Functional regulation of H\(^{+}\)-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment

Jiun-Lin Horng, Li-Yih Lin and Pung-Pung Hwang


**You might find this additional info useful...**

This article cites 44 articles, 23 of which can be accessed free at:

http://ajpcell.physiology.org/content/296/4/C682.full.html#ref-list-1

This article has been cited by 8 other HighWire hosted articles, the first 5 are:

1. Acid secretion by mitochondrion-rich cells of medaka (*Oryzias latipes*) acclimated to acidic freshwater
   Chia-Cheng Lin, Li-Yih Lin, Hao-Hsuan Hsu, Violette Thermes, Patrick Prunet, Jiun-Lin Horng and Pung-Pung Hwang
   [Abstract] [Full Text] [PDF]

2. Rheg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water
   Tin-Han Shih, Jiun-Lin Horng, Stan-Tai Liu, Pung-Pung Hwang and Li-Yih Lin
   [Abstract] [Full Text] [PDF]

3. New insights into ion regulation of cephalopod molluscs: a role of epidermal ionocytes in acid-base regulation during embryogenesis
   Marian Y. Hu, Yung-Che Tseng, Li-Yih Lin, Po-Yen Chen, Mireille Charmantier-Daures, Pung-Pung Hwang and Frank Melzner
   [Abstract] [Full Text] [PDF]

4. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms
   Pung-Pung Hwang, Tsung-Han Lee and Li-Yih Lin
   [Abstract] [Full Text] [PDF]

5. Functional plasticity of mitochondrion-rich cells in the skin of euryhaline medaka larvae (*Oryzias latipes*) subjected to salinity changes
   Wan-Ping Shen, Jiun-Lin Horng and Li-Yih Lin
   *Am J Physiol Regul Integr Comp Physiol*, April, 2011; 301 (4): R858-R868.
   [Abstract] [Full Text] [PDF]

Updated information and services including high resolution figures, can be found at:

http://ajpcell.physiology.org/content/296/4/C682.full.html

Additional material and information about *AJP - Cell Physiology* can be found at:

http://www.the-aps.org/publications/ajpcell

This information is current as of April 17, 2012.
Functional regulation of H\(^+\)-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment

Jiun-Lin Horng,1* Li-Yih Lin,2* and Pung-Pung Hwang1

1Institute of Cellular and Organismic Biology, Academia Sinica; and 2Department of Life Science, National Taiwan Normal University, Taipei, Taiwan, Republic of China

Submitted 10 November 2008; accepted in final form 6 February 2009

Horng JL, Lin LY, Hwang PP. Functional regulation of H\(^+\)-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment. Am J Physiol Cell Physiol 296: C682–C692, 2009. First published February 11, 2009; doi:10.1152/ajpcell.00576.2008.—It is important to maintain internal pH homeostasis in biological systems. In our previous studies, H\(^+\)-ATPase-rich (HR) cells were found to be responsible for proton secretion in the skin of zebrafish embryos during development. In this study, zebrafish embryos were exposed to acidic and basic waters to investigate the regulation of HR cell acid secretion during pH disturbances. Our results showed that the function of HR cells on the skin of zebrafish embryos can be upregulated in pH 4 water. p63 and PCNA immunostaining results also showed that additional HR cells in pH 4 water may be differentiated not only from ionocyte precursor cells but also newly proliferating epithelial stem cells.

ionocytes; acid-base regulation; ion regulation; proliferation; differentiation

INTERNAL pH is tightly controlled in all biological systems. In mammals, the kidneys play a central role in acid-base homeostasis by reabsorbing bicarbonate, excreting acids, and Regenerating bicarbonate in the proximal tubules and collecting ducts. The importance of these mechanisms has been highlighted by a number of inherited or acquired disorders in humans leading to syndromes of incomplete or complete renal tubular acidosis (8, 16). Under systemic acidosis, the density of H\(^+\)-ATPases in the apical membrane of \(\alpha\)-intercalated cells in the collecting duct increases, thus greatly enhancing the rate of proton transport into the urine (7, 46). Additionally, the number of \(\alpha\)-intercalated cells also increases while the number of base-secreting \(\beta\)-intercalated cells is reduced without a change in the total number of intercalated cells, suggesting conversion of one cell type to another for enhancing net acid secretion (3, 34, 42).

