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Effects of hypothermia on gene expression in zebrafish gills: upregulation in differentiation and function of ionocytes as compensatory responses

Ming-Yi Chou[1,2], Chung-Der Hsiao[1,3], Shyh-Chi Chen[1], I-Wen Chen[1], Sian-Tai Liu[1] and Pung-Pung Hwang[1,2,*]

[1]Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, 115 Taiwan, [2]Institute of Fishery Science, National Taiwan University, Taipei, 10617 Taiwan and [3]Department of Bioscience Technology, Chung Yuan Christian University, Chung Li, 32023 Taiwan

*Author for correspondence (e-mail: pphwang@gate.sinica.edu.tw)

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SUMMARY

Ectothermic vertebrates are different from mammals that are sensitive to hypothermia and have to maintain core temperature for survival. Why and how ectothermic animals survive, grow and reproduce in low temperature have been for a long time a scientifically challenging and important inquiry to biologists. We used a microarray to profile the gill transcriptome in zebrafish (*Danio rerio*) after exposure to low temperature. Adult zebrafish were acclimated to a low temperature of 12°C for 1 day and up to 30 days, and the gill transcriptome was compared with that of control fish in 28°C by oligonucleotide microarray hybridization. Results showed 11 and 22 transcripts were found to be upregulated, whereas 56 and 70 transcripts were downregulated by low-temperature treatment for 1 day and 30 days, respectively. The gill transcriptome profiles revealed that ionoregulation-related genes were highly upregulated in cold-acclimated zebrafish. This paved the way to investigate the role of ionoregulatory genes in zebrafish gills during cold acclimation. Cold acclimation caused upregulation of genes that are essential for ionocyte specification, differentiation, ionoregulation, acid–base balance and the number of cells expressing these genes increased. For instance, epithelial Ca^{2+} channel (EcaC; an ionoregulatory protein) mRNA increased in parallel with the level of Ca^{2+} influx, revealing a functional compensation after long-term acclimation to cold. Phosphohistone H3 and TUNEL staining showed that the cell turnover rate was retarded in cold-acclimated gills. Altogether, these results suggest that gills may sustain their functions by producing mature ionocytes from pre-existing undifferentiated progenitors in low-temperature environments.

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Key words: acclimation, cold, differentiation, ionocyte, transcriptome, transporter.

INTRODUCTION

Temperature is one of the most important environmental factors, as it determines the distribution, behaviors and physiological responses of animals. Temperature also affects longevity in insects (Sohal and Allen, 1986), fish (Guderley, 1990; Malek et al., 2004), primates and humans (Buffenstein, 2005; Roth et al., 2002). At the molecular and cellular levels, low temperatures strongly reduce the rates of enzymatic reactions, diffusion and transport, induce the denaturation and misaggregation of proteins, inhibit transcription and translation, disrupt cellular cytoskeletal elements, and change membrane permeability (Sonna et al., 2002). Cold stress also alters the properties of the lipid bilayer including phase transitions and fatty acid composition (Hsieh and Kuo, 2005).

In contrast to endothermic mammals, ectothermic vertebrates such as fish can survive in a wide range of thermal environments, and thus fish cells may utilize different strategies to cope with thermal fluctuations. Many physiological responses, including changes in lipid composition (Hazel, 1979), increases in pump activity and specific Na^+/K^+-ATPase activity (Schwarzbaum et al., 1992a; Schwarzbaum et al., 1992b) and oxygen consumption (Raynard and Cossins, 1991), have been extensively reported when fish are exposed to cold stress, however there still remain several interesting issues for further studies.

Ambient temperatures have a direct and critical impact on the physiological functioning of fish; hence, fish are excellent experimental organisms in which to investigate responses to environmental stresses (Cossins and Crawford, 2005). To cope with fluctuating temperatures, fish can increase the quantity of enzymes required for different physiological tasks, or can express protein isoforms that are more suitable for changing thermal conditions (Cossins et al., 2002; Driedzic and Ewart, 2004; Johnston et al., 1990; Watabe, 2002). Cold stress triggers a complex program of gene expression and biochemical responses in different tissues (Gracey et al., 2004; Ju et al., 2002; Malek et al., 2004; Vornanen et al., 2005). The gills are the first target organ for environmental stress because they are directly exposed to the ambient environment. Fish gills have multiple functions including gas exchange, ion regulation, nitrogen balance and acid–base adjustment. Gill ionocytes are the major response site for osmo- and pH regulation (Evans et al., 2005), and the cell cycle of gill cells is about 4–5 days (Tsai and Hwang, 1998), indicative of a higher cell turnover rate of tissues with complex and critical physiological activities. Moreover, gills showed great morphological plasticity when acclimated to different ambient salinity, ionic composition, oxygen and temperature conditions (Chang and Hwang, 2004; Chang et al., 2001; Sollid and Nilsson, 2006). Therefore, gills may provide a suitable target for physiological responses to environmental changes.

Zebrafish (*Danio rerio*), a tropical teleost species, can survive in low-temperature (18°C) environments for 1 year and show normal motor activity compared to control fish at 28°C (Malek et al., 2004).

Tang and colleagues also reported that zebrafish can survive in water less than 12°C for at least 48 h (Tang et al., 1999). In contrast to other species, zebrafish are small, and the genetic resources of this model species are well known and abundant (Malek et al., 2004). Zebrafish are therefore suitable for use as a model animal to study the mechanisms of acclimation to low temperatures in fish. The purpose of this study was to use zebrafish as a model to investigate the effects of low temperature on the functions of gill cells. We used microarray technology to compare the gill transcriptome between acute and chronic low-temperature treatments. A group of upregulated genes related to ion balance and acid–base regulation mechanisms in branchial ionocytes were identified. We demonstrated that elevation of Ca^{2+} influx was accompanied by an increase of *trpv6* (epithelial Ca^{2+} channel) expression. Moreover, we found cell proliferation and apoptosis in zebrafish gill cells after cold stress. These data provide functional genomic and physiological evidences for cold acclimation in fish gills.

