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Effects of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo

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Tseng D-Y, Chou M-Y, Tseng Y-C, Hsiao C-D, Huang C-J, Kaneko T, Hwang P-P. Effects of stanniocalcin 1 on calcium uptake in zebrafish (*Danio rerio*) embryo. *Am J Physiol Regul Integr Comp Physiol* 296: R549–R557, 2009. First published December 10, 2008; doi:10.1152/ajpregu.90742.2008.—Stanniocalcin (STC) formerly called hypocalcin or teleocalcin, is a 50-kDa disulfide-linked homodimeric glycoprotein that was originally identified in fish and secreted from the corpuscles of Stannius (CS). One of the main functions of STC-1 is Ca^{2+} uptake inhibition; however, the mechanisms remain unknown. In the present study, we provide molecular evidence to elucidate how zebrafish STC-1 regulates Ca^{2+} uptake in zebrafish embryos. In a wide variety of tissues including the kidney, brain, gill, muscle, and skin, *zstc-1* was expressed. Incubating zebrafish embryos in low-Ca^{2+} (0.02 mM) freshwater stimulated whole body Ca^{2+} influx and zebrafish epithelial Ca^{2+} channel (zECaC) mRNA expression, while downregulated *zstc-1* expression. A morpholino microinjection approach was used to knockdown the zSTC-1 protein, and the results showed that the Ca^{2+} content, Ca^{2+} influx, and zECaC mRNA expression all increased in morphants. These data suggest that zSTC-1 negatively regulates ECaC gene expression to reduce Ca^{2+} uptake in zebrafish embryos.

Ca^{2+} uptake; Ca^{2+} influx

CALCIUM IS AN ESSENTIAL ELEMENT for all animals. It is important for bone structure, and it serves a variety of intracellular and extracellular roles (20). For terrestrial vertebrates, food is the main source of Ca^{2+}, while aquatic vertebrates obtain Ca^{2+} from water (14). Ca^{2+} absorption occurs in epithelia, including the kidney, intestine, placenta, mammary gland, and gill, and the currently accepted model proposes that Ca^{2+} enters epithelial cells through an apical epithelial calcium channel (ECaC) and is extruded from the basolateral side to the blood compartment through a plasma membrane calcium ATPase (PMCA) and a sodium calcium exchanger (NCX) (20). Recent evidence from zebrafish gill indicates that specific isoforms of Ca^{2+} transporters, zebrafish (z)ECaC, zPMCA2, and zNCX1b, are responsible for Ca^{2+} uptake in gill/skin ionocytes and that ECaC is the major regulatory target for this mechanism during environmental challenge (27, 36).

The endocrine control of transepithelial Ca^{2+} transport has been well studied. Most studies focused on calciotropic hormones including parathyroid hormone, 1,25-dihydroxyvitamin D3 (20), prolactin, and cortisol (16, 37, 41). For instance, parathyroid hormones have been demonstrated to enhance Ca^{2+} uptake and reduce Ca^{2+} efflux in fish (17). However, the hypocalcemic hormone was also involved in Ca^{2+} uptake regulation (53). Stanniocalcin (STC), formerly called hypocalcin or teleocalcin, was originally identified in fish and was also suggested to participate in Ca^{2+} homeostasis (53). STC is a homodimeric glycoprotein that is secreted from the corpuscles of Stannius (CS), which are attached to the kidneys (56). A human orthologue of fish STC, STC-1, was found by mRNA differential display of genes related to cellular immortalization, a key aspect of the cancer cell phenotype (7), and independently by random sequencing of a fetal lung cDNA library (35).

In humans, the functions of STC-1 have been proposed to be similar to antihypercalcemic actions in fish (55). Indeed, STC-1 was found to decrease Ca^{2+} uptake across mammalian intestines and kidney (29, 58, 60). STC-1 has been isolated and sequenced from several fish species (1–4, 18, 33, 43, 53, 59). The secretion of STC-1 in fish is tightly regulated by the levels of extracellular Ca^{2+} through a calcium-sensing receptor (CaR) (40). STC-1 inhibits Ca^{2+} uptake in gill and intestine and stimulates phosphate reabsorption in fish kidney (26, 28, 47). However, the mechanisms through which STC-1 regulates fish gill Ca^{2+} uptake are still unknown. Previous studies suggested that reducing gill Ca^{2+} transport by STC may result from regulation of the gill apical membrane Ca^{2+} channel or redirecting blood flow away from the gill (5, 13), but there has been no convincing molecular physiological evidence provided to date to support that.

The purpose of the present study was to test the hypothesis that STC-1 inhibits Ca^{2+} uptake via regulating the expression of ECaC, the key transporter in fish gill/skin Ca^{2+} uptake mechanisms (22, 27). The zebrafish (*Danio rerio*) was selected as the experimental animal because of its extensive genetic database and applicability to several molecular physiological approaches. Specific aims were to investigate *1*) the mRNA expression of zSTC-1 in zebrafish tissues and developing embryos; *2*) localization of zSTC-1 mRNA in different stages of zebrafish embryos; *3*) the effects of environmental Ca^{2+} levels on the expressions of *zstc-1* and *zecac*; *4*) the effects of zECaC knockdown on *zstc-1* expression, Ca^{2+} influx, and Ca^{2+} content; and *5*) the effects of zSCT-1 knockdown on Ca^{2+} influx and expressions of *zecac*, *zpmca*, and *zncx*. The

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Table 1. *Primer sets for quantitative RT-PCR*

Gene	Primer Sequence, 5′-/-3′	Amplicon Size, bp
zecac		
Forward	TCCTTTCCCATCACCCTCT	
Reverse	GCACTGTGGCAACTTTCGT	261
zstc-1		
Forward	CCAGCTGCTTCAAAACAAACC	
Reverse	ATGGAGCGTTTTCTGGCGA	160
pmca2		
Forward	AAGCAGTTCAGGGGTTTAC	
Reverse	CAGATCATTGCCTTGTATCA	69
ncx1b		
Forward	TAAAGTGGCAGCGATACAGG	
Reverse	CAGATCAAGGCGAAGATGG	194
β-actin		
Forward	ATTGCTGACAGGATGCAGAAG	
Reverse	GATGGTCCAGACTCATCGTACTC	173

present study provides the molecular evidences of STC-1 in Ca^{2+} uptake regulation in zebrafish embryos.