In zebrafish, the major acid-base and ion regulatory organ, it has been long proposed that secretion of endogenous acid (H\(^+\) and/or NH\(_4\)\(^+\)) is linked to transepithelial absorption of Na\(^+\), whereas the secretion of endogenous bases (HCO\(_3\)\(^-\)) and/or OH\(^-\) is linked to the transepithelial absorption of Cl\(^-\) (9, 10, 20, 39). Although there is still some debate in some species, H\(^+\)-ATPase has been demonstrated to be the major player in the apical acid secretion pathway in fish gill ionocytes (9, 10, 20, 39). Several studies have been conducted to investigate the regulatory mechanism of H\(^+\)-ATPase during acid-base disturbances. The immunoreactivity of branchial H\(^+\)-ATPase in rainbow trout gills increases (44), as do H\(^+\)-ATPase mRNA transcripts (37). During intravascular infusions of HCl, a large and sustained increase (~4- to 70-fold) of H\(^+\)-ATPase mRNA expression was observed in rainbow trout gills (40). However, only a transient increase in H\(^+\)-ATPase mRNA in rainbow trout (Onchorhyncus mykiss) gills during hypercapnia (38) and no increase and a decrease in H\(^+\)-ATPase mRNA levels were, respectively, noted in Japanese dace (Tribolodon hakonensis) exposed to pH 3.5 water (17) and in Atlantic salmon (Salmo salar) exposed to hypercapnia (41). To investigate the cell responsible for acid excretion during systemic acidosis, Goss and colleagues (14) demonstrated differential H\(^+\)-ATPase protein expression in two types of isolated branchial cell fractions [peanut agglutinin (PNA)\(^+\) and PNA\(^-\) cells], and only PNA\(^-\) cells, which show the characteristics of pavement cells, were found to specifically respond to a hypercapnic stimulus by increasing the expression of H\(^+\)-ATPase. However, how H\(^+\)-secreting cells regulate the cell number, enzyme expression, and acid secretion function in fish responding to acid-base disturbances are still unclear, and an integrative in vivo study is required to explore this critical issue.

The zebrafish embryonic epidermis is composed of three layers: a basa layer, an intermediate stratum, and a microvillar-rich superficial layer (27). Surface epidermal cells are replaced by cells from the intermediate layer upon death or injury, and thus skin cell proliferation and differentiation homeostatic mechanisms must be in place to ensure maintenance of this tissue (27). In the mammalian epidermis, p63, which marks the stem cell population (36, 49), is critically required for mammalian epidermal development (21, 28, 50). In zebrafish, only \(\Delta\)Np63, an isoform of p63, is expressed in a single layer of embryonic skin (4, 26), and epidermal cells of embryos in which \(\Delta\)Np63’s function is blocked fail to undergo proliferation after 20 h postfertilization (hpf), suggesting that \(\Delta\)Np63 maintains epidermal cells in a proliferative state (26).

A recent study (30) identified a novel type of ionocyte, H\(^+\)-ATPase-rich (HR) cells, which is responsible for proton secretion in the skin of zebrafish embryos during development. Knockdown of the translation of H\(^+\)-ATPase largely impairs proton secretion by embryos, demonstrating the importance of H\(^+\)-ATPase in acid-base regulation of zebrafish (18). Based on these facts, the present work used zebrafish embryos as an in

* J.-L. Horng and L.-Y. Lin contributed equally to this work.