MATERIALS AND METHODS

Experimental animals and low-temperature acclimation

The *AB* strain of zebrafish (*Danio rerio* Hamilton) were originally obtained from the University of Oregon, and were kept in the zebrafish stock center at Academia Sinica, Taipei, Taiwan. Fish were reared in local tap water at 28°C and a photoperiod regime of 14 h:10 h L:D. Fish were incubated in several experimental tanks placed inside a water bath to maintain a constant temperature. The water qualities such as pH, dissolved oxygen and ion concentration were monitored daily, and were maintained by circulation with a filtration system, aeration and renewal of some of fresh water every 3 days. For acclimation of adult zebrafish to 12°C, the temperature was gradually reduced from 28°C at a gradient of 3°C h^{-1} in order to prevent temperature shock and reduce mortality. After 30 days of acclimation, surviving (80% survival rate) fish appeared to be feeding and behaving normally compared with control fish.

A total of 192 surviving fish were sacrificed for the subsequent microarray and quantitative reverse-transcription polymerase chain reaction (qRT–PCR) analysis. In order to obtain sufficient quantity of RNA, the whole gills from both sides of six individuals (three male and three female fish) were pooled as a sample. For microarray experiments, 18 fish were incubated in three different experimental tanks (i.e. N=3) at 12°C for 1 day and another 18 fish were incubated for 30 d. Control groups with 18 fish for 1 d and another 18 fish for 30 d were both incubated in 28°C. For qRT–PCR, 30 fish were incubated in 5 different experimental tanks (i.e. N=5) at 12°C for 1 day and another 30 were incubated for 30 days. Another 60 fish for the controls were treated as described above except the acclimation temperature.

For whole-mount *in situ* hybridization, immunohistochemistry and TUNEL assay, four fish (N=4) were used for each test group. In western blotting experiment, 48 fish, 24 for cold treatment and 24 for control (gills from six fish were pooled as a sample, i.e. N=4), were sacrificed for protein isolation. Fish were anesthetized with 100–200 mg l^{-1} of buffered MS-222 (3-aminobenzoic acid ethyl ester; Sigma, St Louis, MO, USA) before sampling following, guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFiZOOHP2007086).

RNA extraction

Dissected and pooled gill tissues were homogenized in 5 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). After chloroform extraction, RNA precipitation and ethanol washing, the RNA samples were purified and treated with DNase1 to remove the genomic DNA by using RNeasy Mini Kit (Qiagen, Huntsville, AL, USA). The quantity and quality of total RNA were assessed by spectrophotometry and agarose gel electrophoresis, respectively.

Microarray hybridization and data analysis

The commercial zebrafish 14K oligonucleotide set (MWG Biotech AG, Ebersbach, Germany) were obtained and were printed on an UltraGAPS-coated slide (Corning, New York, NY, USA) with the use of the OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI, USA) according to the manufacturer's instructions. The 14 067 oligonucleotides represent 9666 genes (7009 singlet genes and 2657 redundant genes), and the redundancy of this chip is 31%. The detailed description of the oligonucleotide information can be obtained on the Ocimun Biosolution website (http://www.ocimumbio.com/web/default.asp).

cDNA probes were synthesized by reverse transcription of 20 μg total RNA using a SuperScript indirect cDNA labeling system (Invitrogen) and were labeled with Cy5 (cold treatment groups) and Cy3 (control groups) (Amersham Bioscience, Buckinghamshire, UK), respectively. The zebrafish 14K OciChip array chip was pretreated with 1% bovine serum albumin (BSA; fraction V), 4× SSC buffer and 1% sodium dodecylsulfate (SDS) at 42°C for 45 min, and then hybridized overnight in a cocktail containing 5× Denhardt's solution, 6× SSC, 0.5% SDS, 50% formamide, 50 mmol l^{-1} sodium phosphate and 2 μg l^{-1} yeast tRNA. Slides were washed with 2× SSC and 0.1% SDS (5 min), 1× SSC and 0.1% SDS (5 min), 0.5× SSC (5 min), and twice with 0.1× SSC (2 min each). Scanning was performed with a Genepix scanner (Molecular Devices, Sunnyvale, CA, USA). The acquired images were analyzed using Genepix and Genespring software (Aglient Technologies, Foster City, CA, USA). The measurements of spots were filtered by flags, and the Lowess normalization was performed after subtraction of the median background. Each experiment contained three biological replicates (including one dye swap) with different samples. In total six chips (three chips for 1 day and three chips for 30 days) were used for microarray hybridization experiments, and two (one chip for 1 day and one chip for 30 days) of the six chips were used for dye swap. Thus, 12 biological replicates were used for hybridization including dye swaps. The differentially expressed genes were selected from those with at least two of three significant signals (ratio >2 or <0.5), and then the Significant Analysis of Microarray method was used to determine statistical significances. Data were submitted to NCBI Gene Expression Omnibus (series accession no. GSE7853).