MATERIALS AND METHODS

Experimental animals. Zebrafish (*D. rerio*) were kept in local tap water ([Ca^{2+}] = 0.2 mM) at 28.5°C under a 14:10-h light-dark photoperiod at the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. Experiments were performed in accordance with guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFiZOOHP2007086).

Acclimation experiment. Artificial freshwaters high-Ca^{2+} (2 mM) and low-Ca^{2+} (0.02 mM) were prepared with double-deionized water (model Milli-RO60; Millipore, Billerica, MA) supplemented with adequate $CaSO_4 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, NaCl, K_2HPO_4, and $KH_2PO_4 \cdot Ca^{2+}$ concentrations (total Ca^{2+} levels measured by absorption spectrophotometry) of the high- and low-Ca^{2+} media were 2 and 0.02 mM, respectively, but the other ion concentrations of the three media were the same ([Na^+], 0.5 mM; [Mg^{2+}], 0.16 mM; [K^+], 0.3 mM) as those in local tap water. Variations in the ion concentrations were maintained within 10% of the predicted values. Zebrafish fertilized eggs were transferred to high- and low-Ca^{2+} media, respectively, and incubated thereafter until sampling.

RNA extraction. Tissues were homogenized in 0.5 ml Trizol reagent (Invitrogen, Carlsbad, CA), mixed with 0.1 ml chloroform, and thoroughly shaken. After centrifugation at 4°C and 12,000 *g* for 30 min, the supernatants were obtained and mixed with an equal volume of isopropanol. Pellets were precipitated by another centrifugation at 4°C and 12,000 *g* for 30 min, washed with 70% alcohol, and stored at −20°C until use. The quantity and quality of total RNA were assessed by the absorbances at 260 and 280 nm and by gel electrophoresis.

RT-PCR analysis. Total RNAs extracted from zebrafish tissues and embryos were treated with DNase I (Promega, Madison, WI) to remove DNA contamination. After DNase I digestion, phenol-chloroform extraction and purification were performed to stop the reaction. For cDNA synthesis, 5 μg of total RNA were reverse-transcribed in a final volume of 20 μl containing 0.5 mM dNTPs, 2.5 μM oligo$(dT)_{18}$, 5 mM dithiothreitol, and 200 units of PowerScript reverse transcriptase (Invitrogen) for 1.5 h at 42°C and followed by a 15-min incubation at 70°C. For PCR amplification, 1 μl of cDNA was used as a template in a 25-μl final reaction volume containing 0.25 μM dNTP, 1.25 units of Gen-Taq polymerase (Genemark, Taipei, Taiwan), and 0.2 μM of each primer. Thirty-five cycles were performed for each reaction. The amplicons were sequenced to ensure that the PCR products were the desired gene fragments.

Verification of gene expression using quantitative real-time RT-PCR. To obtain sufficient RNA, 50 embryos were pooled as a sample and then were reverse-transcribed to synthesize cDNA. Real-time quantitative (q) RT-PCR was used to analyze the expressions of 4 transcripts: *zecac, zstc-1*, *pmca2*, and *ncx1b*. The primers were listed in Table 1. As an internal control, primers for β-actin were designed and amplified in parallel with the genes of interest. 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) according to the manufacturer's instructions. Primer targeting was designed using Primer Express 2.0 software (Applied Biosystems, Wellesley, MA). Reactions in the 96-well format were performed with a ABI Prism 7000 sequence detection system (Perkin-Elmer, Applied Biosystems).

RNA probe synthesis. The full length of zebrafish *zstc-1* fragment was obtained by PCR and inserted into the pGEM-T Easy vector (Promega). Purified plasmids were then linearized by restriction enzyme digestion, and in vitro transcription was performed with T7 and SP6 RNA polymerases (Roche, Penzberg, Germany), respectively, 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 membrane was incubated with an alkaline phosphatase-conjugated anti-dig antibody and stained with nitro blue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

Whole mount in situ hybridization. Zebrafish embryos were fixed with 4% paraformaldehyde overnight at 4°C and then washed several times with PBS. Fixed samples were rinsed with PBST (PBS with 0.2% Tween 20, 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na_2HPO_4, and 0.002 mM KH_2PO_4; pH 7.4). After a brief washing with PBST, embryos 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^+ (HyB supplemented with 500 μg/ml yeast tRNA and 50 μg/ml 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. Embryos 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 2× 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

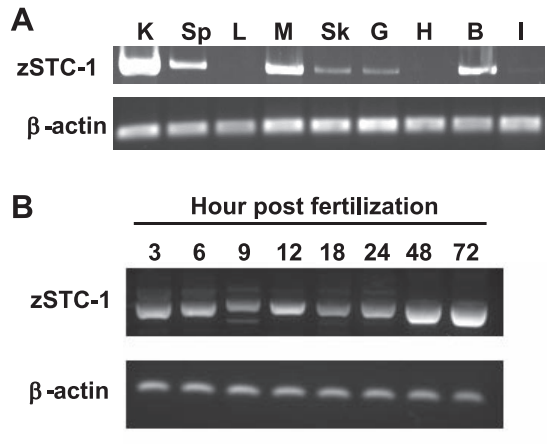

Fig. 1. mRNA expressions of zebra Stanniocalcin (zSTC-1) in various zebrafish tissues and developing embryos. zSTC-1 mRNA expression patterns were analyzed by RT-PCR. *A*: *zstc-1* was detected in several tissues including the kidney, spleen, muscle, skin, gill, and brain in adult fish. *B*: in zebrafish embryos, *zstc-1* was first detected at 3 hours postfertilization (hpf). K, kidney; Sp, spleen; L, liver; M, muscle; Sk, skin; G, gill; H, heart; B, brain; I, intestine.

