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# Salinity-dependent expression of a $Na^+$, $K^+$, $2Cl^-$ cotransporter in gills of the brackish medaka *Oryzias dancena*: A molecular correlate for hyposmoregulatory endurance

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ABSTRACT

This study used the brackish medaka (*Oryzias dancena*) to characterize $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC) expression from the genetic to cellular level in gills. Using RT-PCR to survey tissue distribution of *nkcc1a*, *1b*, and *2*, we report that gills of brackish medaka prominently express *Odnkcc1a*. The full-length cDNA of *Odnkcc1a* was cloned from gill tissue. *In situ* hybridization indicates that *Odnkcc1a* was localized to mitochondrion-rich (MR) cells. Higher mRNA levels of *Odnkcc1a* were found in gills from seawater (SW) and brackish water (BW) medaka when compared to freshwater (FW) fish. Furthermore, higher amounts of NKCC1a-like protein were detected by the monoclonal antibody in gills of SW and BW medaka compared to FW medaka. Double immunofluorescence staining revealed that NKCC1a-like protein colocalizes with $Na^+$, $K^+$–ATPase on the basolateral membrane of MR cells in BW and SW fish. In addition, transfer of brackish medaka from SW to FW revealed that expression of NKCC1a-like protein in gills was retained until 7 days, which is a likely mechanism for maintaining hyposmoregulatory endurance. The study illustrates salinity-dependent expression of NKCC1a in branchial MR cells from brackish medaka and suggests a critical role for NKCC1a in hyposmoregulatory endurance of this fish.



## 1. Introduction

The $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC), which is a member of the SLC12A family, is an integral membrane protein responsible for transporting one $Na^+$, one $K^+$, and two $Cl^-$ ions simultaneously from the external to the internal side of epithelial cells (Russell, 2000; Hebert et al., 2004; Gamba, 2005). Two distinct isoforms of NKCC cotransporters (NKCC1 and NKCC2) have been cloned from mammals. NKCC1, generally called the secretory isoform, is expressed ubiquitously and has functional roles in keeping $Cl^-$ above electrochemical equilibrium in order to regulate cell volume (Hoffmann and Dunham, 1995) as well as secreting epithelial salts (Haas and Forbush, 2000). NKCC1 is activated by cell shrinkage in response to hyperosmotic medium (Kregenow, 1981; Hoffmann et al., 1983). On the other hand, NKCC2 is the absorptive isoform localized to the apical membrane of epithelial cells. Mammalian NKCC2 proteins are involved in $Cl^-$ absorption in the intestine and thick ascending limb of Henle's loop (Gamba et al., 1994; Payne and Forbush, 1994, 1995; Igarashi et al., 1995; Starremans et al., 2003).

Euryhaline teleosts exhibit robust hyperosmoregulatory and hyposmoregulatory abilities that maintain plasma osmolality within narrow physiological ranges in hyposmotic and hyperosmotic environments, respectively (Kaneko et al., 2008). Gills exposed to the external medium are the major osmoregulatory organ of euryhaline teleosts. Several studies have described the ion transporter systems of mitochondrion-rich cells (MR cells, also called chloride cells) in gills (Marshall, 2002; Hirose et al., 2003; Evans et al., 2005; Hwang and Lee, 2007). MR cells express a $Na^+$, $K^+$–ATPase (NKA) on their basolateral membrane, which provides the driving force allowing secondary ion transporters to absorb ions in fresh water (FW) and secrete ions into seawater (SW) (Wood and Marshall, 1994; Evans et al., 1999; Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007). In SW teleosts, the model of $Cl^-$ secretion in gills of MR cells involved basolateral NKA and NKCC and an apical chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR), actively transporting $Cl^-$ from blood to the cells (Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007; Evans, 2008).

Two isoforms of NKCC1 (i.e., NKCC1a and NKCC1b) have been cloned from eel and tilapia species (Cutler and Cramb, 2002; Hiroi et al., 2008). In teleosts, the *nkcc1a* gene is expressed ubiquitously in various organs, while *nkcc1b* is expressed prominently in brain. In addition, expression of *nkcc2* was found in the intestine and kidney (Cutler and Cramb, 2008; Hiroi et al., 2008). In previous studies, branchial expression of *nkcc1* was demonstrated and found to increase upon hyperosmotic challenges in several euryhaline teleosts (Cutler and Cramb, 2002; Tipsmark et al., 2002; Scott et al., 2004,

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2005). Tse et al. (2006) reported an abundance of *nkcc1* in isolated MR cells and pavement cells from gills of Japanese eel. This experiment revealed that MR cells express more *nkcc1* gene than pavement cells. There is no direct evidence, however, identifying the cells expressed *nkcc1* in the whole tissue of gills of euryhaline teleosts.

In teleosts, the distribution and expression of branchial NKCC protein were detected by a monoclonal anti-human NKCC1 antibody (T4; Lytle et al., 1995). The molecular weights of immunoreactive bands from gill lysates were in the range of 120–190 kDa. Increased amounts of NKCC protein were reported in several euryhaline species upon hyperosmotic challenge (Tipsmark et al., 2002; Scott et al., 2004; Wu et al., 2003; Hiroi and McCormick, 2007). In addition, some studies reported that levels of NKCC protein in gills declined when fish were transferred from SW to FW (Lorin-Nebel et al., 2006; Tipsmark et al., 2004, 2008a,b). On the other hand, immunohistochemical staining with the T4 antibody on gills revealed that NKCC is localized to the basolateral membrane of MR cells in SW-acclimated fish (Wu et al., 2003; Hiroi and McCormick, 2007; Katoh et al., 2008).

