to drive the electrogenic Na⁺/HCO₃⁻ transport of basolateral kNBC1 (Purkerson and Schwartz, 2007). On the other hand, Esaki and colleagues (Esaki et al., 2007) reported that HR cells express only a slightly lower level of NKA than Na⁺-K⁺-ATPase-rich (NaR) cells, implying a role of NKA in providing intracellular low-Na⁺ and negative gradients to drive the basolateral transport pathways in HR cells.

Regulation of NHE and H⁺-ATPase

Interestingly, environmental situations appear to cause differential expression and function of the related transporters and enzymes in zebrafish HR cells. Acclimation to low-Na⁺ FW caused upregulation of zNHE3b and downregulation of H⁺-ATPase, while an acidic environment induced reverse responses, downregulation of zNHE3b and upregulation of H⁺-ATPase, in zebrafish HR cells (Yan et al., 2007). Knockdown of zCA2-like a or zCA14b also caused stimulation of zNHE3b and suppression of H⁺-ATPase (Lin et al., 2008). Taking all these findings together, a model for Na⁺-uptake and acid-base regulation in zebrafish HR cells was proposed (Yan et al., 2007): in low-Na⁺ environments, apical H⁺-ATPase is downregulated to maintain an intracellular H⁺ gradient to facilitate Na⁺ uptake *via* apical zNHE3b, which is the dominant player in regulating the internal Na⁺ balance. In acidic environments, however, H⁺-ATPase, the dominant player, is upregulated to enhance acid secretion to maintain the internal acid-base balance, and zNHE3b is greatly downregulated because the high ambient H⁺ does not favour its operation. This reflects partitioning of the apical zNHE3b and H⁺-ATPase in HR cell functions to meet different physiological requirements in various harsh environments. However, it is notable that the situation may be species specific. In rainbow trout gills, NHE2 and H⁺-ATPase are respectively expressed in PNA⁺ and PNA⁻ cells (Ivanis et al., 2008), and hypercapnia treatment was found to upregulate the expression of the two transporters in the respective cells (Galvez et al., 2002; Ivanis et al., 2008).

NaR cells

NaR cells in zebrafish embryonic skin/gills were first identified by Pan and coworkers (Pan et al., 2005) based on their strong and

specific immunoreactions with a heterologous antibody (α 5) against 1020 amino acids of the avian α subunit core polypeptide (Developmental Studies Hybridoma Bank of Iowa University; Fig. 2A). The function and expression patterns of transporters in NaR cells appear to be more conclusive compared with those in HR cells (as described above) and Na⁺-Cl⁻-cotransporter (NCC) cells (see below). According to the current model in mammals, active transcellular Ca²⁺ transport is carried out through the operation of apical epithelial Ca²⁺ channels (ECaC, TRPV5 and/or TRPV6), and the basolateral plasma membrane Ca²⁺-ATPase (PMCA) and Na⁺/Ca²⁺ exchanger (NCX) (Hoenderop et al., 2005). A model of the capability of physiological regulation has been similarly proposed in fish gills (Flik et al., 1995). However, it was not until the study by Pan and colleagues (Pan et al., 2005) that molecular evidence for the existence of an orthologue of mammalian ECaC in fish skin/gill ionocytes was provided. Using similar approaches to those used in the studies on zebrafish NHE and CA (Yan et al., 2007; Lin et al., 2008), one isoform of ECaC, six isoforms of PMCAs (zPMCA1a, zPMCA1b, zPMCA2, zPMCA3a, zPMCA3b, and zPMCA4) and seven isoforms of NCX (zNCX1a, zNCX1b, zNCX2a, zNCX2b, zNCX3, zNCX4a and zNCX4b) were identified in zebrafish, and triple fluorescence ISH and ICC were conducted to demonstrate the colocalization of zECaC, zPMCA2 and zNCX1b mRNAs in a portion of skin/gill NaR cells (Pan et al., 2005; Liao et al., 2007). Acclimation to a low-Ca²⁺ environment caused stimulation of zebrafish Ca²⁺-uptake function and a concomitant upregulation in skin/gill zECaC expression, but no effect on the expression of either zPMCAs or zNCXs (Pan et al., 2005; Liao et al., 2007), supporting the current Ca²⁺-uptake model, in which ECaC may act as a major regulatory target for this mechanism during environmental challenge. In another study by Craig and colleagues (Craig et al., 2007), both ECaC mRNA and ECaC protein levels in gills were higher in zebrafish acclimated to soft FW (0.051 mmol l⁻¹ Ca²⁺) than those in control FW (0.946 mmol l⁻¹ Ca²⁺). This molecular physiological evidence also agrees with previous physiological studies by Flik and colleagues. Stanniocalcin, a hypocalcaemic hormone, showed no significant effect on basolateral Ca²⁺ transport in North American eel (*Anguilla rostrata*) gills (Verboost et al., 1993b), but quickly reduced the apical membrane Ca²⁺ uptake in tilapia (*Oreochromis mossambicus*) gills (Verboost et al., 1993a). All these suggest that ECaC is the rate-limiting step and the gatekeeper channel for active Ca²⁺ transport in fish as in mammals (Vennekens et al., 2000). Acclimation to low-Ca²⁺ FW caused upregulation of zECaC but not zPMCA2 or zNCX1b, implying that the steady-state expression of zPMCA2 and zNCX1b may fulfill the requirement for the transepithelial transport machinery under all situations (Liao et al., 2007). This further supports a previous enzyme kinetic study, in which the basolateral Ca²⁺ extrusion mechanisms by PMCA and NCX were found to be far below their maximum capacity in rainbow trout (*Oncorhynchus mykiss*) gills (Flik et al., 1997). Moreover, basolateral localization of NKA in zebrafish NaR cells demonstrated the role of the pump in creating a Na⁺ gradient to drive the exchange by NCX1b.

