Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (Danio rerio)

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Horng J-L, Lin L-Y, Huang C-J, Katoh F, Kaneko T, Hwang P-P. Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (Danio rerio). Am J Physiol Regul Integr Comp Physiol 292: R2068–R2076, 2007. First published February 1, 2007; doi:10.1152/ajpregu.00578.2006.—In the skin of zebrafish embryo, the vacuolar H+-ATPase (V-ATPase, H+-pump) distributed mainly in the apical membrane of H+-pump-rich cells, which pump internal acid out of the embryo and function similarly to acid-secreting intercalated cells in mammalian kidney. In addition to acid excretion, the electrogenic H+-efflux via the H+-ATPases in the gill apical membrane of freshwater fish was proposed to act as a driving force for Na+ entry through the apical Na+ channels. However, convincing molecular physiological evidence in vivo for this model is still lacking. In this study, we used morpholino-modified antisense oligonucleotides to knockdown the gene product of H+-ATPase subunit A (atp6v1a) and examined the phenotype of the mutants. The H+-ATPase knockout embryos revealed several abnormalities, including suppression of acid-secretion from skin, growth retardation, trunk deformation, and loss of internal Ca2+ and Na+. This finding reveals the critical role of H+-ATPase in embryonic acid-secretion and ion balance, as well.

H+-ATPase; HR cell; morpholino-knockdown; Na+, Ca2+

VACUOLAR-TYPE PROTON ATPase (V-ATPase, H+-ATPase, proton pump) is ubiquitous in intracellular compartments of eukaryotic cells, such as those enclosing vacuoles, lysosomes, coated vesicles, secretory granules, and the trans-Golgi network (15, 29). In these organelar membranes, H+-ATPase pumps H+ into the lumen of the organelles in a process coupled with ATP hydrolysis, leading to acidification of organelar compartments. H+-ATPase consists of two major functional sectors known as V1 and V0. The V1 sector comprises at least eight different subunits (A–H), and the three catalytic sites for ATP hydrolysis are formed from the A and B subunits. The V0 sector contains up to five subunits (a, c, c’, c”, and d), and is responsible for proton translocation across the membranes (38).

In a number of differentiated cell types, including osteoclasts (4), kidney, and epididymis epithelial cells (2), H+-ATPases are also enriched in plasma membranes, where they actively secrete H+ from the cell and establish an acidic extracellular compartment. This process is involved in driving bone reabsorption by osteoclasts, bicarbonate reabsorption in kidney, and reproductive tract acidification in mammalian epididymis (38, 40). In mammals, overall body pH (acid-base) homeostasis is controlled mainly by the exhalation of CO2 and by the reabsorption, generation, or secretion of bicarbonate as well as the secretion of acid and acid equivalents by the kidneys. From 70 to 80% of the filtered bicarbonate is reabsorbed in the proximal tubule and up to 40% of proximal tubule bicarbonate reabsorption is mediated by H+-ATPase expressed in the brush-border membrane (40). In the late distal tubule and collecting duct, acid-secreting type A intercalated cells expressing H+-ATPase on the apical side are responsible for the proton secretion and bicarbonate absorption that is stimulated by metabolic acidosis (40). The human disease, distal renal tubular acidosis (dRTA), results from a direct failure of the distal nephron to secrete acid into the tubular lumen and is characterized by the inability to maximally acidify the urine <pH 5.5 during systemic acidosis. Inherited forms of this type of RTA have been shown to be caused by mutations in the B1 or a4 subunit of H+-ATPase (18, 28, 40). To investigate mechanisms of dRTA and the function of H+-ATPase in intact animals, an ideal animal model for reverse-genetic approach is required. However, because of the widespread distribution and critical function of H+-ATPase in cells, mutation of genes encoding H+-ATPase subunits in yeast, Drosophila, and mice often leads to lethality (10, 21, 27).