Several teleost species including zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*), spotted green pufferfish (*Tetraodon nigroviridis*) and fugu (*Takifugu rubripes*) have become model organisms with well-developed databases of genomic sequences for molecular studies. However, the zebrafish is a stenohaline teleost that cannot survive in environments with salinity over 12‰. The other two pufferfish species reproduce with difficulty in the laboratory. On the contrary, Japanese medaka is an easily bred euryhaline teleost that resides naturally in FW (Haruta et al., 1991; Sakamoto et al., 2001; Inoue and Takei, 2002, 2003). It is a model fish with a genomic database and transgenic technology available for functional assays of target genes (Wittbrodt et al., 2002). Another euryhaline medaka species, brackish medaka (*Oryzias dancena*, also designated as *O. melastigma*; Inoue and Takei, 2002, 2003), is closely related to Japanese medaka, but primarily inhabits river mouths and estuaries (Roberts, 1998). Brackish medaka shows better salinity tolerance than Japanese medaka in survival rates of adult fish and hatching rates of embryos (Inoue and Takei, 2002, 2003). According to Kang et al. (2008), the lowest levels of NKA in MR cells were found in BW-acclimated brackish medaka, and the levels of NKA in fish acclimated to FW and SW were increased in order to maintain ionic homeostasis. These results indicated that hyposmoregulatory ability of brackish medaka was better than that of Japanese medaka when exposed to hyperosmotic environments.

The goal of the present study was to identify the specific NKCC isoform present in gill MR cells that underlies the hyposmoregulatory ability of brackish medaka. We have discovered the isoform of NKCC most prominently expressed in gills of brackish medaka by RT-PCR, and used *in situ* hybridization to characterize the distribution of branchial *nkcc1*. In addition, the $Na^+/Cl^-$ concentrations in plasma, as well as the expression of branchial NKCC from the gene to the cell level, were compared among fish of different salinity groups (i.e. FW, BW and SW). Finally, we measured changes in branchial NKCC1a-like protein abundance for SW-acclimated brackish medaka over time following hyposmotic FW challenge, in order to illustrate the correlation of NKCC protein expression with the hyposmoregulatory endurance of this euryhaline species.

## 2. Materials and methods

### 2.1. Experimental animals

Brackish medaka (*O. dancena*) obtained from a local aquarium were 2.50 ± 0.30 cm in length. For experiments, fish were acclimated to seawater (SW: $[Na^+]$ 483.00 ± 12.70 mM; $[K^+]$ 11.381 ± 0.07 mM; $[Ca^{2+}]$ 15.34 ± 0.16 mM; $[Mg^{2+}]$ 67.87 ± 1.45 mM; $[Cl^-]$ 572.89 ± 24.24 mM), brackish water (BW: $[Na^+]$ 156.11 ± 4,50 mM; $[K^+]$ 5.72 ± 0.06 mM; $[Ca^{2+}]$ 9.29 ± 0.28 mM; $[Mg^{2+}]$ 30.34 ± 1.29 mM; $[Cl^-]$ 270.60 ± 12.07 mM) or local fresh tap water (FW: $[Na^+]$ 0.22 ± 0.01 mM; $[K^+]$ 0.04 ± 0.01 mM; $[Ca^{2+}]$ 0.68 ± 0.01 mM; $[Mg^{2+}]$ 0.28 ± 0.01 mM; $[Cl^-]$ 0.14 ± 0.01 mM) at 28 ± 1 °C with a daily 14 h:10 h L:D photoperiod for at least 1 month before experiments. Water was continuously circulated through fabric-floss filters and was partially refreshed every 2 weeks. Fish were fed daily with a diet of commercial pellets. Following the experiment, fish were not fed and were anaesthetized with MS-222 (100–200 mg/L) before sampling. The facilities and protocols for experimental animals were approved by the Animal Care and Utility Committee of National Chung-Hsing University (approval no. 96–48).

### 2.2. Plasma analysis

The method of collecting plasma from brackish medaka acclimated to FW, BW, and SW was described by Kang et al. (2008). $[Na^+]$ was measured with a Hitachi Z-8000 polarized Zeeman atomic absorption spectrophotometer (Tokyo, Japan). $[Cl^-]$ was determined by a protocol modified from the ferricyanide method (Franson, 1985) using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 460 nm.

### 2.3. Total RNA extraction

Using the method of Kang et al. (2008), total RNA samples from various tissues was extracted using RNA-Bee™ (Tel-Test, Friendwood, TX, USA) following the manufacturer's instructions. Genomic DNA was eliminated using the RNA clean-up protocol of the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA).