The expression and function of Ca²⁺ transporters in zebrafish skin/gill ionocytes are supported by studies of other species. In tilapia (*O. mossambicus*), mRNAs of ECaC, PMCA2 and NCX1b were all co-localized in MR cells (with NKA as the marker), and ECaC expression was also stimulated by a low-Ca²⁺ environment (B. K. Liao, A. N. Deng and P.-P.H., unpublished data). Similarly, in rainbow trout, ECaC mRNA and protein were localized in gill cells (Shahsavariani et al., 2006) and were upregulated by soft FW

(20–30 mmol l⁻¹ Ca²⁺) or hypercapnia (Shahsavarani and Perry, 2006). However, localization of ECaC in gill cell populations appears to be much more complicated in rainbow trout than in zebrafish and tilapia. The mRNA and protein of ECaC were localized in PNA⁺ and PNA⁻ MR cell populations as well as pavement cells (Shahsavarani et al., 2006). On the other hand, the Ca⁺-uptake capacity in isolated rainbow trout gill PNA⁺ cells was about 3-fold higher than that in PNA⁻ cells, indicating the major role of PNA⁺ cells in the trout gill Ca⁺-uptake mechanism (Galvez et al., 2006), while ECaC mRNA levels in PNA⁻ cells were lower, although non-significantly, than in PNA⁺ cells (Shahsavarani et al., 2006). Taken together, it may be that only PNA⁺ cells express ECaC and basolateral transporters (PMCA and NCX) and thus can achieve the entire transepithelial Ca⁺ pathway, although no data for the existence PMCA and NCX in trout gill cells are available so far. Indeed, it was also found that not all ECaC-expressing cells co-expressed both zPMCA2 and zNCX1b in zebrafish gill NaR cell populations (Liao et al., 2007), which may be analogous to trout PNA⁺ cells (see below). Fish gill cells that express only one or two (never all) of the three major Ca²⁺ transporters may be in the process of terminal differentiation as Hsiao and coworkers (Hsiao et al., 2007) reported in zebrafish embryonic skin ionocytes. Alternatively, the Ca²⁺ transporter(s) expressed in these cells may be involved in intracellular Ca²⁺ homeostasis or other cellular events (Prasad et al., 2007; Reppel et al., 2007), and this needs to be clarified in further investigations. Whether NaR cells are responsible for ion-transport functions other than Ca²⁺ uptake is another issue that should be considered.