Validation of differentially expressed genes by quantitative reverse-transcription polymerase chain reaction (qRT–PCR)

We used *cold-inducible RNA binding protein* (*cirbp*, BC057481) and *high-mobility group box 1* (*hmgb1*, NM_199555) to test the general responses to cold. To confirm the differentially expressed genes collected from the microarray analysis, we selected nine transcripts including *Ca^{2+} transporting, cardiac muscle, fast twitch 1* (*atp2a1*, NM_001007029), *Ca^{2+} ATPase, cardiac muscle, fast twitch 1 like* (*atp2a1l*, NM_001077533), *tubulin alpha 8 like 2* (*tuba8l2*, NM_200691), *GTP binding protein 4* (*gtpbp4*, NM_199851), *Na^{+}/K^{+}-ATPase, alpha 1a.4 polypeptide* (*atp1a1a.4*, NM_131689), *glycogen synthase kinase binding protein* (*gbp*, NM_131442), *V-ATPase subunit A* (*atp6v1a*, NM_201135), *keratin 18* (*krt18*, NM_178437) and *annexin A2a* (*anxa2a*, NM_181761) to validate their relative expression levels by quantitative RT–PCR (N=5). We also measured the mRNA expressional levels of ionocyte-related genes, such as *forkhead box I3a* (*foxi3a*, NM_198917), *N-*

myc downstream-regulated gene 1 (*ndrg1*, NM_213348), *carbonic anhydrase II* (*ca2*, NM_199215), *delta C* (*dlc*, NM_130944), *Na^+/K^+-ATPase, beta 1b polypeptide* (*atp1b1b*, NM_131671), *Na^+/K^+-ATPase, alpha 1a.2 subunit* (*atp1a1a.2*, NM_131687), *Na^+/Cl^- cotransporter* (*slc12a3*, EF591989), *carbonic anhydrase 15a isoform* (*ca15a* EF591981), *glial cells missing homolog 2* (*gcm2*, NM_001005603), and epithelial Ca^{2+} channel (*trpv6*, NM_001001849), to systematically analyze the expressional profiles of ionoregulatory genes. As an internal control, primers for β-actin (NM_131031) were designed and amplified in parallel with the genes of interest. Quantitative reverse-transcription PCR (qRT–PCR) was carried out using a SYBR Green dye (Qiagen, Hilden, Germany)-based assay with an ABI Prism 7000 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA, USA) according to the manufacturer's instructions. Primer targeting was designed using Primer Express 2.0 software (Applied Biosystems). The primer sequences are listed in supplementary material Table S1.

RNA probe synthesis

Two zebrafish nucleotide fragments from, *trpv6* and *ca2*, were obtained by PCR and inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). Purified plasmids were then linearized by restriction enzyme digestion, and *in vitro* transcription was carried out with T7 or SP6 RNA polymerase (Roche, Penzberg, Germany) in the presence of digoxigenin (Dig)-UTP. Dig-labeled RNA probes were examined with RNA gels and a dot-blot assay to confirm the quality and concentration. For the dot-blot assay, the synthesized probes and standard RNA probes were spotted onto nitrocellulose membranes according to the manufacturer's instructions (Dig RNA labeling kit; Roche Diagnostics, Mannheim, Germany). After cross-linking and blocking, the membranes were incubated with an alkaline phosphatase-conjugated anti-dig antibody and stained with nitro blue tetrazolium (NBT; Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche).

Whole-mount *in situ* hybridization

Zebrafish gills were fixed with 4% paraformaldehyde overnight at 4°C, and then washed several times with phosphate-buffered saline (PBS). Fixed samples were rinsed with PBST (PBS with 0.2% Tween 20, 1.4 mmol l^{-1} NaCl, 0.2 mmol l^{-1} KCl, 0.1 mmol l^{-1} Na_2HPO_4, and 0.002 mmol l^{-1} KH_2PO_4; pH 7.4). After a brief washing with PBST, gill filaments were incubated with hybridization buffer (HyB) containing 60% formamide, 5× SSC, and 0.1% Tween 20 for 5 min at 65°C. Prehybridization was performed in HyB^+ (the hybridization buffer supplemented with 500 μg ml^{-1} yeast tRNA and 50 μg ml^{-1} heparin) for 2 h at 65°C. After prehybridization, samples were hybridized in 100 ng of the RNA probe in 200 μl of HyB^+ at 65°C overnight. Gills were then washed at 65°C for 10 min in 75% HyB and 25% 2× SSC, for 10 min in 50% HyB and 50% 2× SSC, for 10 min in 25% HyB and 75% 2× SSC, for 10 min in 2× SSC, and twice for 30 min each in 0.2× SSC at 70°C. Further washes were performed at room temperature for 5 min in 75% 0.2× SSC and 25% PBST, for 5 min in 50% 0.2× SSC and 50% PBST, for 5 min in 25% 0.2× SSC and 75% PBST, and for 5 min in PBST. After serial washings, gill filaments were incubated in blocking solution containing 5% sheep serum and 2 mg ml^{-1} BSA in PBST for 2 h and then incubated in the 1:10 000-diluted alkaline phosphatase-conjugated anti-dig antibody for another 16 h at 4°C. After the reaction, samples were washed with PBST plus blocking reagent and then stained with NBT and BCIP.

Measurement of whole body Ca^{2+} influx

Whole body Ca^{2+} influx was measured following the method of Chang and Hwang (Chang and Hwang, 2004) with some modifications. Control and cold-acclimated fish were transferred to 20 ml [$^{45}Ca^{2+}$]-containing medium for 5.5 h incubation. Following the incubation, 200 μl water medium were sampled with addition of 2 ml counting solution (Ultima Gold, Packard, Waltham, MA, USA), and then the radioactivities of the solutions were determined with a LS6500 beta counter (Beckman, CA, USA). The $^{45}Ca^{2+}$ influx was calculated using the following formula:

$$J_i = (Q_i \times V_i - Q_f \times V_f)/[1/2 \times (SA_i + SA_f) \times t \times W] ,$$

where J_i is the influx (nmol g^{-1} h^{-1}), Q_i and Q_f (c.p.m. ml^{-1}) refer to the initial and final radioactivities in the tracer media; V_i and V_f (ml) refer to the initial and final volumes of the tracer media; SA_i and SA_f (c.p.m. $nmole^{-1}$) are the specific activities of the incubation medium, t is the incubation time (h), and W is the body wet mass of zebrafish (g).