### 2.4. RT-PCR analysis of $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC) isoforms in different tissues

Expression of NKCC1a (SLC12a2a), NKCC1b (SLC12a2b) and NKCC2 (SLC12a1) in various organs of brackish medaka was examined with RT-PCR. Primers were designed to identify specific regions of the three NKCC isoforms according to the cDNA library in the Ensembl medaka genomic database browser (http://www.ensembl.org/Oryzias_latipes/index.html), as shown in Table 1. Total RNA was extracted from brains, gills, eyes, hearts, intestines, kidneys, livers, muscles, fins, ovaries and testes. The tissues were collected from ten FW- and ten SW-acclimated fishes. RNA samples were stored at −80 °C after isolation. First-strand cDNA was synthesized by reverse transcribing 5 μg of total RNA using 1 μL Oligo-dT (0.5 μg/μL) primer and 1 μL PowerScript™ Reverse Transcriptase (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. For PCR amplification (28 cycles), 1 μL of cDNA was used as a template in a 25 μL final reaction volume containing 0.25 μM dNTPs, 1.25 U Hot

**Table 1**
Primer sequences used for cDNA cloning, RACE, and probe construction for *in situ* hybridization and quantitative real-time PCR of NKCC genes of the brackish medaka.

| Primer name | Primer sequence (5′ to 3′) |
|---|---|
| *nkcc1a*-F | ACACCGTGATGAGCGAGAG |
| *nkcc1a*-R | AAGGTCAGGACGCTCTGGT |
| *nkcc1b*-F | TGGACACAGTGAACTGCACA |
| *nkcc1b*-R | AAGCACTGTGGCCTGAAGTT |
| *nkcc2*-F | CGCATATCATCAAGGCAGAA |
| *nkcc2*-R | GGTGGAAGGTTCCTTGTCAG |
| *nkcc1a*-RACE-F | AGCACAGCCAACCTCATCGTAATAAG |
| *nkcc1a*-RACE-R | GTTCTCAGGTAGGTGTTGGTGTGG |
| *nkcc1a*-QPCR-F | CTCTCCACTTCAGCCATCG |
| *nkcc1a*-QPCR-R | ACCACATACATGGCAACAGC |
| *nkcc1a*-Probe-F | CTGGCTGATGAAGAA**CC**AGA |
| nkcc1a-Probe-R | GGTACGGCTCAATCATCTCC |
| *β-actin*-F | CTGGACTTCGAGCAGGAGAT |
| *β-actin*-R | AGGAAGGAAGGCTGGAAGAG |

start EX-Taq polymerase (Takara, Shiga, Japan),and 0.2 μM of each primer. PCR products were subcloned into the pOSI-T vector (Genemark, Taipei, Taiwan), and amplicons were sequenced for confirmation. β-actin (Kang et al., 2008) was used as an internal control for all tissues (Table 1).

### 2.5. cDNA cloning of a full-length NKCC1a gene from gill and sequence analysis

cDNA for cloning and RACE was made from total RNA of gills from four fish using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) and SMART RACE cDNA amplification kit (Clontech) following the manufacturer's protocol. For PCR amplification, 2 μL of cDNA was used as a template in a 50-μL reaction containing 0.25 mM dNTPs, 2.5 U Hot start EX-Taq polymerase (Takara), and 0.2 μM of each primer. Primer sets were designed from the bioinformatics method in Table 2. PCR products were subcloned into the pOSI-T vector (Genemark) and amplicons were sequenced to confirm PCR products. Specific primers for 5′ and 3′ RACE were designed from partial sequences obtained from the PCR using the primer sets listed in Table 1. RACE PCR products were also subcloned into the pOSI-T vector (Genemark) and sequenced. Sequence alignments were performed with CLUSTALW. Prediction of transmembrane segments was done on the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Potential N-glycosylation sites and phosphorylation sites were predicted by the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/), respectively. Amino acid sequences for NKCC from different organisms were aligned by CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and a phylogenetic tree was constructed using MEGA 4.1. This tree was built using the Neighbor-joining method with the pairwise deletion gaps calculating option.

### 2.6. Quantitative real-time PCR (qPCR)

NKCC1a mRNA was quantified with an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR reactions contained 8 μL of cDNA (1000×), 2 μL of either 5 μM nkcc1a-QPCR primer mixture or 5 μM β-actin primer mixture (Kang et al., 2008), and 10 μL of 2× SYBR Green PCR MasterMix (Applied Biosystems). The methods of real-time PCR analysis and the calculated formula for the target gene were described by Kang et al. (2008). Primer sets for real-time PCR are shown in Table 1. Primers for β-actin were used as an internal control in all samples.

### 2.7. Antibodies

Primary antibodies used in this study include: (1) a mouse monoclonal antibody (α5; Developmental Studies Hybridoma Bank) raised against the α-subunit of the avian $Na^+$, $K^+$–ATPase (NKA); (2) mouse monoclonal antiserum (T4; Developmental Studies Hybridoma Bank) raised against the C-terminus of human NKCC; (3) rabbit polyclonal antiserum (#11) was raised against 565 amino acids of the α-subunit of the NKA (Hwang et al., 1998); and (4) mouse monoclonal antibody (8226, Abcam, Cambridge, UK) against residues 1–100 of human β-actin. Alkaline phosphatase-(AP) conjugated sheep anti-digoxigenin (dig) Fab fragments (Roche, Mannheim, Germany) were used to detect the dig-labeled probe by whole-mount *in situ* hybridization. The secondary antibody for immunoblots was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA). For double immunofluorescence staining, the secondary antibodies were AlexaFluor-488-conjugated goat anti-mouse IgG and AlexaFluor-546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA).