Address for reprint requests and other correspondence: P.-P. Hwang, Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan, Republic of China (e-mail: pphwang@gate.sinica.edu.tw).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
vivo model to test the hypothesis of whether zebrafish can regulate cell differentiation and H\(^+-\)ATPase expression and function in HR cells to adjust the acid-base balance mechanism in response to environmental pH disturbances. In the present study, we measured the HR cell number, cell apical membrane, and cell function under systemic acidosis induced by pH 4 freshwater (FW). Moreover, markers for epidermal stem cells (p63) and dividing cells (PCNA) were used to label the skin of zebrafish embryos to further investigate the origin of the increased number of HR cells.

**MATERIALS AND METHODS**

**Zebrafish.** Mature zebrafish (AB strain) were reared in tanks with circulating tap water at 28°C. Fertilized eggs were incubated in artificial FW, which contained 0.4 mM NaCl, 0.2 mM MgSO\(_4\), 0.08 mM K\(_2\)HPO\(_4\), 0.005 mM KH\(_2\)PO\(_4\), and 0.2 mM CaSO\(_4\) (pH 6.8). The ionic composition of the zebrafish solution reflects that of the local tap water. Experimental protocols were approved by the Academia Sinica Institutional Animal Care and Use Committee (Approval no. RFZOOHP2007086).

**Acclimation to different pH environments.** For the experiments on acclimation to different pH environments, artificial FW was supplemented with 300 \(\mu\)M MES (Sigma), 300 \(\mu\)M MOPS (Sigma), or 300 \(\mu\)M tricine to prepare, respectively, pH 4, 7, and 10 artificial FW. Zebrafish eggs were transferred to artificial FW at different pH values at 28°C for 96 h, and these were sampled for further analyses. Survival rates among pH 4-, 7-, and 10-exposed embryos exhibited no significant differences. During acclimation, experimental media were changed daily to guarantee optimal water quality.

**Whole mount immunocytochemistry.** For double staining of concanavalin A (ConA) and H\(^+-\)ATPase, live larvae were preincubated in FW containing 0.5 mg/ml Alexa 568-conjugated ConA (or Alexa 488-conjugated ConA) (Molecular Probes, Eugene, OR) for 10 min. After being washed in normal FW for 3 min, ConA-labeled larvae were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 h at 4°C. After being rinsed with PBS, larvae were postfixed and permeabilized with 70% ethanol at −20°C for 10 min. After being washed with PBS, samples were incubated with 3% BSA for 30 min to block nonspecific binding. Samples were then incubated overnight at 4°C with a polyclonal antibody against the A subunit of killifish H\(^+-\)ATPase (24). After being rinsed with PBS for 20 min, larvae were further incubated in goat anti-rabbit IgG conjugated with Alexa 488 (or Alexa 568, diluted 1:100, Molecular Probes) for 2 h at room temperature (−26–28°C). For double staining of Na\(^+-\)K\(^+-\)ATPase and H\(^+-\)ATPase, embryos were fixed and permeabilized as described above. Embryos were then incubated overnight at 4°C with an α5 monoclonal antibody against the α-subunit of avian Na\(^+-\)K\(^+-\)ATPase (diluted 1:200 with PBS, Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) and a polyclonal antibody against the A subunit of killifish H\(^+-\)ATPase. After being rinsed with PBS for 20 min, larvae were further incubated in goat anti-rabbit IgG conjugated with Alexa 488 and goat anti-mouse IgG conjugated with Alexa 568 (diluted 1:100, Molecular Probes) for 2 h at room temperature. For the immunostaining of p63 (diluted 1:100, monoclonal antibody, BioGenex, San Ramon, CA) or PCNA (diluted 1:100, polyclonal antibody, CalBiochem, Tokyo, Japan), embryos were fixed and treated with the processes described above. Observations and image acquisitions were made using a Leica TCS-SP5 confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). For quanti-

![Fig. 1. Confocal whole mount immunocytochemical and scanning electron microscopy (SEM) images of skin H\(^+-\)ATPase-rich (HR) cells in zebrafish embryos at 96 h postfertilization (hpf) incubated in control freshwater (FW). A: double staining of concanavalin A (ConA; red) and H\(^+-\)ATPase (green). B: H\(^+-\)ATPase. C: spotty pattern of ConA signal at apical openings. A–C are from the same image. D: SEM image of the skin surface showing the “alveolar-type apical opening” with many small pits in HR cells. Arrows indicate the apical openings of HR cells. Scale bars = 10 μm in A–C and 8 μm in D.](image-url)
fication of the density and size of HR cells, three areas (250 × 250 μm each) on the yolk sac surface of one embryo were chosen for counting. For measurements of the size of ConA-label apical openings, image analysis with Image-Pro Plus (version 4.5, Media Cybernetics, Bethesda, MD) was conducted.