NCC cells Apical NCC

Renfro (Renfro, 1975) found that the flounder's urinary bladder transported Na⁺/Cl⁻ *via* an electroneutral pathway, for the first time providing evidence for the existence of an NCC. The NCC, a thiazide-sensitive membrane protein, is specifically expressed in the apical membranes of mammalian distal convoluted tubules (DCTs), which are responsible for the reabsorption of 5% to 10% of the total filtered Na⁺/Cl⁻ reabsorbed by the kidneys (Reilly and Ellison, 2000). Recent studies have opened a new window for elucidating the role of NCC orthologues in Na⁺/Cl⁻ uptake mechanisms in fish skin/gills. In zebrafish, Hwang and colleagues first reported that an NCC isoform (zNCCg) had been cloned and localized in a group of cells in embryonic skin/gills (Hwang and Lee, 2007). Double ISH and ICC experiments demonstrated specific expression of zNCCg mRNA in a novel type of ionocyte, NCC cells, which differ from the previously reported HR and NaR cells (Fig. 2C,D), and loss-of-function experiments with zNCCg morpholinos revealed significant decreases in both Cl⁻ influx and Cl⁻ content in the morphants (Wang et al., 2009). These results indicate the function of NCC cells, in which zNCCg may be the major player in the apical Cl⁻ uptake pathway, although ICC localization of the zNCCg protein in zebrafish NCC cells remains to be confirmed. Recent studies on tilapia support this notion. Hiroi and coworkers cloned an orthologue of zNCCg from tilapia and used a homologous antibody to specifically localize the NCC isoform in the apical membrane of type-II MR cells but not in other MR cell types (Hiroi et al., 2008; Inokuchi et al., 2008). Acclimation to FW or deionized FW caused an increase in the density of NCC-expressing type-II MR cells and a concomitant upregulation of NCC mRNA in tilapia adult gills and embryonic yolk-sac membranes, compared with the SW controls (Hiroi et al., 2008; Inokuchi et al., 2008). Hiroi and colleagues' studies could

not distinguish between the uptake of Na⁺, Cl⁻ or both ions as the role of NCC in tilapia. However, zebrafish NCC cells are unlike to be involved in Na⁺-uptake mechanisms. Esaki and coworkers (Esaki et al., 2007) provided convincing *in vivo* evidence of the Na⁺-uptake function (monitored with Sodium Green) in zebrafish embryonic HR cells only, and serial molecular physiological studies also demonstrated that HR cells are the major cell type responsible for Na⁺ uptake (Hornig et al., 2007; Yan et al., 2007; Lin et al., 2008) as discussed above.

The next question concerns how NCC cells accomplish the overall transepithelial Cl⁻ uptake pathway. Na⁺ concentrations ranging from 35 to 77 mmol l⁻¹ in the lumen of mammalian DCTs (Good et al., 1984) and low intracellular Na⁺ resulting from basolateral NKA provide a sufficient Na⁺ gradient to drive the operation of the apical NCC in DCT cells (Reilly and Ellison, 2000). In contrast, in ambient FW (local tap water in Taipei), zebrafish normally have much lower Na⁺/Cl⁻ levels, creating ion gradients that may not be favourable for the operation of NCC in apical membranes of NCC cells in zebrafish. Hiroi and colleagues (Hiroi et al., 2008) proposed that basolateral NKA may maintain a low intracellular Na⁺, at least in the apical region, thus providing a gradient to drive the operation of the NCC in tilapia MR cells. Another possibility is that zebrafish and tilapia NCCs may differ from the mammalian orthologue in transport kinetics or stoichiometry. All these issues remain to be resolved.

Basolateral Cl⁻ transport

Pathways for the basolateral Cl⁻ exit from zebrafish NCC cells should also be taken into consideration with the overall transcellular Cl⁻ transport machinery. The final step, basolateral Cl⁻ exit, in human renal Cl⁻ reabsorption is mainly achieved *via* Cl⁻ channels (ClCs) composed of the pore-forming unit ClC-Kb, and the β-subunit barttin (Lang et al., 2005). From zebrafish genetic databases, nine orthologues of mammalian ClCs were identified. Very few studies have investigated ClCs in other fish species. In tilapia, ClC3 and -5 were cloned and found to be expressed in various organs including gills, but they were suggested to function as intracellular Cl⁻ channels based on an *in vitro* functional analysis (Miyazaki et al., 2002). Recently, higher protein expression analysed by an anti-rat ClC3 antibody was found in FW pufferfish gills than in SW ones (Tang and Lee, 2007). On the other hand, a cystic fibrosis transmembrane conductance regulator (CFTR) was also proposed to be a candidate for basolateral Cl⁻ exit based on its basolateral localization in pavement and MR cells in killifish operculum (Marshall, 2002). In NCC cells, basolateral NKA may provide the intracellular negative gradient for driving the basolateral Cl⁻ channels to transport Cl⁻ out of NCC cells, as in mammalian DCT cells. Further studies are needed to draw definitive conclusions on the mechanisms for basolateral Cl⁻ exit from NCC cells.