**Table 2**
Plasma osmolality, and $Na^+/Cl^-$ concentrations of the brackish medaka acclimated to fresh water (FW), brackish water (BW), and seawater (SW).

| Environment | FW | BW | SW |
|---|---|---|---|
| Plasma osmolality (mOsmol $kg^{-1}$) | 283 ± 4.65* | 313.25 ± 3.45 | 313.33 ± 0.62 |
| Plasma $Na^+$ concentration (mmol $kg^{-1}$) | 100.98 ± 1.53* | 120.09 ± 1.75 | 126.09 ± 3.73 |
| Plasma $Cl^-$ concentration (mmol $kg^{-1}$) | 132.23 ± 1.67* | 147.96 ± 2.79 | 152.32 ± 2.91 |

The data of plasma osmolality was modified from Kang et al. (2008). The plasma $Na^+$ and $Cl^-$ concentrations were analyzed with Tukey's multiple-comparison test following one-way ANOVA ($p<0.05$, $n=8$). The asterisks indicated significant differences among groups.

### 2.8. Whole-mount in situ hybridization (WISH) of gills

A cDNA fragment of *Odnkcc1a* (815 bp) was amplified with *Odnkcc1a*-Probe-F and R primers (Table 1) by PCR and inserted into the pOSI-T vector (Genemark). After amplifying the target gene by PCR with T7 and SP6 primers, products were subjected to *in vitro* transcription with T7 and SP6 RNA polymerases (Takara), respectively. Dig-labeled RNA probes were examined in RNA gels to confirm their quality and concentration. Four pairs of gills from FW- and SW-acclimated brackish medaka were excised and fixed in 4% PFA in DEPC-PBS solution (1.4 mM NaCl, 0.2 mM KCl, 0.1 mM $Na_2HPO_4$ and 0.002 mM $KH_2PO_4$) at 4 °C for 2 h, and then washed with DEPC-PBS. Subsequently, gills were treated with methanol at −20 °C overnight. Samples were then washed with DEPC-PBST (PBS with 0.1% Tween-20) twice for 10 min. Samples were prehybridized with hybridization buffer (60% formamide, 5× SSC, 0.1% Tween-20, 500 μg/mL yeast tRNA and 50 μg/mL heparin) for 2 h at 65 °C, and then hybridized with probe (40 ng) in buffer overnight at 67 °C. Samples were washed in a series of HyB/SSC at 67 °C and then washed in a series of SSC/PBST buffers at room temperature. Samples were blocked with 5% sheep serum in 2 mg/mL BSA (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 2 h and then incubated with an anti-dig Fab (1:5000 in blocking solution) at 4 °C overnight. After being washed with DEPC-PBST at room temperature 8 times for 15 min each, gills were treated with staining buffer (0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl and 0.1% Tween-20) twice for 10 min and then developed after incubation with the NBT/BCIP kit (Zymed, South San Francisco, CA, USA) for 1.5 h in the dark. The reaction was stopped by PFA and subsequently washed twice with methanol. Finally samples were stored in DEPC-PBST at 4 °C in the dark until further examination and analysis. Micrographs were taken with a digital camera (Nikon COOLPIX 5000, Tokyo, Japan).

### 2.9. Cryosectioning

Gills from FW-, BW- and SW-acclimated brackish medaka were excised and fixed immediately in a mixture of methanol and DMSO (v/v: 4/1) at −20 °C for 3 h for double immunofluorescence staining. Samples were washed with PBS, equilibrated with O.C.T. (Sakura Tissue-Tek, Torrance, CA, USA) overnight at 4 °C and then mounted for cryosectioning. Sections were cut at 5-μm thickness with a Cryostat Microtome (Microm HM 505E, Walldorf, Germany) at −25 °C. Sections were placed on 0.01% poly-L-lysine (Sigma) coated slides and kept in slide boxes at −20 °C before immunofluorescence or immunohistochemical staining.

### 2.10. Immunohistochemical detection of NKA immunoreactive (NKA-IR) cells in whole-mount in situ hybridized gills

Cryosections of *in situ* hybridized gills were washed with PBS and then observed under a microscope (Olympus BX50), and micrographs were taken with a digital camera (Nikon COOLPIX 5000). Sections

were then stained with a monoclonal antibody (α5) to label the NKA α-subunit, followed by a commercial kit (PicTure™, Zymed) for visualizing the immunoreaction. After staining, sections were mounted in Clearmount™ (Zymed), covered with cover slips, and secondary micrographs were taken in the same region of this gill filament. Comparing two micrographs of the same gill filament region revealed the location of NKA immunoreactive cells in the *in situ*-hybridized gill filaments.