**Scanning electron microscopy.** Embryos were fixed at 4°C in cacodylate-buffered 2.5% glutaraldehyde overnight. After being rinsed with 0.1 M cacodylate buffer, specimens were postfixed with 2% osmium tetraoxide in 0.1 M cacodylate buffer for another 2 h. After being rinsed with cacodylate buffer and dehydration with ethanol, specimens were critical point dried with liquid CO2 in a critical point drier (Hitachi HCP-2, Tokyo, Japan) and sputter coated for 3 min with a gold-palladium complex in a vacuum evaporator (Cressington, Watford, UK). Coated specimens were examined by scanning electron microscopy (FEI Quanta 200, Eindhoven, The Netherlands). For the quantification of density and size of alveolar-type apical openings, five areas (90 × 78 μm each) on the yolk sac surface of one embryo were chosen for counting. For measurements of the size of ConA-label apical openings, image analysis with Image-Pro Plus (version 4.5, Media Cybernetics) was conducted.

**Cell death detection.** Cell death in zebrafish was detected by TUNEL. Embryos were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight, washed in PBS, and stored in ethanol at −20°C. After rehydration in PBS, embryos were assayed with an in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN). For the positive control, embryos were first treated with 100 units DNase I for 20 min at 37°C and then assayed by TUNEL.

**Scanning ion-selective electrode technique.** The scanning ion-selective electrode technique (SIET) was used as previously described.

**Fig. 2.** SEM observations of HR cells on the yolk sac skin of zebrafish embryos at 96 hpf acclimated to pH 4 (A), pH 7 (B), and pH 10 (C) FW. The size of the apical openings (arrows) of HR cells was significantly larger in embryos acclimated to pH 4 than to the other pH levels. Insets show magnified images. Scale bars = 10 μm in A–C and 3 μm in the insets.

**Fig. 3.** Comparison of the size and density of apical openings in HR cells in zebrafish embryos at 96 hpf acclimated to pH 4, 7, and 10 FW. Size and density were measured with SEM. Both the size (A) and density (B) of HR cell apical openings were significantly increased in pH 4-exposed embryos, and neither parameter differed between pH 7- and pH 10-exposed groups. Values in A are averages of 233, 176, and 169 apical openings from 6 embryos (n = 6) in pH 4, 7, and 10 FW, respectively, whereas values in B are average cell densities from 6 embryos/group. Values are means ± SD. a,bSignificant differences at the P < 0.05 level (one-way ANOVA followed by Tukey’s pairwise comparison).
Briefly, SIET was used to measure extracellular $H^+$ flux and activity at the surface of an intact zebrafish larva. Microelectrodes with tip diameters of $3–4\mu m$ were pulled from glass capillary tubes (no. TW 150-4 with 1.12 mm inner diameter and 1.5 mm outer diameter, World Precision Instruments, Sarasota, FL) with a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, CA). These were then baked in covered dishes at 200°C overnight and vapor silanized with dimethyl chlorosilane (Fluka, Buchs, Switzerland) for 30 min, and the covers were removed before being further baked at 200°C for at least 1 h. Capillaries were backfilled with a 1-cm column of 100 mM KCl-H$_2$PO$_4$ (pH 7.0) and then frontloaded with a 20- to 30-$\mu m$ column of liquid ion exchanger.