50% PBST, for 5 min in 25% 0.2× SSC and 75% PBST, and for 5 min in PBST. After serial washings, embryos were incubated in blocking solution containing 5% sheep serum and 2 mg/ml 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 nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

For double labeling, fluorescence staining was conducted with the commercial kit, TSA Plus Fluorescence Systems (Perkin-Elmer). The hybridization signals detected by the DIG-labeled RNA probes were amplified through fluorescein-tyramide signal amplification.

Immunohistochemistry. Zebrafish embryos were fixed in 4% paraformaldehyde for 12 h at 4°C. After being washed in PBS, fixed embryos 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. Embryos were then incubated with a polyclonal antibody against hypocalcin, a 54-kDa product purified from the rainbow trout CS (24) diluted at 1:600 at 4°C for 16 h. Samples were washed twice in PBS for 10 min each and then incubated with 1:200 PBS-diluted goat anti-rabbit IgG conjugated with Alexa Fluor 633 (Invitrogen) at 4°C for 16 h. Images were acquired with a confocal laser scanning microscope (TCS-SP5; Leica Lasertechnik, Heidelberg, Germany) for monitoring Alexa Fluor 633.

Microinjection of antisense morpholino oligonucleotides. The morpholino oligonucleotide (MO) was obtained from Gene Tools (Philomath, OR). The zSTC-1-morpholino (5′-AAATCCGCTTTTCAGGAGCATGTCT-3′) and zECaC-morpholino (5′-ACCAGATATGGCGGGGTGGCATGATT-3′) were prepared with 1× Danieau solution [in mM: 58 NaCl, 0.7 KCl, 0.4 $MgSO_4$, 0.6 $Ca(NO3)_2$, 5.0 HEPES pH 7.6]. Standard control oligo (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as the control. The MO solution containing 0.1% phenol red was injected into one- to two-cell stage zebrafish embryos at 4 or 2 ng/embryo with an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). In zSTC-1 and zECaC morphants, no significant morphological phenotype was found. The morphants showed normal morphology during development and the mortality was very low (<2%).

Plasmid construction. To generate the pcDNA3.1+zSTC-1:GFP constructs, the corresponding zSTC-1 coding region (747 bp) was PCR amplified with the following pairs of primers: forward, 5′-GGATCCATGCTCCTGAAAAGCGGCTTTCTT-3′; reverse, 5′-GAATCCAGGACTTCCCACGATGGAGCGTTT-3′; the zSTC-1 PCR amplicon was cloned into a pGEM-T easy vector (Promega) with