Immunohistochemistry

Zebrafish gills were fixed in 4% paraformaldehyde for 12 h at 4°C. After being washed in PBS, fixed gills were treated with 100% methanol for 10 min at –20°C and subsequently subjected to blocking with 3% BSA at room temperature for 60 min. Gill filaments were then incubated with a polyclonal antibody against a short amino acid sequence containing phosphorylated Ser 10 of histone H3 of human origin (phosphohistone H3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:200, at 4°C for 16 h. Samples were washed twice in PBS for 10 min each and then incubated with 1:200 PBS-diluted Rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature.

Western blotting

The gills were homogenized in homogenization solution (100 mmol l^{-1} imidazole, 5 mmol l^{-1} EDTA, 200 mmol l^{-1} sucrose, 0.1% sodium deoxycholate; pH 7.6) and subjected to polyacrylamide gel electrophoresis (PAGE) in 8×10 cm sodium dodecylsulfate (SDS)-polyacrylamide (8%) gels at 100 V for 2 h. Protein was loaded at 30 μg per well. Separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) with a transfer electrophoresis unit (SE 600, Hoffer) at 100 V for 2 h. After blocking for 1.5 h in 5% nonfat dried milk, the blots were incubated with phosphohistone H3 antibodies (overnight, diluted 1:1000). After incubation, the membranes were washed in PBST and reacted for 90 min with an alkaline-phosphatase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; diluted 1:3000). Blots were extensively washed with PBST and then developed with 0.015% NBT and 0.007% bromochloroindolyl phosphate in a reaction buffer containing 100 mmol l^{-1} Tris, 100 mmol l^{-1} NaCl and 5 mmol l^{-1} $MgCl_2$ (pH 9.5).

TUNEL assay

The *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) was used for TUNEL detection of DNA fragmentation. Zebrafish gills were fixed in 4% paraformaldehyde and stored in methanol at –20°C. Zebrafish gills were incubated with 3% H_2O_2 to block endogenous peroxidases, washed, and treated with 10 g ml^{-1} proteinase K at, 37°C for 15 min. Then, gill samples were incubated with fluorescein-conjugated nucleotides and terminal deoxynucleotidyl transferase at 37°C for 1 h. After application of

the anti-fluorescein antibody conjugated with peroxidase at 37°C for 30 min, the 3,3′-diaminobenzidine (DAB) staining method was performed at 25°C for 2–5 min.

Equipment (image acquisition)

Bright-field and fluorescence images were acquired with a Zeiss Axioplan 2 Imaging MOT microscope (Carl Zeiss, Göttingen, Germany). The *z*-axis series images were captured with a Zeiss Axiocam HRm cool CCD (Carl Zeiss) with AxioVision LE Rel 4.3 software (Carl Zeiss) and then merged by Helicon Focus 4.01.1 (Helicon Soft Ltd, Kharkov, Ukraine).

Statistical analysis

Significant analysis of microarray (SAM) method was used for microarray analysis, and one-way ANOVA was used for the other analysis. For qRT-PCR analysis, controls did not show significant differences between 1 day and 30 days, therefore only the 1 day data was used for the subsequent comparisons.

RESULTS

General cold responses in zebrafish gill

Two cold-inducible genes, *cirbp* and *hmgb1*, were used to test the general cold responses in zebrafish gill. The expression of *hmgb1* mRNA was strongly upregulated by cold treatment for 1 day and 30 days, whereas *cirbp* was induced only by 1 day cold treatment (Fig. 1).

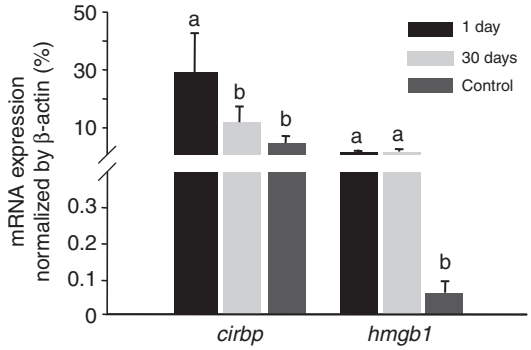

Fig. 1. Expression of *cirbp* and *hmgb1* mRNA in zebrafish gills. After cold treatment for 1 day or 30 days, expression of *cirbp* and *hmgb1* mRNAs was strongly induced. The values were normalized to β-actin. Values are means ± s.d. (*N*=4 or 5). Different letters above the bars indicate significant differences (one-way ANOVA, Tukey's pair-wise comparison).

Overview of gill transcriptome in cold-acclimated gills

In the short-term cold-acclimation experiment, 11 transcripts were found to be upregulated by low-temperature treatment, while 56 were downregulated. By contrast, long-term acclimation caused 22 transcripts to be upregulated and 70 were downregulated. The regulated genes are listed in Table S2 in the supplementary material. We selected five cold-induced genes (*atp2a1*, *atp2a1l*, *gtpbp4*, *tuba8l2* and *atp6v1a*) and four cold-suppressed genes (*krt18*, *anxa2a*, *atp1a1a.4* and *gbp*) to perform quantitative reverse-transcription polymerase chain reaction (qRT-PCR). As a result, the changes in mRNA levels obtained from the qRT-PCR were consistent with the microarray data (Fig. 2).