Other pathways

Anion exchanger

Compared with Na⁺- and Ca²⁺-uptake mechanisms, those for Cl⁻ uptake are largely unclear. As discussed above, NCC cells may be one of the possible ionocytes responsible for Cl⁻ uptake *via* the apical NCC and basolateral ClC and NKA. On the other hand, it was proposed that the apical Cl⁻/HCO₃⁻ exchanger is the major player in the mechanism of Cl⁻ uptake linked with basal secretion in fish gills, and anion exchanger (AE) 1 was one of the candidates proposed (Evans et al., 2005; Hwang and Lee, 2007; Evans, 2008). However, ISH and ICC data for the existence of AE1 in teleost gills

(Sullivan et al., 1996; Wilson et al., 2000) were controversial when considering the specificity of the antibody or probe used and the absence of evidence for AE1 on the apical membrane of other acid/base-secreting epithelia (Perry and Gilmour, 2006; Tresguerres et al., 2006). In our preliminary experiments on zebrafish, mRNA of an AE1 paralogue was localized in a specific group of skin/gill cells (Fig. 2E), and zAE1 mRNA expression was not affected by low-Cl⁻ FW (Fig. 3) that has previously been reported to stimulate Cl⁻ uptake in fish gills (Lin and Hwang, 2001; Chang et al., 2003). This implies that zAE1 might not be associated with the functions of apical Cl⁻ uptake/base secretion in zebrafish skin/gill ionocytes. This issue requires further exploration.

Pendrin

Another candidate for apical Cl⁻ uptake/base secretion is pendrin (SLC26A), which is localized in the apical region of B-type intercalated cells of the cortical collecting duct and which functions in renal Cl⁻ reabsorption and bicarbonate secretion (Royaux et al., 2001; Wagner, 2007). In the stingray (*Dasyatis sabina*), Piermarini and coworkers (Piermarini et al., 2002) used an anti-pendrin (SLC26A4) antibody to indicate immunoreactions in the apical region of gill ionocytes that are rich in basolateral H⁺-ATPase. Our preliminary experiments identified as many as 11 members of the SLC26A family from zebrafish, and seven of them were detected by RT-PCR in zebrafish gills. At least three isoforms, SLC26A3, SLC26A4 and SLC26A6, were localized by ISH and/or ICC in a group of ionocytes in zebrafish gills (S. F. Perry, personal communication). This sheds new light on another transport pathway for Cl⁻ uptake/base secretion in zebrafish gill ionocytes.

Comparison of ionocytes between species

It has long been a challenging and important issue for fish physiologists to explore whether fish develop pleomorphic ionocytes (or MR cells) to achieve the different ion-transport functions for the internal ionic and osmotic homeostasis. Pleomorphic ionocytes in various species have been identified based on different criteria depending on the study (Hirose et al., 2003; Hwang and Lee, 2007), but generalizing a comprehensive working model of fish ion-regulatory mechanisms by comparing the pleomorphic ionocytes among species would be of physiological and evolutionary significance. In the past decade, most studies on pleomorphic ionocytes focused particularly on FW fish, probably because FW environments are more diverse

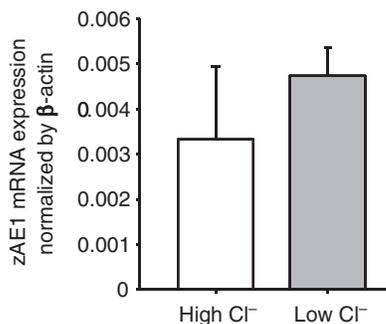


Fig. 3. Effects of environmental Cl⁻ levels on zAE1 mRNA expression in zebrafish gills. Zebrafish were acclimated to high Cl⁻ (10 mmol l⁻¹ Cl⁻) and low Cl⁻ (0.04 mmol l⁻¹ Cl⁻) for 1 week. Quantitative RT-PCR analysis did not show a significant difference between the two groups (Student's *t*-test, *P*>0.05). Means ± s.d. (*N*=4).