Owing to the dilute nature of the external medium relative to the body fluids, freshwater-adapted teleosts must cope with the continual loss of salts and the entry of water across their permeable surface. For internal homeostasis, freshwater teleosts have to actively absorb ions from the environment and regulate the internal pH via their gills (12, 17, 20). In these mechanisms, transport of internal H+ or HCO3− to the water was suggested to couple with the uptake of Na+ or Cl− from the environment. Na+/H+ exchanger was first suggested as a primary mechanism of acid secretion coupling with the Na+ uptake in freshwater teleost gills. Considering the thermodynamic requirements for freshwater Na+ uptake in teleost gills, a new scheme for ion uptake driven by H+-ATPase was proposed (9, 12). Electrogenic H+ efflux via the H+-ATPases in the apical membrane generates a negative intracellular electrical potential, which in turn acts as a driving force to
allow Na⁺ entry from the water down an electrochemical gradient through apical Na⁺ channels (9, 12, 25). Bafilomycin A₁, a specific inhibitor for H⁺-ATPases, was found to reduce the Na⁺ uptake by ~80% in young tilapia and 70% in young carp (14). Recently, H⁺-ATPase was also proposed as providing some driving force to operate apical Cl⁻/HCO₃⁻ exchangers for Cl⁻ uptake in freshwater fish gills (5, 26). Apparently, roles of H⁺-ATPase in ion regulation of teleost gills are still under debate.

In our previous work, we identified a novel H⁺-secreting cell [H⁺-ATPase-rich cells (HR cells)] in the skin of zebrafish embryo and characterized its function with electrophysiological technique (24). H⁺-ATPase was expressed abundantly in the apical membrane of HR cells and was shown to pump H⁺ out of the embryo during development. The acid-secreting function of these HR cells in aquatic zebrafish was quite similar to the intercalated cell in mammalian nephron. In the present study, we further investigate the function of H⁺-ATPase in zebrafish with a reverse genetic approach. Morpholino-modified antisense oligonucleotides were used to knockdown the gene product of H⁺-ATPase subunit A (atp6v1a), and the phenotype of the mutants were examined with morphological and physiological approaches. Based on this work, we aimed to see the role of H⁺-ATPase in ion regulation mechanisms of fish skin/gill and to establish a potential in vivo animal model for further studies on human dRTA disease.

**MATERIALS AND METHODS**

**Zebrafish.** Mature zebrafish (AB strain) were reared in circulating MOPS buffer (Sigma, St. Louis, MO), and 0.1 M Tricine (3-aminobenzoic acid ethyl ester; Sigma), pH 6.8. The microelectrode system was attached to an Olympus upright microscope (model BX-50WI) equipped with a charge-coupled device camera. The Nemustain properties of each microelectrode were measured by placing the microelectrode in a series of standard pH solutions (pH 6, 7, and 8). By plotting the voltage output of the probe against the log H⁺ concentration, a linear regression yielded a Nemustin slope of 57.9 (SD 2.5) (n = 10).

**Surface pH of zebrafish embryos.** The measurement was performed at room temperature (24–26°C) in a small plastic recording chamber filled with 1 ml of “recording solution” that contained “zebrafish solution,” 300 µM MOPS buffer (Sigma, St. Louis, MO), and 0.1 mg/ml Tricine (3-aminobenzoic acid ethyl ester; Sigma), pH 6.8. An anesthetized embryo was positioned in the center of the chamber with its lateral side contacting the base of the chamber. To record the surface pH surrounding the embryos, the probe was moved to six selected locations. The voltage output signals (in mV) were recorded every 3.0 s and averaged for 30 min. In the double stain of concanavalin A (ConA) and Na⁺,K⁺-ATPase, live embryos were preincubated in zebrafish solution containing 0.5 mg/ml Texas Red-conjugated ConA (Molecular Probes, Eugene, OR) for 10 min. After washing, the ConA-labeled embryos were fixed and immunostained with α5-monomoclonal antibody as described above.