Fig. 4. Confocal double immunocytochemistry of ConA (green) and $H^+$-ATPase (red) and size comparisons of ConA signals in yolk sac skin of embryos at 96 hpf acclimated to pH 4, 7, and 10 FW. A: pH 4. B: pH 7. C: pH 10. D: comparison of the sizes of ConA-labeled apical openings of HR cells in embryos exposed to different pH waters. Values are averages of 153, 186, and 147 ConA-labeled apical openings from 6 embryos ($n = 6$) of pH 4, 7, and 10 FW, respectively. The size of HR cell apical membranes significantly increased in pH 4-exposed embryos. Values are means ± SD. a,b,cSignificant differences at the $P < 0.05$ level (one-way ANOVA followed by Tukey’s pairwise comparison). Scale bars = 30 $\mu m$.

Fig. 5. Confocal double immunocytochemistry of $H^+$-ATPase (green; marker for HR cells) and Na$^+$-K$^+$-ATPase [red; marker for Na$^+$-K$^+$-ATPase-rich (NaR) cells] and ionocyte density comparisons in yolk sac skin of embryos at 96 hpf acclimated to pH 4, 7, and 10 FW. A: pH 4. B: pH 7. C: pH 10. D and E: comparisons of cell densities of HR and NaR cells in embryos exposed to different pH waters. Both HR and NaR cell densities in pH 4-exposed embryos significantly increased. Values are means ± SD; $n = 10$ embryos. a,bSignificant differences at the $P < 0.05$ level (one-way ANOVA followed by Tukey’s pairwise comparison). Scale bars = 30 $\mu m$. 
record the background values of the medium. Voltage outputs were converted to H⁺ concentrations according to the three-point calibration (described above). In this study, Δ[H⁺] was used to represent measured H⁺ gradients between the target point on the skin surface and the background.

To detect local H⁺ fluxes generated by specific cells, the probe was moved to a position ~2–3 μm above the surface spot of interest. At every spot, voltage differences (in μV, which were further converted to H⁺ fluxes) were measured by probing orthogonally to the measured surface at 10-μm intervals. The calculation of H⁺ flux has been presented in a previous report (11). Briefly, voltage gradients obtained from ASET software were converted into concentration (activity) gradients using the following equation:

\[ \Delta C = C_b \times \frac{10^{\Delta V_0} - 1}{s} \]  

(1)

where \( \Delta C \) is the concentration gradient between the two points (measured in mmol·l⁻¹·cm⁻²); \( C_b \) is the background ion concentration, which was calculated as the average of the concentration at each point (measured in mmol/l); \( \Delta V \) is the voltage gradient obtained from ASET software (in μV); and \( s \) is the Nernst slope of the electrode.

The concentration gradient was subsequently converted into (extracellular) ion flux using Fick’s law of diffusion with the following equation:

\[ J = D(\Delta C)/\Delta X \]  

(2)

where \( J \) is the net flux of the ion (in pmol·cm⁻²·s⁻¹), \( D \) is the diffusion coefficient of the ion (9.4 × 10⁻⁵ cm²/s for H⁺); \( \Delta C \) is the concentration gradient (in pmol/cm³), and \( \Delta X \) is the distance between the two points (measured in cm).

**Measurement of whole body ion contents.** Ten zebrafish larvae were briefly rinsed in deionized water, pooled as one sample, and the weighed. HNO₃ (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⁺, K⁺, and Ca²⁺ content was measured with an atomic absorption spectrophotometer (Z-8000, Hitachi, Tokyo, Japan). For Cl⁻ content measurements, samples were homogenized with 1 ml deionized water and centrifuged at 14,000 rpm for 30 min. The supernatant collected was supplemented with Hg(SCN₄)₂ (0.3 g in 95% ethanol) and NH₄Fe(SO₄)₂·12H₂O (30 g in 135 ml of 6 N HNO₃) solutions for analysis. The Cl⁻ concentration was measured by the ferricyanide method (12) with a double-beam spectrophotometer (U-2000, Hitachi). Standard solutions of Na⁺, K⁺, Cl⁻, and Ca²⁺ from Merck (Darmstadt, Germany) were used to make the standard curves.