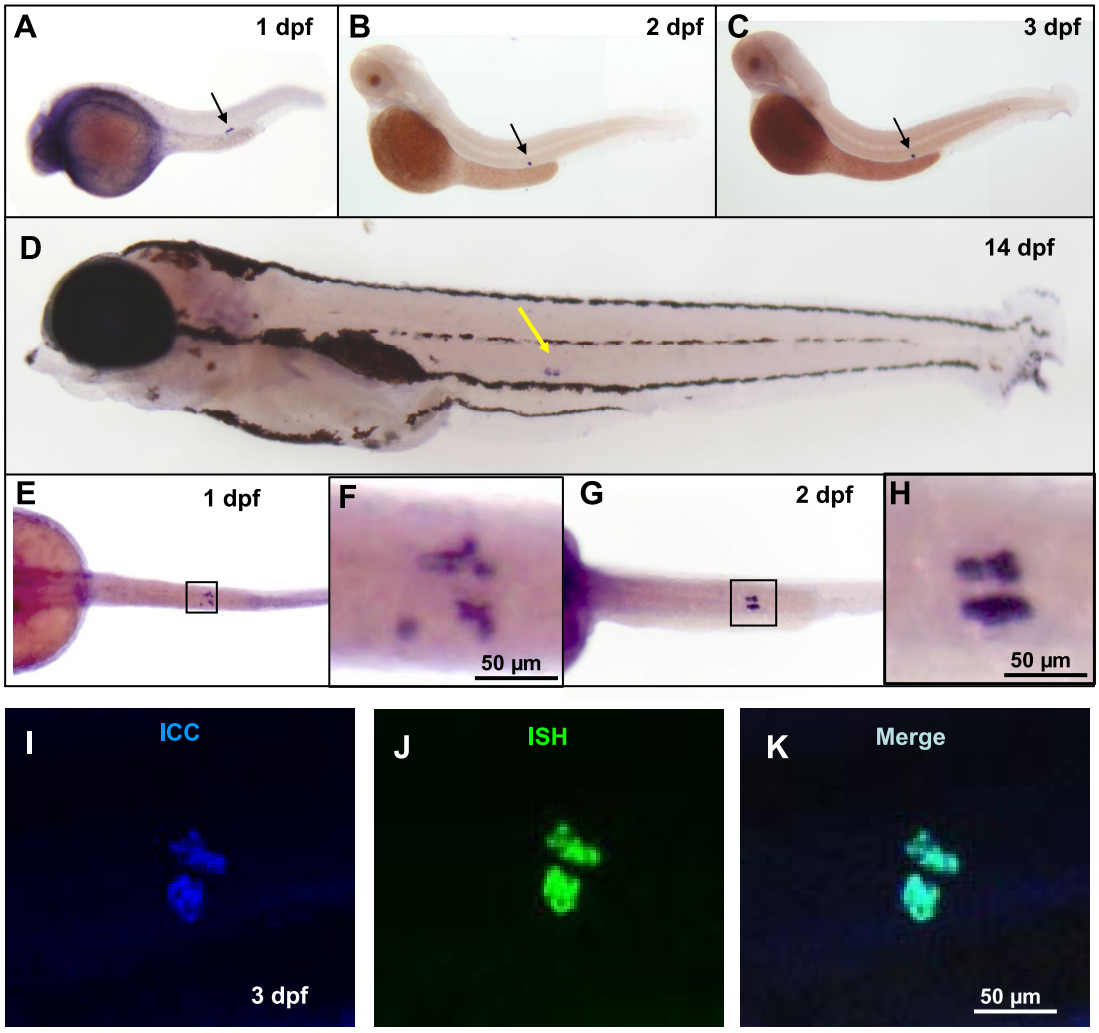

Fig. 2. In situ hybridization (ISH) and immunocytochemistry (ICC) of zSTC-1. *A–D*: *zstc-1* mRNA was expressed in corpuscles of Stannius of zebrafish embryos throughout development from 1 to 14 days postfertilization (dpf). *E–H*: *zstc-1*-expressing cells expressed at the presumed location of the corpuscles of Stannius at 1 dpf, and thereafter they aggregated as a specific group of cells. *I–K*: signals of the protein and mRNA of zSTC-1 were colocalized in the same cells. The arrow indicates the position of corpuscles of Stannius.

*Bam*HI and *Eco*RI sites and was then subcloned into a pcDNA3.1+GFP vector at the *Bam*HI and *Eco*RI sites. The final pcDNA3.1+zSTC-1:GFP construct was in-frame fused with the green fluorescent protein (GFP) reporter. The corresponding *Bam*HI and *Eco*RI sites are underlined.

Capped-mRNA injection. All constructs cloned in the pcDNA3.1+GFP XLT vectors were linearized by *Xba*I, and capped-mRNA was transcribed using an SP6 message RNA polymerase kit (Ambion, Huntington, UK). Capped-mRNAs were injected into embryos at the one-cell stage at 250 pg/embryo. In the rescue and MO effectiveness experiments, 4 ng zSTC-1 MOs and 250 pg capped-mRNA were coinjected into each one- to two-cell stage embryo.

Measurement of the whole body Ca^{2+} content. Zebrafish embryos were anesthetized with MS-222, dechorionated, and briefly rinsed in deionized water, and 10 individuals were pooled as one sample. HNO_3 (13.1 N) was added to samples for digestion at 60°C overnight. Digested solutions were diluted with double-deionized water, and the total Ca^{2+} content was measured with an atomic absorption spectrophotometer (model Z-8000; Hitachi). Standard solutions from Merck (Darmstadt, Germany) were used to make the standard curves.

Measurement of whole body Ca^{2+} influx. By following previously described methods (9) with some modifications, zebrafish embryos were dechorionated, rinsed briefly in deionized water, and then transferred to a 4-ml $^{45}Ca^{2+}$ (Amersham, Piscataway, NJ; with a final working specific activity of 1–2 mCi/mmol)-containing medium for a subsequent 4-h incubation. After incubation, embryos were washed several times in isotope-free water medium. Three embryos were pooled into one vial, anesthetized with MS-222, and then digested with tissue solubilizer (Solvable; Packard, Meriden, CT) at 50°C for 8 h. The digested solutions were supplemented with counting solution (Ultima Gold; Packard), and the radioactivities of the solutions were counted with a liquid scintillation beta counter (model LS6500; Beckman, Fullerton, CA). The Ca^{2+} influx was calculated using the following formula: $J\text{in} = Q_{\text{embryo}} \cdot X_{\text{out}}^{-1} \cdot t^{-1} \cdot W^{-1}$, where J in is the influx (pmol·mg^{-1}·h^{-1}), Q_{embryo} is the radioactivity of the embryo (cpm per individual) at the end of incubation, X_{out} is the specific activity of the incubation medium (cpm/pmol), t is the incubation time (hours), and W is the average body wet weight of different stage embryos (mg).

Statistical analysis. Values are presented as the means ± SD and were compared using Student's *t*-test. A significant difference of $P < 0.05$ and $P < 0.001$, respectively, was found between treatment and control groups. One-way ANOVA with Tukeys comparisons was also conducted depending on experiments.

RESULTS

Expression patterns of zstc-1 in various tissues of zebrafish and developing embryos. Specific primers were designed according to the zebrafish *stc-1* sequence (GenBank accession no. BC056310), and full-length cDNA of the zSTC-1 gene was cloned and sequenced. The total nucleotide length was 1228 bp with an open reading frame of 750 bp that encodes a protein of 249 amino acids. The cloned sequence is identical to the sequence (GenBank accession no. BC056310) in the database.

Expression of *zstc-1* was evaluated by RT-PCR (with β-actin as the internal control). As shown in Fig. 1*A*, *zstc-1* was expressed in several tissues including the brain, spleen, skin, gill, and kidney in adult fish (Fig. 1*A*). The RT-PCR analysis was repeated with three different sets of samples, and the results were similar. In zebrafish embryos, *zstc-1* was first detected at 3 h postfertilization (hpf) and continued to be expressed throughout development (Figs. 1*B*).

Localization of zSTC-1 mRNA and protein in developing zebrafish. zstc-1 expression throughout development was also examined by whole mount in situ hybridization. The zSTC-1 mRNA signal was first detected in a population of cells located in proximity to nephric ducts, the presumed location of the CS, at 1 day postfertilization (dpf) (Fig. 2, *A, E*, and *F*), and the specific expression of zSTC-1 mRNA appeared only at the location of the CS throughout development, at 1–14 dpf (Fig. 2, *B–D, F–H*). It was noted with detailed observations that the *zstc-1*-expressing cells occurred scattered at the presumed location of the CS at 1 dpf (Fig. 2, *E* and *F*), and thereafter they aggregated as a specific group of cells, i.e., the CS, from 2 dpf (Fig. 2, *G* and *H*).