To better understand the transcriptome in cold-acclimated gills, we functionally categorized the differentially expressed genes according to their gene ontology (GO). Only two-thirds of the differentially expressed genes could be annotated with GO terms. The other un-annotated differentially expressed genes, encoding novel genes or expressing sequence tags, may be involved in as yet uncharacterized pathways of cold acclimation in zebrafish (supplementary material Table S2).

There were more cold-suppressed genes than cold-induced genes. The largest GO category of both cold-suppressed and cold-induced transcripts was cellular metabolism; however, the components of cellular metabolism differed between the suppressed and induced groups.

Effects of low temperature treatment on expression of ionocyte-related genes and whole body Ca^{2+} influx

According to the data set of the cold-induced transcripts, *atp6v1a* is involved in branchial ion transport and acid–base regulation mechanisms in gill ionocytes. Because cold has been reported to affect transport mechanisms and disrupt ion and acid–base homeostasis (Hochachka, 1986; Hochachka, 1988), it was hypothesized that zebrafish may globally activate ion and acid–base balance mechanisms to compensate for the passive lose of ions and the imbalance in acid–base regulation. To test this hypothesis, the mRNA expression levels of ionocyte-related genes including *ca2*, *atp6v1a*, *atp1b1b*, *atp1a1a.2*, *slc12a3*, *ca15a* and *trpv6* were examined. As expected, all the ionocyte marker genes were significantly upregulated during cold acclimation (summarized in Table 1). A further question asked was whether cold stress affects the spatial distribution of ionocyte marker gene expression. In whole-mount *in situ* hybridization experiments, expression of *trpv6* and *ca2* mRNAs (markers of gill ionocytes) were evidently increased, and notably, *trpv6*- and *ca2*-expressing cells were found in the lamellae in addition to the gill filaments (Fig. 3), whereas in the control group, these cells only appeared in gill filaments. In further experiments, the function of one of these genes, *trpv6*, was analyzed. Whole body Ca^{2+} influx in zebrafish was retarded about 60% by 12°C treatment for 9 h compared with the control group at 28°C, but was able to recover to the normal level after subsequent acclimation to 12°C for 30 days (Fig. 4).

The differentiation of gill ionocytes was also investigated to determine whether they are affected during acclimation to low temperature. As shown in Table 1, expressions of *dlc*, *ndrg1*, *gcm2* and *foxi3a* mRNAs, which may be involved in ionocyte differentiation (Hsiao et al., 2007; Jones et al., 1995; Yu et al., 1999), were all upregulated after acclimation to low temperature (Table 1).

Effects of temperature reduction on cell proliferation and apoptosis in zebrafish gills

For evaluation of cell proliferation rate, an M-phase cell cycle marker, phosphohistone H3, was used for immunohistochemistry and western blot in gills. Cell proliferation rate appeared to slow down after low temperature treatment for 30 days (Fig. 5A). Western blot result indicated that the intensity of phosphohistone H3 immuno-reacted band in low temperature-treated gills was much weaker than that of control gills, supporting a lower proliferation rate in immunohistochemistry data (Fig. 5B). We also compared TUNEL assay in control and low temperature treated gills. The apoptotic cells in cold treatment group were much less than in control group (Fig. 5C).

DISCUSSION

General cold responses in zebrafish gills

Fish experience environmental changes and activate physiological mechanisms to acclimatize to the fluctuations of the environment. Gracey et al. (Gracey et al., 2004) reported that cold-inducible RNA-

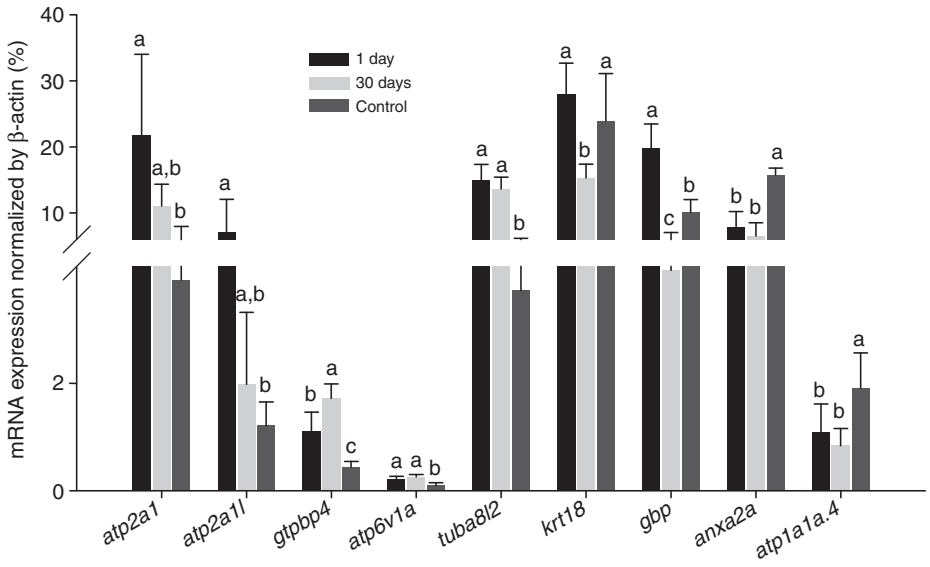

Fig. 2. Validation of the microarray data by real-time RT–PCR. Nine selected genes were subjected to real-time RT–PCR, and their relative expression levels in the control, 1 day and 30 days cold-acclimated groups were compared. Basically, the expression levels detected by real-time RT–PCR were consistent with the microarray data. The values were normalized to β-actin. Values are means ± s.d. (*N*=4 or 5). Different letters above the bars indicate significant differences (one-way ANOVA, Tukey's pair-wise comparison).