than SW ones in terms of ionic composition, pH, hardness, etc. In early studies, different populations or subtypes of ionocytes were mostly identified based on their anatomic location in gills and/or the ultrastructural characteristics of cytosol or apical openings (Hwang and Lee, 2007). Pisam and colleagues (Pisam et al., 1987) identified two types of MR cells, α and β cells, in gills of several species acclimated to FW based on the transmission electron microscopic ultrastructure and the location in gill filaments. By examining the effects of environmental salinity or ion levels and exogenous hormones on the density of α and β MR cells (Pisam et al., 1993), β MR cells were suggested to be associated with Ca²⁺ uptake while α MR cells exerted dual functions: Cl⁻ secretion in SW and Na⁺ uptake in FW (Pisam et al., 1993; Pisam et al., 1995), but no ion-influx data are available to support their inference. Hwang's group classified three subtypes of MR cells, wavy-convex, shallow-basin and deep-hole types, in FW tilapia gills according to the morphologies of the apical openings (Lee et al., 1996), and subsequent physiological studies suggested roles for the three types of cells in Cl⁻ uptake, Ca²⁺ uptake and Cl⁻ secretion, respectively, based on the correlations between the gill cell density of the three ionocyte types and ion (Na⁺, Cl⁻ and Ca²⁺) influxes (Tsai and Hwang, 1998; Chang et al., 2001; Chang et al., 2003). However, further functional analyses of those ionocytes are difficult due to limitations in the applicability of molecular physiological approaches to these anatomically or morphologically identified ionocytes. Moreover, comparison of pleomorphic ionocytes between species is complicated because the same criteria in anatomy or morphology cannot be applied to different species.

Recently, particularly in zebrafish, rainbow trout and tilapia, different types of ionocytes were identified and characterized with specific molecular markers, and their functions were further analysed by various cellular and molecular physiological approaches. As discussed above in zebrafish skin/gills, at least three types of ionocytes, HR cells, NaR cells and NCC cells, have been identified by double/triple ISH and/or ICC for the related ion transporters and enzymes, and their respective ion-transport functions of Na⁺ uptake/acid secretion, Ca²⁺ uptake and Cl⁻ uptake were demonstrated based on a loss-of-function approach with specific transport (or enzyme) morpholinos or on correlations of a transporter's expression and ion influxes. Ionocytes in zebrafish provide an alternative platform for further molecular physiological investigations of the functions and their regulation in fish ion-uptake and acid-base balance mechanisms. As shown in Fig. 4, we attempted to make models for other species analogous to the model of zebrafish ionocytes, from the point of view of expression patterns of transporters. In tilapia, type-II and type-III MR cells are analogous to zebrafish NCC cells and HR cells, respectively. However, localization of H⁺-ATPase (with an anti-bovine adrenal medulla V-ATPase subunit A antibody) in tilapia embryonic skin was found to be confined to the apical membrane of pavement cells in a previous study (Hiroi et al., 1998), and this needs to be confirmed because different results were obtained using an anti-killifish V-ATPase A subunit antibody (Kato et al., 2003) (T. Kaneko and J. Hiroi, personal communication). Moreover, ECaC, the mRNA of which was specifically expressed in zebrafish NaR cells, was localized using a homologous antibody to most tilapia MR cells (T. Kaneko, personal communication).

The case for rainbow trout is more complicated. PNA⁻ and PNA⁺ MR cells, which were isolated with PNA, express higher H⁺-ATPase and NKA, respectively (Galvez et al., 2002), and show different ion-transport functions: PNA⁻ exhibited a bafilomycin-

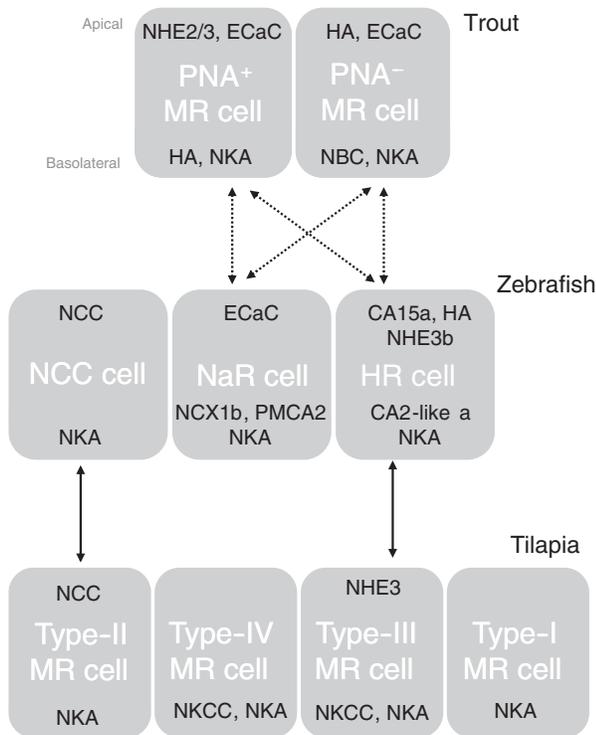


Fig. 4. Comparison of ionocytes between zebrafish, rainbow trout and tilapia. Solid and dotted lines indicate higher and lower homology, respectively, among ionocytes of the different species. CA, carbonic anhydrase; MR, mitochondria rich; NCX, Na⁺/Ca²⁺ exchanger; NKCC, Na⁺-K⁺-Cl⁻ cotransporter; PNA, peanut lectin agglutinin.