**Western blot analysis.** Twenty embryos were pooled as one sample and homogenized. Protein of 50 mg/well was loaded to a 10% SDS-PAGE at 100 V for 2 h. After separation, proteins were transferred onto polyvinylidene difluoride membrane (Millipore) at 100 V for 2 h. After being blocked for 1.5 h in 5% nonfat milk, the blots were incubated with a polyclonal antibody against the A-subunit of H⁺-ATPase (overnight, 4°C, diluted 1:1,000) and with an alkaline-phosphatase-conjugated goat anti-rabbit IgG (diluted 1:5,000, room temperature; Jackson Laboratories) for another 2 h. The blots were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium. Intensities of the immunoreactive bands were measured by densitometry (Chemilab, Syngene, UK) and were converted to numerical values to compare the relative amounts of H⁺-ATPase.

<table>
<thead>
<tr>
<th>Artificial Medium</th>
<th>[Na⁺]</th>
<th>[Cl⁻]</th>
<th>[Ca²⁺]</th>
<th>[K⁺]</th>
<th>[Mg²⁺]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Na⁺</td>
<td>0.500–0.509</td>
<td>0.478–0.555</td>
<td>0.182–0.197</td>
<td>0.504–0.533</td>
<td>0.392–0.396</td>
<td>6.79–6.82</td>
</tr>
<tr>
<td>Low Na⁺</td>
<td>0.010–0.011</td>
<td>0.437–0.535</td>
<td>0.178–0.188</td>
<td>0.496–0.514</td>
<td>0.400–0.404</td>
<td>6.82–6.87</td>
</tr>
<tr>
<td>Normal Cl⁻</td>
<td>0.561–0.579</td>
<td>0.554–0.561</td>
<td>0.171–0.185</td>
<td>0.232–0.238</td>
<td>0.241–0.242</td>
<td>6.76–6.82</td>
</tr>
<tr>
<td>Low Cl⁻</td>
<td>0.567–0.599</td>
<td>0.003–0.010</td>
<td>0.178–0.183</td>
<td>0.232–0.235</td>
<td>0.235–0.239</td>
<td>6.81–6.85</td>
</tr>
<tr>
<td>Normal Ca²⁺</td>
<td>0.537–0.571</td>
<td>0.562–0.573</td>
<td>0.181–0.195</td>
<td>0.214–0.226</td>
<td>0.224–0.232</td>
<td>6.85–6.9</td>
</tr>
<tr>
<td>Low Ca²⁺</td>
<td>0.563–0.574</td>
<td>0.559–0.568</td>
<td>0.011–0.015</td>
<td>0.218–0.232</td>
<td>0.221–0.235</td>
<td>6.84–6.89</td>
</tr>
</tbody>
</table>

Values are ranges in millimoles.
background measurements were taken outside the biologically generated gradients at a distance of 5 mm from the surface of the embryos. The ability to generate a pH gradient in embryos is presented as the net acid load (ΔpH) over the skin, i.e., the pH of recording sites (locations 1–6) minus the background. A ΔpH of <0 means an acid pH around the fish.

Microinjection of antisense morpholino oligonucleotide. The morpholino oligonucleotide (MO) was obtained from Gene Tools (Philomath, OR). The morpholino against H^+-ATPase subunit A (BC055130) begins at 19 bp and spans the ATG ending at the +6 nucleotide position (5′-ATCCATCTTGTGTGTTAGAAAACTG-3′) and was prepared with sterile water. Standard control oligo was used as the control, 5′-CCTCTTACCTCAGTTACAATTTATA-3′. This standard control oligo provided by Gene Tools has no target and no significant biological activity. The MO solution contains 0.1% phenol red used as a visualizing indicator and was injected into zebrafish embryos at the one- to four-cell stages using an IM-300 microinjector system (Narishigi Scientific Instrument Laboratory, Tokyo, Japan). Antisense MO at 2–12 ng/embryo and control oligo at 8 –12 ng/embryo was injected. Antisense MO-injected embryos at 72 hpf were examined and counted under a stereo microscope. Body length of wild-type (WT) embryos was measured 3.31 mm (SD 0.11). The body length of MO-injected embryos 3.20 mm (1 SD away from the mean of WT embryos) was defined as small-size embryo. Based on results of control oligo injection, MO at 4 – 8 ng/embryo was used for the following experiments.