binding protein (*CIRBP*) and high-mobility group proteins (*HMG1*, *HMGT1*, *HMG4* and *NHPX*) were strongly induced by cold treatment in carp and concluded that *CIRBP* may play an important role in the response to cold (Gracey et al., 2004). In human THP-1 cells, *CIRBP* was also proposed as being a marker for cold shock (Sonna et al., 2006). *CIRBP*, which is induced by both cold and hypoxia, could stabilize RNA structure and increase translation rate (Fujita, 1999; Gracey et al., 2004; Sonna et al., 2006; Wellmann et al., 2004). In zebrafish, we observed that *cirbp* was highly upregulated in gills after cold treatment. This implies that *cirbp* may be a mediator of cold adaptation in zebrafish. Moreover, the expression of *hmgb1* mRNA was dramatically elevated (Fig. 1). HMGB1 binds to DNA without sequence specificity, and facilitates transcription, replication, and DNA transposition (Bianchi and Manfredi, 2004). Recently, HMGB1 was reported to be a cytokine that may participate in immune responses (Yang et al., 2005). Cold was found to affect the DNA secondary structure (Delrow et al., 1998); therefore increases in Hmgb1 expression in zebrafish gills may reasonably be considered to stabilize the DNA structure and as a result, to promote the transcription rate for compensation during cold acclimation. Taken altogether, zebrafish exhibit some of the same cold acclimation mechanisms as other species.

Upregulated genes in cold-acclimated gills

The largest GO category of cold-induced transcripts was cellular metabolism, which could be further subdivided into several subgroups including glycolysis, regulation of transcription, nucleosome assembly, proteolysis, protein polymerization, chromatin assembly or disassembly, protein amino acid dephosphorylation, and protein polymerization (supplementary material Table S2). The number of upregulated genes in zebrafish acclimated to cold for 30 days were more and the functions of these genes were diverse compared to fish acclimated for only 1 day. Most previous studies examined the time course changes of transcriptome in fish after thermal or hypoxic stress, but overlooked the time-dependent modulation of physiological responses (Gracey et al., 2004; Gracey et al., 2001; Ton et al., 2003). In annual killifish (*Austrofundulus limnaeus*) liver, small and large molecular chaperons were differentially stimulated in response to seasonal temperature changes and daily fluctuating temperatures, respectively (Podrabsky and Somero, 2004). These studies proposed the gradual enhancement and achievement of compensatory mechanisms of gill functions following cold acclimation.

In general, the genes induced by cold in zebrafish gills are involved in various functions including cellular metabolism, ion transport, carbohydrate metabolism, antigen processing and presentation, immune response and signal transduction. Similar gene expression profiles were also reported in the heart of rainbow trout (Vornanen et al., 2005) and various organs of carp (Gracey et al., 2004) after cold acclimation. In rainbow trout heart, genes related to protein synthesis and intermediary metabolism were most strongly upregulated by cold, whereas the transcription regulation-related genes were upregulated in carp gill, kidney, brain, heart, muscle, liver and intestine. These studies suggest that some cold responses occur in a variety of organs and are conserved among organisms.

Downregulated genes in cold-acclimated gills

In zebrafish gills there were more genes that were suppressed under cold stress than were upregulated (supplementary material Table S2). Based on the GO analysis, subgroups of cold-suppressed transcripts were those involved in DNA repair, protein folding, protein amino acid phosphorylation and response to oxidative stress. The downregulated genes differed significantly between the

Table 1. mRNA expression levels (real-time RT–PCR) of several ionocyte-related genes in cold-acclimated and control zebrafish gills

Gene name	1 day acclimation	30 days acclimation	Control
atp1a1a.2	24.02±6.62[a]	13.22±2.77[b]	16.61±2.04[b]
atp1b1b	315.6±8.14[a]	171.4±23.0[b]	149.4±29.1[b]
slc12a3	10.39±1.58[a]	3.12±0.81[b]	2.61±0.91[b]
trpv6	2.96±1.19[b]	6.90±4.39[a]	1.51±0.53[b]
atp6v1a	0.29±0.08[a]	0.36±0.06[a]	0.14±0.07[b]
ca2	0.24±0.09[b]	0.91±0.25[a]	0.28±0.07[b]
ca15a	80.73±7.31[b]	103.0±4.05[a]	78.21±14.72[b]
ndrg1	7.50±2.33[a]	6.95±1.20[a]	3.18±1.05[b]
foxi3a	1.30±0.19[a]	1.26±0.65[a]	0.47±0.22[b]
gcm2	1.73±0.48[a]	2.06±0.25[a]	1.07±0.29[b]
dlc	4.38±1.14[a]	3.18±0.50[b]	2.32±0.82[b]

The values were normalized to β-actin. Values are means ± s.d. (*N*=4 or 5). Different superscript letters indicate significant differences (one-way ANOVA, Tukey's pair-wise comparison).