### 2.11. Double immunofluorescence staining and confocal microscopy

Cryosections of gills from FW-, BW- and SW-acclimated brackish medaka were rinsed in PBS and incubated in 5% bovine serum albumin (Sigma) and 2% Tween-20 (Merck, Hohenbrunn, Germany) in PBS for 30 min. Cryosections were then washed in PBS and incubated with primary polyclonal antibodies (#11; 1:100) overnight at 4 °C. Cryosections were then washed several times with PBS, incubated with secondary antibody (AlexaFluor 546 goat anti-rabbit IgG) at room temperature for 2 h and washed several times with PBS. After the first staining, cryosections were incubated with PBS-diluted primary monoclonal antibody (T4; 1:50) for 2 h at room temperature. Cryosections were then washed several times with PBS and incubated with secondary antibody (AlexaFluor 488 goat anti-mouse IgG) at room temperature for 2 h followed by several PBS washes. Cryosections were then mounted in Clearmount™ (Zymed) and examined using a Zeiss LSM 510 inverted laser scanning microscope (Hamburg, Germany).

### 2.12. Preparation of gill homogenates

Four pairs of gills were excised and stored in a microcentrifuge tube at −80 °C before use. The tissue was rapidly thawed and homogenized in 500 µL SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole and 0.1% sodium deoxycholate, pH 7.5) containing protease inhibitors (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) (v/v: 50/1). Homogenization was performed in microcentrifuge tubes with a Polytron PT12000E (Lucerne, Switzerland) at the maximal speed for 25 rotations on ice. Homogenates were then centrifuged at 5000 *g* and 4 °C for 5 min. Supernatants were collected and BCA protein assay reagents were used (Pierce) to determine protein concentrations, with BSA (Sigma) as a standard.

### 2.13. Immunoblotting

The prestained molecular weight marker was from Fermentas (SM0671; Hanover, MD, USA). Aliquots containing 10 µg of gill homogenate were heated at 65 °C for 20 min and fractionated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. Proteins were transferred from unstained gels to PVDF (Millipore, Bedford, MA, USA) using a tank transfer system (Bio-Rad, Mini protean 3). Blots were pre-incubated for 2 h in PBST containing 5% (w/v) non-fat dried milk and subsequently incubated at 4 °C with primary antibody (T4) diluted with PBST (1:500) overnight, followed by a 1 h reaction with horseradish peroxidase-conjugated antibody (1:5000 dilution). Blots were developed using the ECL kit (Pierce). Immunoblots were photographed and imported as TIFF files into the ID image analysis software package (MCID Analysis Evaluation 7.0). Results were converted to numerical values in order to compare relative intensities of immunoreactive bands.

### 2.14. Declining hyposmoregulatory endurance and dynamic expression of NKCC in brackish medaka in response to hyposmotic challenge

Four groups of SW-acclimated medaka with 15 individuals in each group were exposed to FW for 3 (group 1), 7 (group 2), 14 (group 3) and 21 (group 4) days. These groups were then transferred directly to SW for 48 h. Survival rates indicating hyposmoregulatory endurance were compared among these groups. Meanwhile, the survival rate of the control group in which FW-acclimated fish were transferred directly to SW was compared to those of the other groups. Experiments were repeated six times. Since the experiment was designed to clarify the relationship between hyposmoregulatory endurance and the expression of branchial NKCC, SW-acclimated brackish medaka were transferred to FW and sampled at 1, 3, 7, 14 and 21 days post-transfer to analyze changes in NKCC protein expression. In this time-course experiment, fish from the control group were transferred from SW to SW with identical sampling time points and immunoblotting analyses.

### 2.15. Statistical analysis

Values were compared using one-way ANOVA followed by Tukey's pairwise method or Dunnett's test, and $p<0.05$ was set as the significance level. Values are expressed as the mean ± S.E.M. (the standard error of the mean) unless stated otherwise.

## 3. Results

### 3.1. Cloning and characterization of $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC) isoforms

Three NKCC isoforms (*Odnkcc1a*, GQ862972; *Odnkcc1b*, GQ502445; and *Odnkcc2*, GQ862971) were cloned from brackish medaka using primers designed according to Japanese medaka databases (Table 1). RT-PCR analysis of different organs revealed that *Odnkcc1a* (1441 bp) is expressed ubiquitously in several organs, *Odnkcc1b* (703 bp) is found in the brain, eye, ovary and testis, and *Odnkcc2* (757 bp) is expressed mainly in kidney, intestine, brain, liver, ovary and testis (Fig. 1). These results indicate that gills of brackish medaka prominently express *Odnkcc1a*.

A 3426 bp full-length NKCC1a cDNA encoding an 1142 amino acid protein was isolated from gills of brackish medaka by RACE-PCR. The cDNA contained 313 bp of 5′ UTR and 1469 bp of 3′ UTR. The deduced amino acid sequence of NKCC1a aligned with those from other species revealed 12 predicted transmembrane domains (Fig. 2), and four potential N-linked glycosylation sites were found. Phosphorylation sites for Sterile 20 protein-related proline alanine-rich kinase (SPAK) and oxidative stress response 1 kinase (OSR1) were also identified (Fig. 2), and consisted of 27 serine, 14 threonine and 4 tyrosine residues (Fig. 2). In addition, medaka NKCC1a showed 75% similarity to the human epitope for the T4 monoclonal antibody (Fig. 2).