Acclimation to different pH environments. For the experiments of acclimation to different pH environments, zebrafish solution was supplemented with 300 μM MES (Sigma) or 300 μM MOPS (Sigma) to prepare pH 4, 5.5, and 7 artificial freshwater. WT and MO-injected zebrafish eggs were transferred to different artificial freshwater media at 28°C and were sampled at 120 hpf for the whole body Na^+/Cl^−/Ca^{2+} content measurements. During all acclimation experiments, larvae were not fed; media were changed daily to guarantee optimal water quality.

Measurement of whole body Na^+/Cl^−/Ca^{2+} content. Ten zebrafish larvae were briefly rinsed in deionized water and then pooled as one sample and weighed. HNO_3 at 13.1 N was added to the samples for digestion at 60°C overnight. Digested solutions were diluted with double-deionized water, and the total Na^+/Ca^{2+} content was measured with an atomic absorption spectrophotometer (model Z-8,000; Hitachi). For Cl− content measurements, samples were homogenized with 1 ml deionized water and centrifuged at 14,000 rpm for 30 min. Supernatant was collected and thereafter was added Hg(SCN4) (0.3 g in 95% ethanol) and NH_4Fe(SO_4)_{12} H_2O (30 g in 135 ml 6 N NO_3) solutions for analysis. Cl− concentration was measured by the Ferricyanide method (16) with a double-beam spectrophotometer (model U-2000; Hitachi). Standard solutions of Na^+/Cl^−/Ca^{2+} measurements were transferred to either of six artificial freshwater media at 28°C and were sampled at 120 hpf for the whole body Na^+/Cl^−/Ca^{2+} content measurements. During all acclimation experiments, larvae were not fed; media were changed daily to guarantee optimal water quality.

Fig. 1. Confocal laser scanning micrographs of whole mount immunocytochemistry of Na^+-K^+-ATPase and H^+-ATPase on the yolk sac of zebrafish embryos at 24 (A), 48 (B), and 72 hpf (hours post-fertilization) (C and D). The embryos were double stained for Na^+-K^+-ATPase (Texas Red, red) and H^+-ATPase (FITC, green). Scale bar: 100 μm.

Fig. 2. Density changes of H^+-ATPase-rich cells (HR cells) following development. Means (SD) (n = 10 zebrafish embryos). One-way ANOVA (Tukey’s pairwise comparison) was conducted among the different stages. a,b,c,dDifferent letters indicated significant difference.
from Merck (Darmstadt, Germany) were used to make the standard curves.

Statistical analysis. Values are presented as the means (SD) and were compared using Student’s t-test or one-way ANOVA (Tukey’s pairwise comparison). The frequencies of survival rate between WT and antisense MO-injected (or control oligo-injected) embryos were conducted by χ² test.

RESULTS

Expression of H⁺-ATPase-rich cells on the yolk sac during development. The anti-Na⁺-K⁺-ATPase and anti-H⁺-ATPase antibodies stained specific epithelial cells on the yolk sac, respectively. Na⁺-K⁺-ATPase and H⁺-ATPase-immunoreactive cells [Na⁺-K⁺-ATPase-rich cells (NaR cells) and HR cells, respectively] were first detected on the yolk sac of whole mount-prepared zebrafish embryos at 24 hpf (Fig. 1A). At the early stage of development, the fluorescence signal was weak and only concentrated in the apical regions of HR cells (Fig. 1, A and B). The H⁺-ATPase-immunoreactive fluorescence signal was stronger and spread to the subapical region after hatching (Fig. 1, C and D). The HR cells initially appeared scattered on top of the yolk, and following embryonic development, their distribution extended evenly to the entire yolk sac skin but never to the skin covering the head or body somites. The density of HR cells on the yolk sac was counted and showed dramatic increase during early development (Fig. 2). Compared with the zebrafish embryos at 24 hpf, the 72-hpf embryos developed about three times higher the number of HR cells, and 96-hpf embryos about four times higher (Fig. 2).