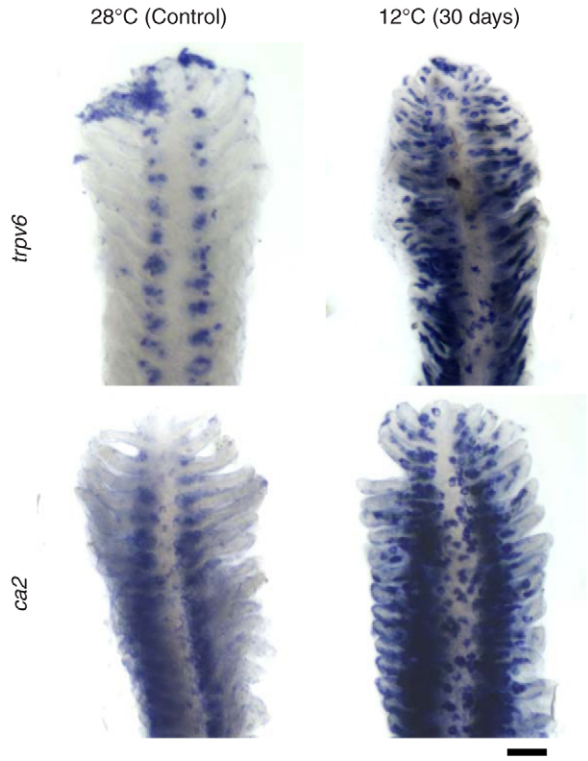

Fig. 3. *In situ* hybridization of *trpv6* and *ca2* in zebrafish gills. Expression of *trpv6* and *ca2* mRNA increased significantly in 30 day cold-acclimated gills. *trpv6*- and *ca2*-expressing cells were found in the gill filaments and the lamellae in the cold-acclimated group, whereas in the control group these cells only appeared in gill filaments. Scale bar, 50 μm.

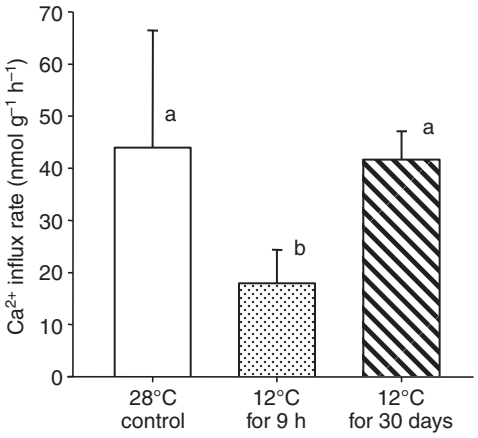

Fig. 4. The effects of cold treatments on Ca^{2+} influx in zebrafish. Ca^{2+} influx decreased after 9 h exposure to 12°C but recovered after acclimation at 12°C for 30 days. Values are means ± s.d. (*N*=5). Different letters above the bars indicate significant differences (one-way ANOVA, Tukey's pair-wise comparison).

1 day and 30 days acclimated groups. Cellular metabolism was still the largest GO category in the downregulated gene lists of both groups. In the 1 day group, the six genes (*ercc3*, *polb*, *cct3*, *mespb*, LOC571699 and zgc:110755) in the subgroup of DNA-dependent metabolism were the most abundant in the cellular metabolism category. Calcium ion transport-related genes (*guca1b*, *actn4*, *calb2l* and *tnnc1*) were also downregulated (supplementary material Table S2).

In the 30 day group, the most intriguing finding was that six intracellular transport genes (*ndel1b*, *zbtb16*, *sec23b*, *ucp2*, *slc25a12* and zgc:110821) were downregulated. These transporters are responsible for trafficking of ions and proteins and important for cellular physiology. The expression patterns of these genes was contrary to those of several cell membrane bound transporters, which were induced by cold (discussed below). This suggests that zebrafish activated some gill-specific responses during cold acclimation. In addition, the immune response gene, *mhclze*, which belongs to the major histocompatibility complex (MHC) family were downregulated. MHC proteins play important roles in immune responses to bacterial and fungal pathogens (Ojcius et al., 1994), and consequently, downregulation of these genes at low temperature may increase the chance of infection by low-temperature-related pathogens. In rainbow trout, the mRNA and protein levels of MHC II alpha and beta were downregulated after 2°C of cold treatment (Nath et al., 2006). However, rainbow trout and Atlantic salmon expressed high levels of beta-2-microgobulin in a 2°C environment (Kales et al., 2006), allowing them to maintain their viral recognition machinery at low temperatures.

In cold-exposed carp and trout only a few genes were suppressed (Gracey et al., 2004; Vornanen et al., 2005) in contrast to zebrafish in which there were over 100 downregulated genes. The most reasonable explanation for these differences may be to the hereditary histories of these species. Both carp and rainbow trout are temperate species, which have evolved sufficient mechanisms for adaptation to habitats with temperatures near 0°C (Jain and Farrell, 2003; Sollid and Nilsson, 2006). Zebrafish, however, are considered to be a tropical species, for which a low-temperature environment is much more challenging. Taking all of these into account, the changes in gene expression profiles during acclimation to low temperatures appear to reflect differential evolutionary and environmental adaptations among species.

Gill-specific responses during acclimation to low temperature

With the aid of functional genomics, Gracey and colleagues (Gracey et al., 2004) extended our knowledge and understanding of how cold exposure elicits different responses in various tissues of fish, and other work has supported their findings (Ju et al., 2002; Malek et al., 2004; Tang et al., 1999; Vornanen et al., 2005). However, there was very little integration of the transcriptome analysis into physiological functions. The present study further extended the transcriptome data to the functional analysis of fish gills, which are a multi-function organ and the main extra-renal site responsible for ion balance and acid–base regulation in fish (Evans et al., 2005). Gene expression profiling in gills indicated that ion balance and acid–base regulation, the principal biological functions of gill, were affected by low temperature. A group of ion and acid–base balance-related genes, including transporters (*trpv6*, *atp1b1b*, *atp1a1a.2*, *atp6v1a* and *slc12a3*) and cytosolic enzymes (*ca15a* and *ca2*) were significantly upregulated by cold. Epithelial Ca^{2+} channel (ECaC; *trpv6*) is the key ion channel of Ca^{2+} absorption in zebrafish and rainbow trout (Pan et al., 2005; Shahsavarani et al., 2006; Shahsavarani and Perry, 2006), and V-type H-ATPase (*atp6v1a*) plays a major role in acid secretion in zebrafish embryos (Horng et al., 2007; Lin et al., 2006). Carbonic anhydrase (*ca2* and *ca15a*) also participates in acid–base balance (Claiborne et al., 2002; Georgalis et al., 2006; Hwang and Lee, 2007; Lin et al., 2008). NCC (*slc12a3*) is responsible for chloride uptake in zebrafish (Hwang