According to the phylogenetic tree, NKCCs from vertebrates can be divided into two subclasses: NKCC1 and NKCC2. Furthermore, two

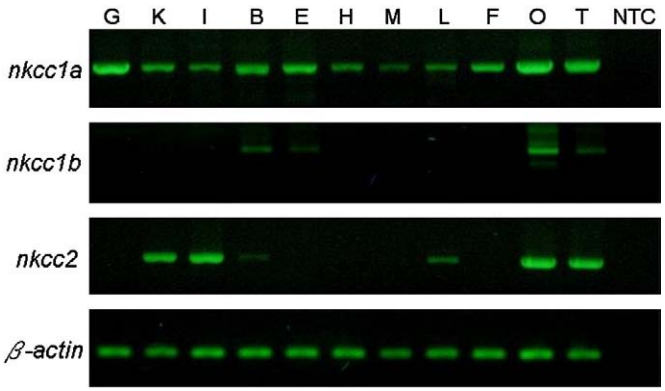



**Fig. 1.** Tissue distribution of three *Odnkcc* genes detected by RT-PCR in brackish medaka. β-actin was used as an internal control. B, brain; E, eye; F, fin; G, gill; H, heart; I, intestine; K, kidney; L, liver; M, muscle; O, ovary; T, testis; NTC, no template control.

isoforms of teleost NKCC1 were found: NKCC1a and NKCC1b. The amino acid sequence of NKCC1a from brackish medaka showed 92% identity to NKCC1a of Japanese medaka and 84–89% identity to the other teleosts, 73–84% identity to NKCC1b of the other teleosts, and 71–73% identity to NKCC1 of the other vertebrates. Only 55–58% identity was found between sequences of medaka NKCC1a and NKCC2 or the outgroup NCC from other species (Fig. 3).

### 3.2. In situ hybridization of nkcc1a in gills

To identify the cell types that prominently express *Odnkcc1a*, an antisense RNA probe was used to detect *Odnkcc1a* in gills from freshwater-(FW-) and seawater-(SW-)acclimated medaka. The results of WISH showed that signals were more prominent in gills of SW-acclimated medaka than in those from FW fish (Fig. 4). *Odnkccla* was mainly found in epithelial cells of the afferent region of the gill filament (Fig. 4C, D). The sense probe revealed no signal in either FW or SW fish (data not shown). Cryosections of gills from SW-acclimated medaka were counterstained with a $Na^+$, $K^+$–ATPase (NKA) α-subunit antibody and revealed that epithelial cells positive for *Odnkcc1a* also showed immunoreactivity for NKA (Fig. 5).

### 3.3. Plasma osmolality and $Na^+/Cl^-$ concentration

The plasma osmolality and $Na^+/Cl^-$ concentrations of the brackish medaka acclimated to FW, BW, and SW were maintained within ranges that tolerate survival in different osmotic environments. The plasma osmolality and $Na^+/Cl^-$ concentrations for FW-acclimated medaka were significantly lower than those for BW- and SW-acclimated fish (Table 2).

### 3.4. Odnkcc1a mRNA abundance quantified by real-time PCR

The branchial *Odnkcc1a* mRNA levels for brackish medaka acclimated to FW, BW, and SW were significantly different between all groups (Fig. 6). The lowest *Odnkcc1a* mRNA level was found in FW-acclimated individuals, and the highest *Odnkcc1a* mRNA level was in the SW group. BW and SW groups were about 2.8- and 4.4-fold higher, respectively, than the FW group. The level in SW fish was thus significantly higher (approximately 1.6-fold) than in the BW group. Therefore, branchial *Odnkcc1a* mRNA levels in brackish medaka increase significantly with environmental salinities.

### 3.5. Immunoblotting and relative amounts of NKCC1a-like protein

Immunoblots of gill proteins from FW-, BW-, and SW-acclimated medaka revealed a single immunoreactive band with a molecular mass from 120 to 170 kDa and centered at 150 kDa (Fig. 7A). Quantification of immunoreactive bands for different groups of brackish medaka revealed that the lowest levels of the NKCC1a-like protein were found in the FW group, with BW and SW individuals being about 2.8- and 9.0-fold higher, respectively (Fig. 7B). Thus, the protein levels in SW fish were significantly higher (approximately 3.2-fold) than in the BW fish. Relative amounts of branchial NKCC1a-like protein therefore increase significantly with environmental salinity.

### 3.6. Immunolocalization of NKCC1a-like protein in the gill

Fig. 8 shows the afferent epithelium in cross-sections of gill filaments from FW-, BW-, and SW-acclimated medaka stained with antibodies against NKCC1a-like protein or the NKA α-subunit. Merged images for BW- and SW-acclimated medaka reveal colocalization of NKCC1a-like protein and NKA α-subunit in the same cells (Fig. 8F, I), which indicates that NKCC1a-like protein is present in the basolateral membrane of NKA-immunoreactive (NKA-IR) cells. On the other hand, double labeling of NKCC1a-like protein and NKA in FW fish showed that NKCC immunoreactivity occurs in apical regions (green) of the NKA-IR cells (Fig. 8C).