External ΔpH gradients around the embryos during development. ΔpH gradients at six different locations surrounding the intact zebrafish embryos were measured at 24, 48, 72, and 96 hpf (Fig. 3A). Higher H⁺ activities were detected by the probe when moving toward and closely approaching the skin surface, showing an outward H⁺ current (i.e., H⁺ secretion). A higher H⁺ gradient (i.e., a more negative ΔpH) indicated H⁺ current from the fish, which increased over time in (Fig. 3B). The locations 1–6 showed different H⁺ gradients, indicating different levels of H⁺ secretion, and the lowest ΔpH (i.e., the highest secretion) occurred at locations 2–4 (Fig. 3B), where the highest density of HR cells was found. On the other hand, locations 1 and 6, with no or very few HR cells, showed the lowest level of H⁺ secretion (Fig. 3B).

The ΔpH surrounding the zebrafish embryo skin also revealed dramatic changes following development (Fig. 3, B and C). Few ΔpH were detected at 24 hpf, indicating no significant H⁺ secretion occurred at this early stage. However, locations 2–4 became...
As shown in Fig. 6, expressed H+ down morphants. However, the phenotype of control embryos survival rate compared with WT embryos (Table 2), indicating 8 ng of control oligo did not show significant difference in and dose-dependent effects. Moreover, embryos injected with still have the signal of ConA (Fig. 6) lacked the signal of H+ATPase in specific cells on the yolk sac (i.e., locations 2–4) during development (Figs. 1 and 2). It was not until 48 hpf that both the H+ gradient at locations 2–4 (Fig. 3, B and C) and HR cell density on the yolk sac significantly increased.

**Effect of H+ATPase knockdown on protein expression and phenotype in zebrafish.** H+ATPase protein expression in 4 ng and 8 ng MO-injected embryos (morphants) was decreased in a dose-dependent manner but was not affected in control-oligo-injected embryos (control embryos) compared with WT embryos (Fig. 4). Morphants showed different levels of defects: some had normal appearance, some were smaller in size, and others were malformed in the tail region (Fig. 5). The ratio of small-size and malformed-tail embryos increased following the increasing amount of the antisense MO injected, showing a dose-to-phenotype severity correlation in H+ATPase knockdown morphants. However, the phenotype of control embryos did not show significant changes compared with WT embryos (Table 2). These indicated that the MO we used showed specific and dose-dependent effects. Moreover, embryos injected with 8 ng of control oligo did not show significant difference in survival rate compared with WT embryos (Table 2), indicating that <8 ng of oligo does not cause nonspecific effect of injection. Therefore, 4–8 ng oligo were used in the following experiments.

**Effect of H+ATPase knockdown on skin ionocytes zebrafish.** As shown in Fig. 6, A and C, WT and control embryos expressed H+ATPase in specific cells on the yolk sac and yolk-extension skin surface at 72 hpf. On the other hand, the morphants (Fig. 6E) lacked the signal of H+ATPase in the same staining condition as the WT and control embryos did but still have the signal of ConA (Fig. 6F), which is an apical opening marker for HR cells in zebrafish (24). H+ATPase knockdown did not affect the expression of Na+K+-ATPase. WT, control, and morphant embryos all developed NaR cells (Fig. 6B, D, F, and G).

**Effect of H+ATPase knockdown on proton secretion function in zebrafish.** In the previous results, we showed that the ∆pH around the zebrafish embryos become more acidic (especially at locations 2–4 of embryos) during development. To investigate whether MO-mediated knockdown of H+ATPase alters the ∆pH generated during development, we examined the H+ gradient at six specific locations as described in Fig. 3 in intact morphants at 48 and 96 hpf. Compared to WT embryos, knockdown of H+ATPase significantly reduced the ∆pH at all recording sites at 48 and 96 hpf (Fig. 7). Particularly at locations 2–4 where the highest density of HR cells appeared, a dramatic decrease in the ∆pH was found in MO-targeted zebrafish embryos.