**Statistical analysis.** Values are presented as means ± SD and were compared using Student’s t-test or one-way ANOVA (Tukey’s pairwise comparison).

**RESULTS**

**Identification of the apical structure of HR cells.** ConA is a lectin that specifically binds to α-mannopyranosyl and α-gluco- pyranosyl residues of glycosylated membrane proteins and

---

**Table 1. Ion contents in embryos at 96 h postfertilization acclimated to freshwater with various pH values**

<table>
<thead>
<tr>
<th>Ion Content, mM</th>
<th>pH 4</th>
<th>pH 7</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>22.4±2.1*</td>
<td>23.9±2.0*</td>
<td>32.5±7.1†</td>
</tr>
<tr>
<td>K⁺</td>
<td>38.4±2.2*</td>
<td>47.7±3.6†</td>
<td>48.0±3.3†</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.5±0.5*</td>
<td>9.8±1.7†</td>
<td>10.2±2.0†</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>18.7±1.6*</td>
<td>20.4±2.5*</td>
<td>18.9±2.4*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 embryos. One-way ANOVA (Tukey’s pairwise comparison) was conducted among different pH groups (*, †significant difference).
has been used as an apical membrane marker of HR cells (18, 30). Using ConA and H⁺-ATPase double staining, ConA signals revealed a spotty distribution at apical openings (Fig. 1, A–C). The spotty ConA signals reflect an “alveolar type” of apical opening, which was composed of small pits under scanning electron microscopy observation (Fig. 1, D). This type of opening was mainly distributed on the embryonic yolk sac skin, where HR cells are located (18, 30). Therefore, this type of opening was identified as the opening of HR cells.

Size and density of HR cells in larvae acclimated to different pH waters. Openings of HR cells (the alveolar type) were examined by scanning electron microscopy to compare their size and density in embryos acclimated to pH 4, 7, and 10 water. The openings of pH 4-exposed embryos were significantly larger in size and higher in density than those of pH 7- and pH 10-exposed embryos (Figs. 2 and 3). However, no significant differences were found between pH 10- and pH 7-exposed embryos (Fig. 3).

Similar results were found with confocal microscopy. The size of the ConA-labeled opening was significantly higher in pH 4-exposed embryos than in pH 7- or pH 10-exposed embryos (Fig. 4). The density of HR cells also increased by ~50% in pH 4-exposed embryos (Fig. 5D). In addition, the density of Na⁺-K⁺-ATPase-rich (NaR) cells (30) also increased by ~40% in pH 4-exposed embryos (Fig. 5E). However, no significant differences in density were found between pH 10- and pH 7-exposed embryos (Fig. 5).

Body length, ionic contents, and acid secretion of embryos acclimated to different pH waters. An electrophysiological approach, SIET, was used to compare the surface Δ[H⁺] of embryos acclimated to pH 4, 7, or 10 FW. The results showed that the Δ[H⁺] of pH 4-exposed embryos was significantly higher than that of pH 7- or pH 10-exposed embryos (Fig. 6C). The body length of pH 4-exposed embryos was shorter than those of pH 7- and pH 10-exposed embryos (Fig. 6, A and B). However, no significant differences were found between pH 7- and pH 10-exposed embryos (Fig. 6). These results indicate that H⁺ secretion was upregulated in pH 4-exposed embryos even though they demonstrated growth retardation.

Whole body Ca²⁺ and K⁺ contents of pH 4-exposed embryos were significantly lower than those of pH 7- or pH 10-exposed embryos (Table 1). The Ca²⁺ content in pH 4-exposed embryos was only ~15% of that in pH 7-exposed embryos. Na⁺ and Cl⁻ contents did not significantly differ.
between pH 4- and pH 7-exposed embryos. In addition, Na⁺ content increased by ~35% in pH 10-exposed embryos compared with pH 7-exposed embryos.