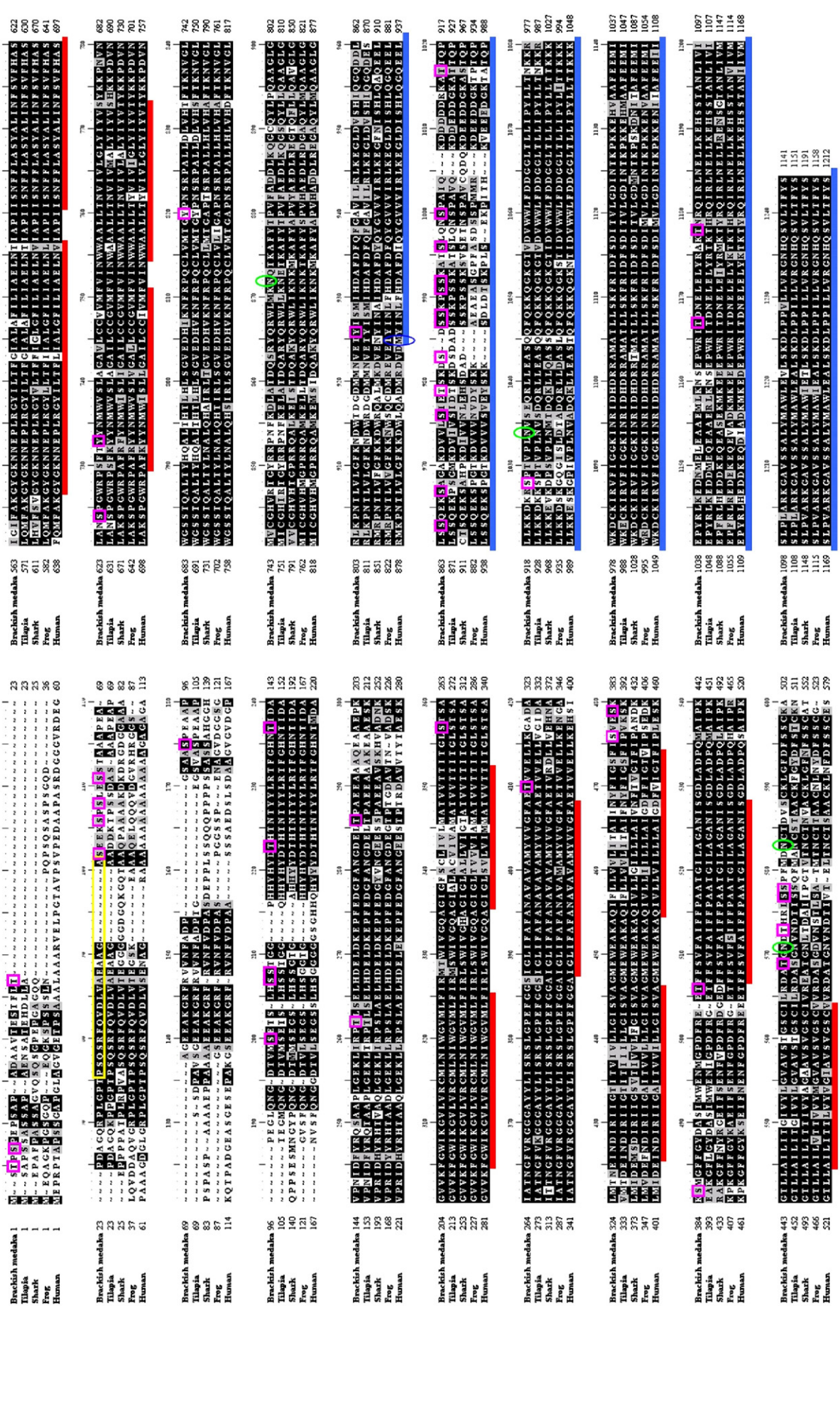
### 3.7. Time course of hyposmoregulatory endurance and NKCC1a-like protein expression in response to hyposmotic challenge

Brackish medaka can survive after transfer from SW to FW. The hyposmoregulatory endurance of SW-acclimated medaka was determined by survival rates when fish were exposed directly to FW for 3, 7, 14 or 21 days and subsequently transferred to SW (Fig. 9). When FW-acclimated medaka were transferred directly to SW (the control group), the survival rate was approximately 5%, indicating almost no hyperosmotic tolerance in these fish (Fig. 9). When the FW exposure periods for SW-acclimated medaka were 3 or 7 days, they had survival rates of about 92 or 77% following SW challenge. The survival rate for the 14-day exposure group was about 57% when transferred back to SW, significantly lower than for the 3- and 7-day groups. The lowest survival rate (18%) was found in the 21-day exposure group, with no significant difference when compared to the control group. These results indicate that the hyposmoregulatory endurance of SW-acclimated medaka was maintained for at least 7 days when fish were exposed to FW.

Representative immunoblots of NKCC1a-like protein from gills of SW-acclimated medaka transferred to FW for 1, 3, 7, 14 and 21 days displayed a single immunoreactive band centered around 150 kDa (Fig. 10A). Compared to the control group (transfer from SW to SW), expression of branchial NKCC1a-like protein in FW-exposed medaka declined significantly (about 4-fold) at 7 days post-transfer, and further decreased to approximately 14-fold lower than controls at 14 and 21 days after transfer (Fig. 10B).