**Effect of H+ATPase knockdown on acid regulation in zebrafish.** To examine the regulating ability in acid environment, WT embryos and morphants were incubated right after injection in pH 4, pH 5.5, and pH 7 artificial freshwaters. At 72 hpf, morphants have a lower survival rate in acidic environment than WT larvae and morphants either in normal-Cl− artificial freshwater (Table 3). Particularly at locations 2–4 where the highest density of HR cells appeared, a dramatic decrease in the ∆pH was found in MO-targeted zebrafish embryos.

Effect of H+ATPase knockdown on acid regulation in zebrafish. To examine the regulating ability in acid environment, WT embryos and morphants were incubated right after injection in pH 4, pH 5.5, and pH 7 artificial freshwaters. At 72 hpf, morphants have a lower survival rate in acidic environment than WT larvae and morphants either in normal-Cl− artificial freshwater (Table 3).

Fig. 3. Light microscopy images of wild-type (WT; A) and morpholino (B–D) injected embryos (morphants) at 72 hpf. B: morphant appearing as a normal WT embryo but shorter body length. C and D: morphants with abnormal development and body shape.
zebrafish. The Ca\(^{2+}\) content was significantly lower in morphants either in normal-Ca\(^{2+}\) or low-Ca\(^{2+}\) artificial freshwater (Fig. 8C).

**DISCUSSION**

In mammals, the acids of body fluid are coming from respiratory acid (CO\(_2\)) and other metabolic acids. The major regulatory organs of acid-base balance are the lungs and the kidneys, which excrete the respiratory and metabolic acids, respectively. In aquatic fish, gills usually account for >90% of compensatory net acid-base transfers (19, 39). Because of the high gill ventilation rate and high solubility of CO\(_2\), the metabolically generated CO\(_2\) can easily leave through the gills. Therefore, the low steady-state arterial PCO\(_2\) and bicarbonate

<table>
<thead>
<tr>
<th>Survival Rate (%)</th>
<th>No. Embryos Analyzed</th>
<th>Percentage of Different Phenotypes*</th>
<th>Normal, A</th>
<th>Small Size, B</th>
<th>Malformation in Tail, C and D</th>
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</thead>
<tbody>
<tr>
<td>Wild-Type Embryos</td>
<td>96</td>
<td>217</td>
<td>99</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>121</td>
<td>85</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>94.2</td>
<td>209</td>
<td>52.1</td>
<td>38.8</td>
<td>9.1</td>
</tr>
<tr>
<td>8</td>
<td>83.6*</td>
<td>70</td>
<td>4.3</td>
<td>54.3</td>
<td>41.4</td>
</tr>
<tr>
<td>12</td>
<td>56.5*</td>
<td>65</td>
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<tr>
<td>Control Oligo</td>
<td>8</td>
<td>91</td>
<td>99</td>
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<td>12</td>
<td>87.2*</td>
<td>96</td>
<td>99</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Significant difference (\(\chi^2\)-test, \(P < 0.05\)) from wild-type embryos. *Phenotypes were classified as A, B, C, and D (see Fig. 5).

Values are nanograms per embryo.
concentration minimize the ability of fish to “blow-off” buffered metabolic acid loads via hyperventilation during metabolic acid-base disturbances (39). Fish are heavily dependent on net exchange of nonvolatile acid-base equivalents with their environment, a task well suited to gills. Fish gills are covered with an epithelium that is in direct contact with the external medium, which is usually large enough to act as an infinite source and sink of solutes (i.e., acid-base equivalents and ions with which they are exchanged). This is an advantage over internal transport tissues, such as the kidneys (8, 12).

Indeed, the acid-excreting function of zebrafish embryo seems not to be performed by embryonic kidney. In our previous study, H^+/H^+/ATPase was not expressed significantly in embryonic kidney from fertilized embryos to 5-day-old larvae, nor did we detect significant acid secretion from the pronephic duct opening (data not shown) (11).