Antiserum was raised against rainbow trout (*Salmo gairdneri*) STC-1, a 54-kDa product purified from the rainbow trout CS (24). zSTC-1 was 70% identical to rainbow trout STC-1 at the amino acid level. We used the STC-1 antiserum and the specific RNA probe of *zstc-1* for whole mount double immunocytochemistry and in situ hybridization of zebrafish embryos. As shown in Fig. 2, *I–K*, the signal of the protein and mRNA of zSTC-1 were colocalized in the same cells of an embryo at 3 dpf, further supporting the specific existence of zSCT-1 in cells of the CS.

Effects of environmental Ca^{2+} levels on zECaC and zSTC-1 mRNA expressions. Acclimation to artificial freshwater containing different levels of Ca^{2+} for 3 days (0–72 hpf) caused no significant effects on hatching or survival, but did induce

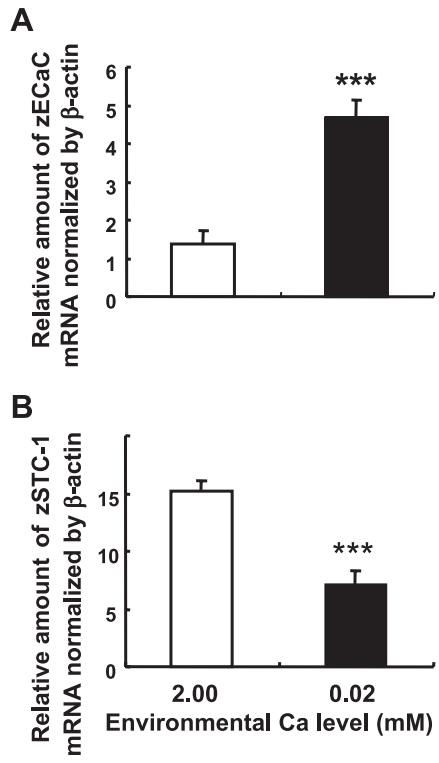

Fig. 3. Effects of environmental Ca^{2+} levels on zebrafish epithelial Ca^{2+} channel (zECaC) and zSTC-1 mRNA expressions. Quantitative real-time PCR was used to evaluate the changes in *zecac* and *zstc-1* gene expressions. zECaC mRNA levels in the low-Ca^{2+} group were notably higher than those in the high-Ca^{2+} group (*A*), while the zSTC-1 mRNA levels were lower than those of the high-Ca^{2+} group (*B*). The values were normalized to β-actin. Means ± SD ($n = 3$). ***Significant difference from the control (Student's *t*-test; $P < 0.001$).

significant changes in both *zecac* and *zstc-1* gene expressions in the developing zebrafish. Changes in *zecac* and *zstc-1* expressions were estimated using quantitative real-time PCR. As shown in Fig. 3, zECaC mRNA levels in the low-Ca^{2+} group were notably higher, by ~3.4-fold, than those in the high-Ca^{2+} group, while the zSTC-1 mRNA levels were 0.5-fold lower than those in the high-Ca^{2+} group.

Effects of the zECaC morpholino on Ca^{2+} influx and zSTC-1 mRNA expression. The zECaC antisense MO and a control MO were injected into one- to two-cell embryos. At 3 dpf, the Ca^{2+} influx, Ca^{2+} content, and zSTC-1 mRNA level in zECaC morphants were significantly lower than those in control zebrafish (Fig. 4).

Effects of the zSTC-1 MO on zSTC-1 translation, zECaC mRNA expression, and Ca^{2+} influx. To examine the role of zSTC-1 in the Ca^{2+} uptake mechanism in zebrafish embryos, we blocked zSTC-1 protein synthesis using specific zSTC-1 MOs. One- to two-cell embryos were respectively injected with the zSTC-1 MO and a control MO. At 2 dpf, injecting the zSTC-1 MO did not affect zSTC-1 mRNA signals (Fig. 5*E*), but did knockdown the translation of zSCT-1 (Fig. 5, *D* and *F*) in morphants, compared with the control MO injection (Fig. 5, *A–C*). The specificity and effectiveness of the zSCT-1 MO were confirmed in a subsequent experiment by injecting embryos with zSTC-1:GFP cRNA. After injecting zSTC-1:GFP cRNA, all embryos (100%, $n = 35$) showed strong GFP expression. When the zSTC-1 MO was coinjected with zSTC-1:GFP cRNA, it was sufficient to abolish GFP expression in all injected embryos (100%, $n = 35$) (Fig. 5, *G* and *H*). Moreover, the Ca^{2+} influxes were measured in the rescue experiments. The zSTC-1 MO injection caused an increase of Ca^{2+} influx, while coinjection with zSTC-1 MO and zSTC-1:GFP cRNA could rescue the effect of zSTC-1 MO (Fig. 5*I*). These results suggest that the zSTC-1 MO can target endogenous zSTC-1 mRNA.

At 2 dpf, the zECaC mRNA level and Ca^{2+} influx in zSTC-1 morphants were ~1.7- and 2.5-fold higher, respectively, than those in control embryos (Fig. 6, *A* and *B*). In accordance with