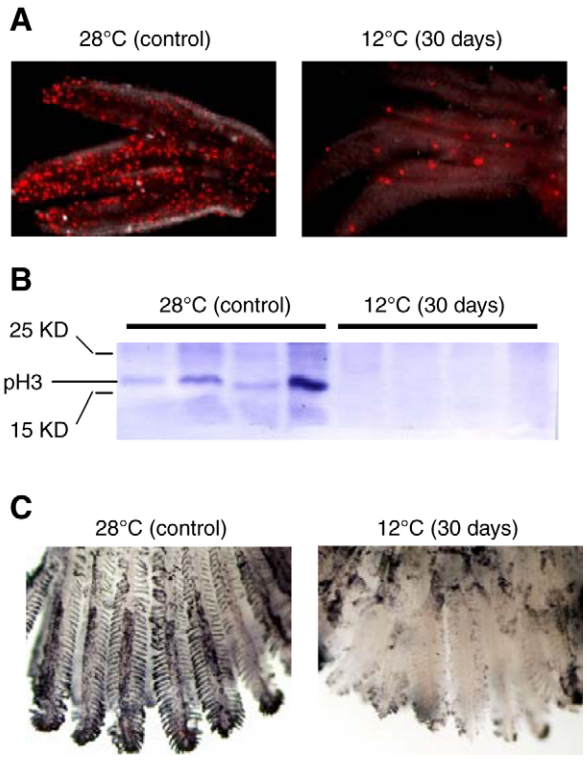

Fig. 5. Cell proliferation and apoptosis in zebrafish gills. (A) Immunohistochemistry of mitotic cells in normal and cold-acclimated gills. (B) Western blot analysis of phosphohistone H3 (pH3) expression in normal and cold-acclimated gills. (C) TUNEL assay for apoptotic cells in normal and cold-acclimated gills. Cold acclimation retarded both cell proliferation and apoptosis.

and Lee, 2007). Na^+/K^+-ATPase (*atp1a1a.2* and *atp1b1b*) is a major driving force for other transporters in mitochondrial-rich cells (Chang and Hwang, 2004; Evans et al., 2005; Lee et al., 1998). Furthermore, the Ca^{2+} influx, as a function of *trpv6*, had recovered after 30 days cold acclimation coincidently with the elevation of *trpv6* mRNA expression, providing the evidence to integrate the molecular findings of this study to physiological function. Taking all these findings into account, it is apparent that ion transporters in gill ionocytes were activated to increase ion uptake and to adjust the pH of body fluids in zebrafish, and these may probably recover the ion and/or acid–base balance that has been disturbed by an acute cold stress.

We examined the expression of *foxi3a*, *dlc*, *gcm2* and *ndrg1* mRNAs to investigate the effects of cold on ionocytes differentiation-related genes. NDRG1 has been reported to be modulated under various conditions such as cellular differentiation (Yu et al., 1999), cell cycle arrest (Piquemal et al., 1999) and hypoxia (Chen et al., 2006). *Glial cells missing* (*gcm*), primarily identified from *Drosophila*, is a genetic switch, controlling glial *versus* neuronal fate (Jones et al., 1995). In both mouse and chicken, *gcm2* is expressed in the pharyngeal pouches and the forming parathyroid gland, whereas in teleosts, *gcm2* is expressed within the pharyngeal pouches and directs development of the internal gill bud (Hogan et al., 2004; Okabe and Graham, 2004). Recent studies demonstrated that a member of the forkhead transcription factor family, *foxi3a*, functions as a master regulator and *dlc* is a lateral inhibitor for ionocyte differentiation in zebrafish embryos (Hsiao et al., 2007). All these cell differentiation-related genes, *foxi3a*, *dlc*, *gcm2* and *ndrg1*, were evidently stimulated after acclimation to cold. Cold stimulates the differentiation of ionocytes in zebrafish gills, supporting our hypothesis described above.

Increases in ionocyte numbers and expression of functional genes were observed in zebrafish gills after cold acclimation. Experiments on phosphohistone H3 and TUNEL assays indicated that both cell proliferation and apoptosis in gills were decreased after cold acclimation. These imply that the increased ionocytes may mainly originate from acceleration of the terminally differentiating pre-existing un-differentiated or immature cells located in the gill rather than from cell proliferation.

In cultured mammalian cells, cold shock retarded cell proliferation that leads to apoptosis (Al-Fageeh et al., 2006; Rieder and Cole, 2002; Sonna et al., 2002). By contrast, in the intestines of hibernating mammals, the rates of both cell proliferation and apoptosis are suppressed (Fleck and Carey, 2005). Similar to hibernating mammals, the overall cell proliferation and apoptosis rates in zebrafish gills declined after cold treatment. The collective suppression of cell proliferation (arrest of the cell cycle) and the delay of apoptosis may result in extension of the cell lifespan. In cold environments, zebrafish need to survive and maintain their normal physiological performance for growth and reproduction. Zebrafish gill ionocytes displayed extended lifespan (due to delayed apoptosis) and sustained cell functions (due to stimulation of pre-existing undifferentiated cells into ionocytes) after cold acclimation. These findings provide new insights into the cellular physiological mechanisms of survival and growth of ectothermic vertebrates in low-temperature environments.

In summary, expression of genes related to ion and acid–base regulation in the gill were stimulated during cold acclimation, suggesting an essential compensatory action for the cold-induced ion imbalance to stabilize normal physiological processes in zebrafish.

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