Although the gill is the major organ for acid secretion, the localization of acid-secreting cells in fish gills is still being debated with a contribution by pavement cells (35, 37, 41), mitochondria rich cells (23), or both (12, 41). In our previous work, a novel proton-secreting cell (HR cell) was found for the first time and characterized in the skin of newly hatched zebrafish larvae (24). In this study, we found the HR cells were first detected in embryo of 24 hpf and slight proton gradient has been detected around the chorion of the unhatched embryos (data not shown). At this stage, the heart of embryo has just been developed and started functioning with beating and blood circulating around the embryo (3), which indicates that a need for exchange of nutrition and waste of the cells cannot be met by diffusion alone (3, 31). Therefore, the appearance of HR cells at this stage may bear the task of acid excretion from the circulation of the embryo.

Following development, particularly right after hatching, the HR cells increased in number and became more mature (increase of H^+/H^+/ATPase expression, Figs. 1 and 2), suggesting that larvae must have more effective acid excretion to maintain acid-base homeostasis to compensate for their accelerating metabolism and additional activity like swimming. Ionocytes, including NaR cells and HR cells, started to appear in zebrafish gills from 72 hpf (Ref. 30 and Horng J-L, Lin L-Y, and Hwang P-P, unpublished data). Following the development of gills, the acid-secreting capability may be gradually transferred from skin to gills where HR cells are located.

H^+/ATPase was highly expressed on the apical membrane and subapical vesicles of HR cells (24). In mammalian renal-collecting duct, the intercalated cells, which are responsible for proton secretion, were found to increase the apical membrane H^+/ATPase expression and the cell number to accelerate net acid secretion after systemic acidosis (40). Excreting acid to maintain acid-base homeostasis was also seen in HR cells in developing zebrafish embryos.

In the present study, we used the reverse genetic approach, morpholino knockdown, to examine the function of H^+/ATPase in zebrafish embryos. Acid secretion in H^+/ATPase morphants was suppressed, and the embryos consequently lost their capability to cope with acidic environment (more than 50% mortality in pH 4), suggesting a critical role of H^+/ATPase in acid-adaptation of fish. In addition, H^+/ATPase knockdown

**Table 3. Effect of H^+/ATPase knockdown on survival rate and phenotype in 72-hpf zebrafish upon acclimation to different pH environments**

<table>
<thead>
<tr>
<th>pH</th>
<th>Survival rate, %</th>
<th>No. Embryos Analyzed</th>
<th>Normal, A</th>
<th>Small Size, B</th>
<th>Malformation in Tail, C and D</th>
</tr>
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<tbody>
<tr>
<td>Wild Type</td>
<td>7</td>
<td>95.5</td>
<td>125</td>
<td>90.0</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>96.1</td>
<td>120</td>
<td>97.5</td>
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<tr>
<td></td>
<td>4</td>
<td>85.5</td>
<td>118</td>
<td>94.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Morphant</td>
<td>7</td>
<td>93.9</td>
<td>44</td>
<td>54.5</td>
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</tr>
<tr>
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<td>22</td>
<td>45.5</td>
<td>54.5</td>
</tr>
</tbody>
</table>

*Significant difference (χ^2-test, P < 0.05) was found between the wild-type and morphant zebrafish in pH 5.5 and pH 4, respectively. Phenotypes were classified as A, B, C, and D (see Fig. 5).
ATPase is not only involved in acid-base regulation and same medium. Means (SD) (n = 6 pooled sample). The Na⁺ content was significantly lower in morphants than in WT larvae after acclimation to normal-Na⁺ medium (L-Na), but no significant difference in the Cl⁻ contents was found between morphants and WT larvae acclimated to either normal-Cl⁻ or low-Cl⁻ medium. The Ca²⁺ content was significantly lower in morphants acclimated to normal-Ca²⁺ (N-Ca) or low-Ca²⁺ (L-Ca) medium. N-Na, normal-Na⁺ artificial freshwater.