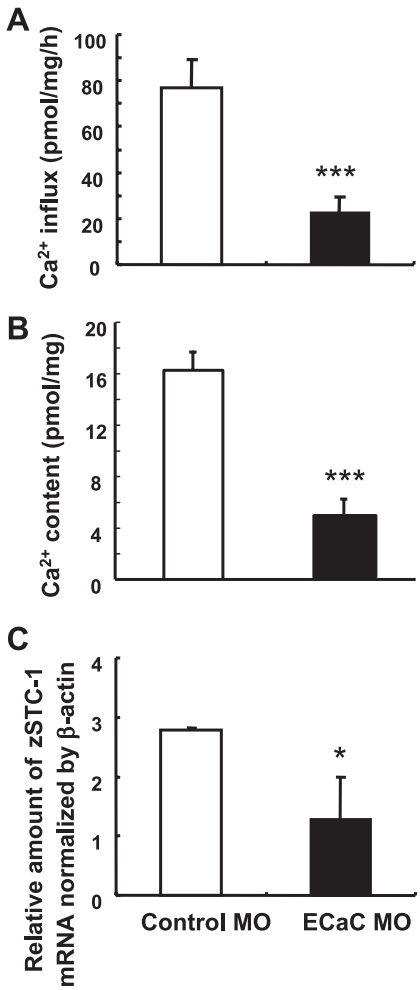

Fig. 4. Effects of the zECaC morpholino (MO) on Ca^{2+} influx (*A*), Ca^{2+} content (*B*), and zSTC-1 mRNA expression (*C*). The zECaC MO (2 ng/embryo) and a control MO were injected into 1- to 2-cell embryos. The Ca^{2+} influx ($n = 8$), Ca^{2+} content ($n = 8$), and zSTC-1 mRNA ($n = 3$) level decreased in zECaC morphants at 3 dpf. The mRNA values were normalized to β-actin. Means ± SD. Significant difference from the control (Student's *t*-test, $*P < 0.05$; $***P < 0.001$).

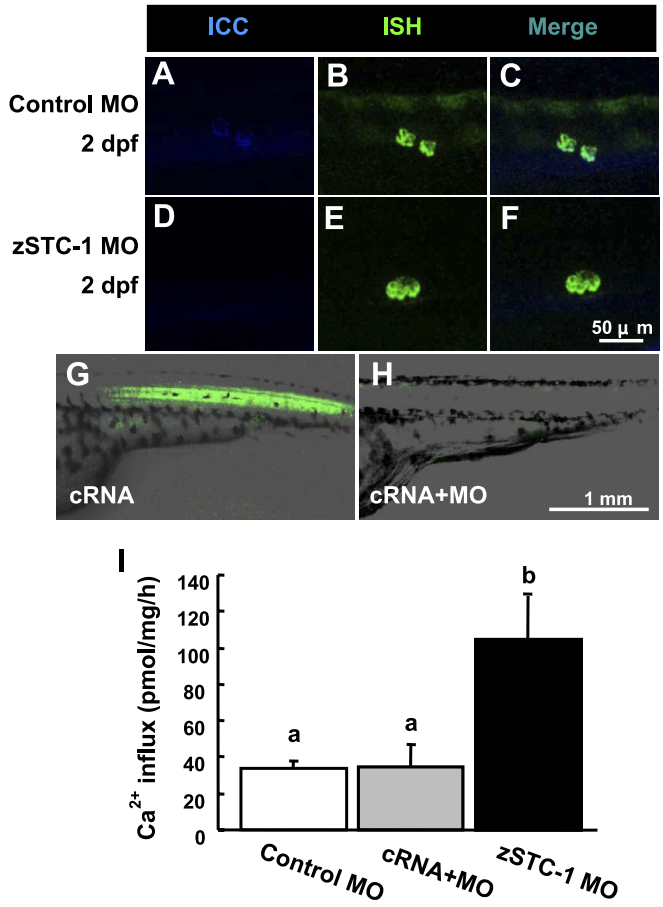

Fig. 5. Effects of the zSTC-1 MO on zSTC-1 translation and Ca^{2+} influx. One- to two-cell embryos were injected with zSTC-1 MO (4 ng/embryo) and a control MO, respectively, and the zSTC-1m RNA and protein expressions were analyzed by in situ hybridization and immunocytochemistry. *A–C*: zSTC-1 protein and mRNA signals could be detected in corpuscles of Stannius of control zebrafish embryos. *D–F*: MO did not affect zSTC-1 mRNA signals, but did knockdown the translation of zSCT-1 in the morphants. *G*: after injection with zSTC-1:GFP cRNA (250 pg/embryo), embryos showed strong green fluorescent protein (GFP) expression. *H*: when the zSTC-1 MO (4 ng/embryo) was coinjected with zSTC-1:GFP cRNA (250 pg), it was sufficient to abolish GFP expression in all injected embryos. *I*: injection with MO only caused an increase in Ca^{2+} influx, while coinjection with zSTC-1:GFP cRNA and MO rescued the defect. Means ± SD ($n = 8$). [a,b]Different letters indicate significant difference (one-way ANOVA, Tukeys comparisons).