**H⁺ fluxes of HR cells in embryos acclimated to different pH waters.** SIET was also used to measure the H⁺ flux at the opening of HR cells. Larger apical openings were visualized in pH 4-exposed embryos by differential interference contrast microscopy with SIET probing (Fig. 7, A and B). Fluorescent ConA staining was further conducted after probing to confirm

![Image](image_url)
the presence of HR cells (Fig. 7, A and B). As shown in Fig. 7, A and B, more cells were labeled with ConA (i.e., HR cells) in pH 4-exposed embryos than in pH 7-exposed embryos. Figure 7, C and D, shows that proton fluxes at HR cells (labeled with ConA) were higher than those at other cells in both groups of zebrafish. Moreover, the H+ flux at each HR cell was obviously higher in pH 4-exposed embryos than in pH 7-exposed embryos (Fig. 7, C–E).

Turnover of skin epithelial cells in embryos acclimated to different pH waters. To determine the turnover of skin epithelial cells of embryos, p63 and PCNA immunostaining and a TUNEL assay were conducted in embryos at 24, 30, and 96 hpf. p63 is a marker of epithelial stem cells, which have been demonstrated to differentiate into skin ionocytes and keratinocytes in zebrafish (19, 23). PCNA, a marker of cell division, is expressed in the S phase of the cell cycle. The TUNEL assay labeled apoptotic cells. As shown in Fig. 8, p63+ cells were distributed all over the skin of embryos. The density of p63+ cells on the yolk sac skin had increased in pH 4-exposed embryos at 30 and 96 hpf (Fig. 8). At 96 hpf, PCNA+ cells on the yolk sac skin had significantly increased in pH 4-exposed embryos (Fig. 9). The z-plane images showed that PCNA+ cells were located at the skin surface of the yolk sac (Fig. 9, A, x-z and y-z, and B, x-z and y-z). However, the density of apoptotic cells on the yolk sac skin did not differ between pH 4- and pH 7-exposed embryos (Fig. 10). In subsequent double immunocytochemistry of p63 and PCNA, some of the p63+ cells were found to be colocalized with PCNA, indicating proliferating epidermal stem cells (Fig. 11, A–C).

DISCUSSION

The skin of zebrafish embryo serves several physiological functions, including gas exchange, NH3/NH4+ excretion, ion uptake, and metabolic acid secretion (18, 30, 33, 35, 43, 48). The present study clearly demonstrates, for the first time, that the function of skin HR cells in zebrafish embryos is upregulated in an acidic environment by increasing the cell number through cell proliferation and differentiation and also by enhancing the acid-secreting function of single cells. These results provide new insights into the functional regulation mechanism of acid secretion in fish during acclimation to environmental pH challenges.

Morphological changes in the apical membrane of mitochondrion-rich (MR) cells, major ionocytes in gills, have been reported in several species of FW fishes in response to ambient ionic compositions (for a review, see Ref. 20). Enlargement of the apical surface of MR cells was found in killifish acclimated to low-NaCl water (24, 25). Both the apical surface and cell density of MR cells increased in tilapia acclimated to low-Ca2+ or low-Ca2+/HCO3− artificial FW (5, 6, 31, 32). There were 30% and 85% reductions in the apical area of MR cells during hypercapnia in rainbow trout and brown bullhead catfish, respectively (15). These correlations between ion uptake and apical surface area/cell numbers provide important but indirect evidence for MR cell functions and regulation during challenges with acidic or ion-deficient FW. In the present study, we provide direct evidence to elucidate that cells’ acid-secreting function (at a single cell level) of the apical surface of active HR cells responds to pH 4 water through acclimation processes.

According to our results, embryos seemed to be more sensitive to acidic than to alkaline waters in terms of HR cell functional enhancement and growth retardation. Moreover, no significant downregulation of acid secretion or HR cell activities was found in embryos developed in alkaline FW (Fig. 6). A similar situation was also found in rainbow trout; metabolic alkalosis had no significant effect on H+-ATPase expression or acidosis-sensitive PNA− MR cells (13). However, in another case, metabolic alkalosis triggered H+-ATPase translocation in hagfish gills, which enhanced HCO3− secretion (45). Further studies are required to investigate if other regulatory mechanisms are induced in zebrafish embryos during alkalosis.