sensitive acid-activated ²²Na⁺-uptake/acid-stimulated response of H⁺-ATPase, while PNA⁺ showed Ca²⁺ uptake (Reid et al., 2003; Galvez et al., 2006; Parks et al., 2007). Based on many previous immunocytochemical, physiological and pharmacological data, the current model for trout gill cells proposes that PNA⁻ and PNA⁺ MR cells are responsible for Na⁺ uptake/acid secretion and Ca²⁺ uptake/base secretion/Cl⁻ uptake, respectively (Tresguerres et al., 2006; Perry and Gilmour, 2006; Evans, 2008; Parks et al., 2008). In this situation, PNA⁻ and PNA⁺ MR cells in rainbow trout gills appear to be analogues of zebrafish HR and NaR cells, respectively; however, things are not so simple. Recently, Perry's group reported the localization of NHE2/3 in PNA⁺ MR cells and acid-stimulated expression of NHE2, and argued against the base secretion function of PNA⁺ MR cells in the current model (Ivanis et al., 2008). Moreover, it is worth noting a recent ISH and ICC study by the same group (Shahsavaran et al., 2006) which reported that rainbow trout gills contain four populations of cells: (1) ECaC positive, (2) NKA positive, (3) ECaC and NKA positive, and (4) ECaC and NKA negative, confirming the broad distribution of ECaC among PNA⁻ and PNA⁺ MR cells as well as pavement cells. On the other hand, Galvez and coworkers (Galvez et al., 2006) demonstrated that PNA⁺ MR cells are the major player in Ca²⁺-uptake function. Taken together, classification of PNA⁻ and PNA⁺ MR cells may be too simplified to fully elucidate the complicated ion-uptake and acid-base regulation mechanisms in trout gills. Molecular markers with greater specificity or other cellular approaches are necessary to determine more subtypes of MR cells, other than PNA⁻/PNA⁺ cells, from trout gills to resolve these issues.

Conclusions and perspectives

In zebrafish skin and gills, at least three types of ionocytes, HR, NaR and NCC cells, have been identified with specific probes or antibodies (Fig. 1). Different sets of ion transporters and enzymes as well as their functional regulation have been explored by various molecular physiological approaches. These have not only provided some new insights into fish ion-regulatory mechanisms but are also enhancing our understanding of mammalian renal transport physiology by providing an *in vivo* working model, as the transporter expression patterns and functions of zebrafish ionocytes are analogous to those of proximal tubular cells, intercalated cells, Ca²⁺ reabsorption cells and distal convoluted cells. However, several important issues remain to be studied in the future (Fig. 1): (1) the identification and functional analysis of basolateral transporters in ionocytes, e.g. AE1 and/or NBC1 in HR cells and CIC in NCC cells; (2) the identification and functional analysis of ENaC equivalents, NHA, pendrin and other unidentified transporters or enzymes in ionocytes; and (3) transport properties and stoichiometry of apical transporters, such as zNHE3b and zNCCg.

List of abbreviations

AE	anion exchanger
CA	carbonic anhydrase
ECaC	epithelial Ca ²⁺ channel
HR	H ⁺ -ATPase rich
ICC	immunocytochemistry
ISH	<i>in situ</i> hybridization
MR	mitochondria rich
NaR	Na ⁺ -K ⁺ -ATPase rich
NCC	Na ⁺ -Cl ⁻ cotransporter
NCX	Na ⁺ /Ca ²⁺ exchanger
NHE	Na ⁺ /H ⁺ exchanger
PMCA	plasma membrane Ca ²⁺ -ATPase
PNA	peanut lectin agglutinin

The original research was financially supported by grants from the National Science Council and Academia Sinica of Taiwan, ROC. The author extends his thanks to Y. C. Tung for her technical and secretarial assistance.

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