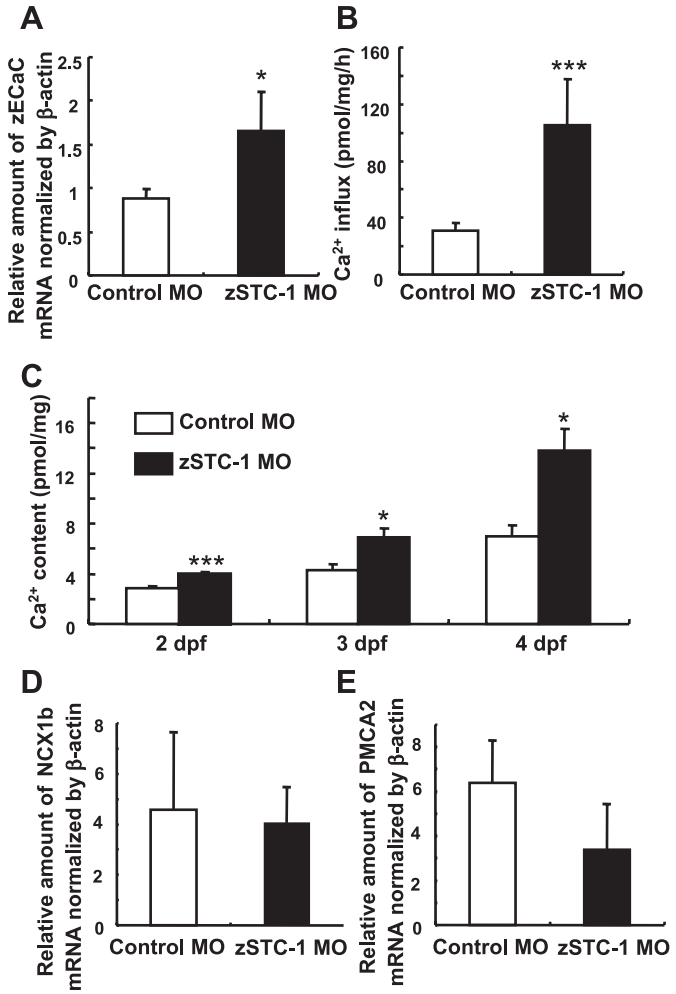

Fig. 6. Effects of the zSTC-1 MO on the Ca^{2+} transport-related gene, Ca^{2+} influx, and Ca^{2+} content. One- to two-cell embryos were injected with the zSTC-1 MO (4 ng/embryo) and a control MO, respectively. *A–C*: The zECaC mRNA ($n = 3$) level and Ca^{2+} influx ($n = 8$) in zSTC-1 morphants at 2 dpf increased, and the whole-body Ca^{2+} content ($n = 8$) in morphants at 2–4 dpf also increased. *D* and *E*: The zebra sodium exchanger-1b (zNCX1b) and zebra plasma membrane Ca ATPase-2 (zPMCA2) mRNA levels were not affected in zSTC-1 morphants at 2 dpf. The mRNA values were normalized to β-actin. Means ± SD. Significant difference from the control (Student's *t*-test, $*P < 0.05$; $***P < 0.001$).

the data of Ca^{2+} influx, the whole body Ca^{2+} content in morphants was ~1.4-, 1.6-, and 2.0-fold those in the respective control at 2, 3, and 4 dpf (Fig. 6*C*). The mRNA expressions of zNCX1b and zPMCA2 were not affected in zSTC-1 morphants at 2 dpf (Fig. 6, *D* and *E*).

Effects of the zSTC-1 MO on zECaC expression in low Ca^{2+}-treated zebrafish embryos. One- and two-cell embryos were injected with the zSTC-1 (4 ng/embryo) and a control MO, respectively, and then were incubated in 0.2 mM or 0.02 mM Ca^{2+} media. In control MO-injected embryos, the zECaC expression at 2 dpf was upregulated after low-Ca^{2+} treatment. In zSTC-1 MO-injected embryos, the zECaC expression was not affected by low Ca^{2+} (Fig. 7).

DISCUSSION

The tissue distribution of the STC-1 transcript in fish and mammals has been extensively studied (2, 6, 7, 18, 43). In fish, it was previously believed that STC-1 is exclusively produced by one specific endocrine gland; however, recent evidence has indicated that STC-1 mRNA is broadly expressed in various tissues including the brain, heart, gill, kidney, gonad, eye, skin, muscles, and intestines (33, 43). In mammals, STC-1 mRNA is also widely expressed (6, 7, 35). In addition to its inhibitory role in Ca^{2+} uptake, STC-1 has been proposed to be associated with neuronal cell differentiation (61, 62), myotube formation in developing skeletal muscles (23), and inhibition of renal phosphate reabsorption (11) based on its universal expression in various tissues. In the present study, zSTC-1 mRNA was expressed in the kidneys, spleen, muscle, skin, gill, and brain (Fig. 1*A*), and these findings are consistent with data from other species, suggesting that zSTC-1 may have putative roles other than Ca^{2+} homeostasis in zebrafish.

zSTC-1 mRNA was expressed in very early stages of zebrafish development. zSTC-1 mRNA was detected by RT-PCR analysis at 3 hpf (Fig. 1*B*). In situ hybridization results showed that zSTC-1 mRNA was dispersed in proximity to nephric ducts at 1 dpf (Fig. 2*F*) and then expressed in the whole CS at 2 dpf (Fig. 2*H*). This mRNA expression pattern is similar to the finding of the protein expression pattern in the CS of chum salmon (*Oncorhynchus keta*) embryos (25). These findings provide molecular evidence to characterize the mRNA and protein expressions during fish embryo development, and suggest that STC-1 may begin functioning in the early stages of development and play important roles in embryogenesis. Studies on mammals also support this notion. The STC-1 gene is highly expressed in developing mouse embryos (23, 50). Based on localization of both the mRNA and protein of STC-1 over the course of development, STC-1 was suggested to play a role in early skeletal patterning and joint formation (45, 46).

Regulation of the Ca^{2+} balance in fish is well documented. Ca^{2+} levels remain at constant levels during embryonic stages, followed by a rapid increase after hatching in tilapia (*Oreochromis mossambicus*) (10, 21). Low-Ca^{2+} environments can stimulate an increase in the Ca^{2+} transport capacity in many species including rainbow trout (38), tilapia (10, 32), zebrafish, goldfish

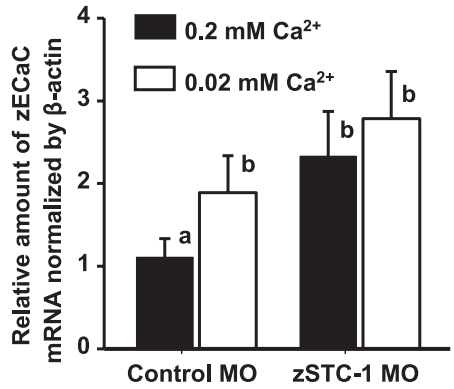

Fig. 7. Effects of the zSTC-1 MO on zECaC expression in low-Ca^{2+}-treated zebrafish embryos. One- to two-cell embryos were injected with the zSTC-1 MO (4 ng/embryo) and a control MO, respectively, and were then incubated in 0.2 mM or 0.02 mM Ca^{2+} media. In control MO-injected embryos, the zECaC expression at 2 dpf was upregulated after low-Ca^{2+} treatment. In zSTC-1 MO-injected embryos, the zECaC expression was not affected by low Ca^{2+}. The values were normalized to β-actin. Means ± SD ($n = 4$). [a,b]Different letters indicate significant difference (one-way ANOVA, Tukeys comparisons).