Also caused developmental defects in embryos, including growth retardation, deformation of tail (Fig. 5), and loss of Ca²⁺ and Na⁺ contents (Fig. 8). It was reported that H⁺-ATPase is not only involved in acid-base regulation and respiration, but also provides driving force for Na⁺ uptake across the gills of some freshwater fish (9, 32, 33). Electrogenic H⁺ efflux via the H⁺-ATPases in the apical membrane may generate a negative intracellular electrical potential, which, in turn, acts as a driving force to allow Na⁺ entry from the water down an electrochemical gradient through apical Na⁺ channels (20, 34). Indeed, our study with reverse genetic method also confirmed that the H⁺-ATPase plays some role in maintaining internal Na⁺ content. The sodium levels were affected only in the embryos raised in low-sodium water. This implies a possibility of other pathways being involved in the Na⁺ uptake mechanism in zebrafish skin. In the H⁺-ATPase knockdown morphants, there was still a group of cells that stained for ConA. This indicates that the ionocytes (i.e., the HR cells in WT embryos) did not express H⁺-ATPase but developed the apical openings and thus might normally express other ion transporters and enzymes, which may provide some pathway other than that mediated by H⁺-ATPase to absorb Na⁺ from environment. These alternative pathways may provide sufficient driving forces for Na⁺ uptake in zebrafish skin exposed to an environment with a higher Na⁺ level (compared with that of the low-Na⁺ condition). Recently, Esaki et al. (13) suggested that Na⁺/H⁺ exchanger is involved in Na⁺ uptake in zebrafish HR cells by using the specific inhibitor EIPA; however, the molecular evidence is still lacking. On the other hand, whether Na⁺ is absorbed from HR cells or other skin epithelial cells is still a challenging question to be answered. So far, our scanning ion-selective electrode technique is still not sensitive enough to find out the specific site of Na⁺ entrance in zebrafish skin.

Furthermore, it is interesting to note that the H⁺-ATPase knockdown also caused a significant loss of whole body Ca²⁺ in embryo acclimated to normal-Ca²⁺ or low-Ca²⁺ water. In dRTA patients, bone disease (rickets or osteomalacia) is common because of the chronic acidosis and low bicarbonate of blood result in obligate leaching of bone (22). Ca²⁺ loss appears to be a common symptom in human dRTA patients and zebrafish H⁺-ATPase-knockdown morphants. Previous studies reported that a respiratory acidosis in rainbow trout could elicit an increase in urine Ca²⁺ excretion (25). In the mammalian cortical collecting system, acidosis also inhibited Ca²⁺ influx (1). These may explain the possible mechanism behind the Ca²⁺ imbalance resulted from the H⁺-ATPase knockdown that would cause internal acidosis.

We also found the morphologies of some NaR cells in morphants were different from those in the WT and control embryos. In comparison, NaR cells were generally round and ellipsoid-shaped in WT and control embryos (Fig. 6, B and D), but they were irregular with lobe-like projections in morphants (Fig. 6, F and G). Because NaR and HR cells are two different kinds of ionocytes, it is quite interesting to see the possible pathways behind the effects of H⁺-ATPase knockdown on the cell morphology of NaR cells. On the other hand, we could not exclude the possibility that the phenotypes of the morphants may partially result from the indirect effects of the H⁺-ATPase knockdown in cells other than HR cells, since H⁺-ATPase is ubiquitously expressed and involved in many cellular processes (15, 29, 38).

dRTA is an inherited human disease caused by mutations in various subunits of H⁺-ATPase, which are highly expressed in renal intercalated cells, and thus the dRTA patients usually fail to secrete acid from distal nephrons. In this study, H⁺-ATPase-knockdown embryos revealed several abnormalities, including
suppression of acid-excretion, growth retardation, and loss of internal Na\(^+\) and Ca\(^{2+}\). Most of these are similar to the symptoms of dRTA patients. Therefore, we suggest that H\(^+\)-ATPase knockdown zebrafish may serve as an in vivo model to elucidate the mechanisms underlying the dRTA syndrome.

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GRANTS

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REFERENCES