During acidic acclimation, the embryonic ion content was examined and showed a remarkable deficiency in Ca2+ content (Table 1), which was also found in H+-ATPase knockdown embryos (18), implying that embryonic acidosis may also

![Fig. 10. Confocal TUNEL assay (red) and density comparison of TUNEL-positive cells in the yolk sac skin of embryos at 96 hpf acclimated to pH 4 and 7 FW. A and B: TUNEL-positive cells on the yolk sac skin of pH 4- and pH 7-exposed embryos. C: DNase treatment (positive control). D: no significant differences in the density of TUNEL-positive cells between pH 4- and pH 7-exposed embryos were observed. Values are means ± SD; n = 5 embryos. P > 0.05 (Student’s t-test). Scale bars = 100 μm.](image-url)
Previous studies (33, 48) that reported reverse regulations of the functions of acid secretion and Na$^+$ uptake in zebrafish HR cells.

In the collecting duct of kidneys, the conversion of β$\text{-}$ to α-intercalated cells under systemic acidosis induced by feeding animals an acidic diet is mediated by hensin. The addition of anti-hensin antibodies prevents the acid-induced translocation of the Cl$^-$/HCO$_3$- exchanger from the apical to basolateral membrane, which is a conversion of β$\text{-}$ to α-intercalated cells (1, 47). Recently, the transformation of β$\text{-}$ to α-intercalated cells was thought to be a terminal differentiation from “proto-epithelium,” a single-layered structure with polarized localization of membrane proteins and lipids, to a “more mature epithelium” that exhibits microvilli and endocytosis on apical membranes (2, 47); hensin has been indicated as being involved in controlling this epithelial terminal differentiation (1, 2). In zebrafish embryos, HR cells are responsible for excreting metabolic acids, similar to α-intercalated cells in mammalian kidneys. The increased HR cell number during systemic acidosis is achieved by cell proliferation and cell differentiation; therefore, it would be interesting, in the future, to investigate if hensin, which is expressed in many tissues, including the kidneys, skin, gastrointestinal tract, lungs, and neurons (2, 47), also mediates this process in zebrafish HR cells.

After acid acclimation, the cell densities of both $p63^+$ and PCNA$^+$ cells increased. The extents of the increases in densities of HR and NaR cells were higher than that of $p63^+$ (or PCNA$^+$) cell density, suggesting that the increased ionocyte cell number may be involved in both the differentiation of precursor cells and proliferation of epithelial stem cells (Figs. 5, 8C, and 9C). Previous studies (19, 23, 29) have shown that during zebrafish development, the skin ionocyte density is regulated by Delta-Notch signaling, which constitutes a system of lateral inhibition used for singling out particular cell types during many different processes of vertebrate and invertebrate development. In zebrafish mindbomb mutants that fail to signal through the Delta/Jagged-Notch pathway due to the absence of a Delta/Jagged ubiquitin ligase (22), both NaR and HR cells significantly increased (19, 23). Although the cellular signaling mechanism behind the increase in HR cell numbers in pH 4-exposed embryos is still unknown, the present study indicates that the epithelial development mechanism can be adjusted during acclimation to different environments.

In summary, the present study, using electron microscopic, immunocytochemical, and electrophysiological approaches, provides direct evidence to demonstrate that apical membrane enlargement in concert with enhanced proton-secreting activity in single HR cells and the increase in the HR cell population are induced to counteract systemic metabolic acidosis. The increase in HR cells may originate from the differentiation of ionocyte precursor cells and the proliferation of epithelial stem cells. Our results provide a clear and comprehensive regulatory mechanism for HR cells under acid environmental challenge.

**ACKNOWLEDGMENTS**

We thank Y. C. Tung and J. I. Wang for the assistance during the experiments and the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica, for assistance in confocal microscopy. We also extend our thanks to Dr. T. Kenako (University of Tokyo) for providing the H$^+$-ATPase antibody.