(*Carassius auratus*), and ayu (*Plecoglossus altivelis*) (9). This enhanced Ca^{2+} uptake is achieved by modulating the Ca^{2+} influx kinetics (9, 37) and concomitant proliferation of mitochondria rich cells (8, 32, 31, 37). Moreover, the increased Ca^{2+} uptake in fish gills/skin was demonstrated to be mediated by the upregulation of the ECaC but not by PMCA or NCX (22, 27, 36). These findings clearly indicate the positive regulation of Ca^{2+} uptake; however, very few studies have examined its negative regulation. Actually, the concept that negative control is also crucial for Ca^{2+} homeostasis is widely accepted (52). STC-1 is a major hypocalcemic hormone and can inhibit gill Ca^{2+} transport (51, 54). However, the target transporter of STC-1 and the mechanism by which STC-1 inhibits Ca^{2+} transport are unknown. The crucial role of ECaC in Ca^{2+} uptake in fish raises the possibility that zSTC-1 negatively regulates ECaC expression to reduce Ca^{2+} uptake in zebrafish.

Upregulation of ECaC expression and the resultant stimulation of Ca^{2+} influx are important physiological responses in zebrafish embryos acclimated to low-Ca^{2+} freshwater (36). On the other hand, high plasma Ca^{2+} levels in rainbow trout (treated with a $CaCl_2$ injection) stimulated STC synthesis in the CS (15). In primary culture of rainbow trout CS cells, a high extracellular Ca^{2+} concentration induced enhanced STC mRNA expression (57), and the half-life of STC mRNA was extended in a high-Ca^{2+} environment (12). In the present study, zECaC mRNA levels in zebrafish acclimated to low-Ca^{2+} freshwater were higher than those of fish acclimated to high Ca^{2+}, while the zSTC-1 mRNA levels were lower in low Ca^{2+} than in high Ca^{2+} (Fig. 3). The contrary mRNA expressions of zECaC and zSTC-1 for the first time provide molecular evidence to support the antihypercalcemic action of STC-1, which has been proposed in fish and mammals (52). This was further supported by the subsequent experiment of zECaC knockdown. The Ca^{2+} uptake, Ca^{2+} content, and *zstc-1* expression were significantly inhibited in zECaC morphants (Fig. 4). It was demonstrated that an intraperitoneal injection of NPS 467 (a positive allosteric modulator of the Ca^{2+}-sensing receptor) stimulates STC secretion in fish (40). Thus, downregulation of *zstc-1* in ECaC morphants may have been due to a decrease in the Ca^{2+} influx that eventually lowered the serum Ca^{2+} concentration. These results suggest that the antihypercalcemic action of *zstc-1* may be involved in regulating zECaC expression. Subsequent experiments supported this point.

In a previous study, van der Heijden et al. (49) reported that the membrane density or affinity of PMCA and NCX in gill cells were not affected after a stanniectomy in an eel and concluded that the increase in the Ca^{2+}-transporting capacity may have been due to an increase in the number and/or size of Ca^{2+}-transporting cells. In isolated rat cardiomyocytes using a patch-clamp approach, STC-1 was found to regulate Ca^{2+} homeostasis by inhibiting the L-channel (42), but so far no evidence was available to indicate the action of STC-1 on ECaC's expression and function. In zSCT-1 morphants, the zECaC mRNA expression level was enhanced due to a lack of zSTC-1, and consequently Ca^{2+} influx and the Ca^{2+} content both increased (Fig. 6); however, no significant changes were found in the mRNA expressions of zPMCA2 or zNCX1b, which were colocalized with *zecac* in zebrafish mitochondria-rich cells (27). Moreover, the zECaC was not stimulated by low-Ca^{2+} treatment in zSTC-1 morphants (Fig. 7). These molecular physiological evidences not only demonstrate that zSTC-1 controls Ca^{2+} homeostasis via negatively regulating the expression of zECaC, but also supports the previous notion that zECaC is the gatekeeper of transepithelial Ca^{2+} transport in fish gills (22, 27, 36) and in mammals (20).

Many studies have indicated that several hormones like vitamin D and cortisol regulate ECaC expression in carrying out Ca^{2+} homeostasis (39, 41, 44, 48). The ECaC promoter contains several consensus vitamin D-responsive elements, which interact with the vitamin D receptor (VDR) and retinoid-x receptor (RXR) (19, 34). The VDR-RXR-coactivator complex interacts with the general transcription apparatus to initiate gene transcription (30). The signal pathways behind zSTC-1's inhibition of zECaC expression are unknown. It will be interesting and challenging to see whether zSTC-1 suppresses zECaC expression via interrupting VDR, RXR, or the coactivator's interaction.

Perspectives and Significance

Calcium homeostasis is strictly controlled in zebrafish during embryonic development. Functional regulation of zECaC, the key transporter of Ca^{2+} uptake, has been proposed to be an important process for internal calcium balance. The present study provides molecular physiological evidence demonstrating that zSTC-1 negatively regulates zECaC expression to control the Ca^{2+} balance in zebrafish. This control pathway is critical and should be early evolved in vertebrates, since vertebrates have been proposed to originate in marine, a high-Ca^{2+} environment. STC-1 was found in many species, implying that the functions of STC-1 on Ca^{2+} regulatory may be conserved among vertebrates during environmental acclimation. It would be evolutionarily important and interesting to see whether the phenomena found in zebrafish also hold in other vertebrates.

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