## 4. Discussion

The present $Cl^-$ secretory model of gill MR cells in seawater teleosts proposes that the $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC) is expressed on the basolateral membrane of epithelial MR cells for transporting $Cl^-$ from circulating plasma into the cytoplasm of cells (Marshall, 2002; Hirose et al., 2003; Hwang and Lee, 2007; Evans, 2008). Inoue and Takei reported that, in hyperosmotic environments, brackish medaka (*O. dancena*) had higher survival rates for adult fish and hatching rates of embryos when compared to Japanese medaka (*O. latipes*). Our previous study found that the plasma osmolality in FW-, BW-, and SW-acclimated brackish medaka was from 283 to 313 mOsm $kg^{-1}$ (Kang et al., 2008). In this study, concentrations of the two most abundant ions ($Na^+$ and $Cl^-$) in plasma from brackish medaka that had acclimated to environments of various salinities were further determined and compared (Table 2). The significantly lower plasma $Na^+$ and $Cl^-$ concentrations found in FW-acclimated brackish medaka ([$Na^+$]: 100 mmol $kg^{-1}$; and [$Cl^-$]: 132 mmol $kg^{-1}$) indicates that the hyperosmoregulatory ability of brackish medaka was activated to keep plasma parameters within a tolerable range. In contrast, the higher but consistent levels of $Na^+$ and $Cl^-$ found in the BW and SW groups illustrated a better hyposmoregulatory ability of those fish in maintaining homeostasis ([$Na^+$]: 123 mmol $kg^{-1}$; and [$Cl^-$]: 150 mmol $kg^{-1}$) in hyperosmotic environments. This pattern was similar to that of other marine euryhaline teleosts such as longhorn sculpin (Hyndman and Evans, 2009), marble goby (Chew et al., 2009), milkfish (Lin et al., 2006b), pufferfish (Kato et al., 2005) and sea bass (Jensen et al., 1998). In addition, our previous study found that expression patterns of branchial $Na^+$, $K^+$–ATPase (NKA) in brackish medaka when acclimated to environments with varying salinities were similar to those of other BW-residing euryhaline teleosts (Kang et al., 2008). Our results indicate that upon hyperosmotic challenge, brackish medaka have better osmoregulatory ability than Japanese medaka. Ho et al. (2008) also claimed that this species was suitable for studying the physiological effects of marine



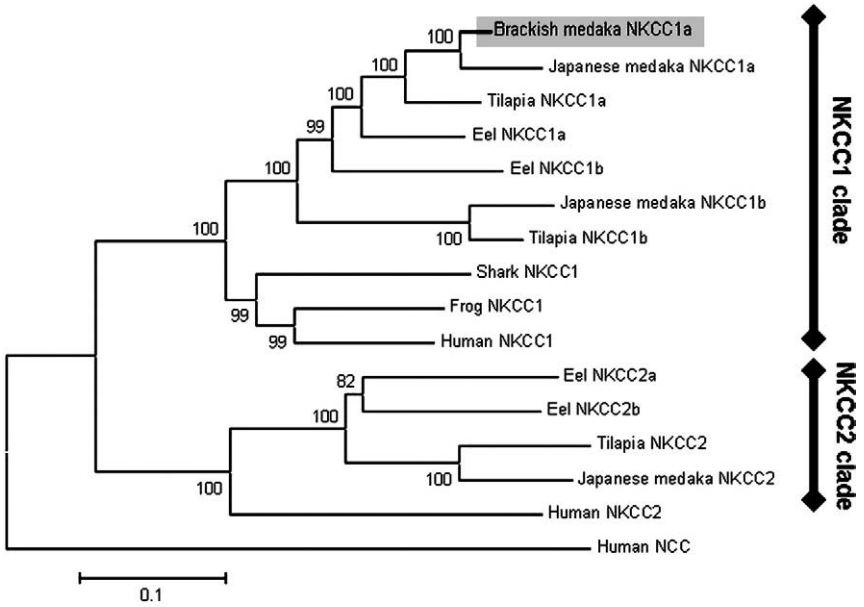



**Fig. 3.** Phylogenetic analysis of full-length amino acid sequences of the $Na^+$, $K^+$, $2Cl^-$ cotransporter (NKCC) from different species, shown as a phylogenetic tree by the neighbor-joining method with 1000 bootstrap replicates. Brackish medaka NKCC1a is shaded. The $Na^+$, $Cl^-$ cotransporter (NCC) of human was used as an outgroup to root the tree. The branches and three clades (NKCC1 clade and NKCC2 clade) were found to be different sub-tree regions. Numbers at the nodes are bootstrap values for 1000 replications, shown as percentages. The GenBank accession numbers are as follows: tilapia (NKCC1a, AAR97731; NKCC1b, AAR97732; NKCC2, AAR97733; NCC, ACB12742), eel (NKCC1a, CAD31111; NKCC1b, CAD31112; NKCC2α, CAD92100; NKCC2β, CAD92101), Japanese medaka (Ensembl protein report NKCC1a, ENSORLT00000024012; NKCC1b, ENSORLT00000021358; NKCC2, ENSORLT00000003346), shark (P550113), frog (NP_001116071) and human (NKCC1, NP_001037; NKCC2, NP_000329; NCC, NP_001119580).

pollution. Therefore, plasma parameters indicate that, for illustrating the expression of NKCC in the $Cl^-$ secretory model for branchial MR cells, brackish medaka is a suitable animal model with effective hyposmoregulatory ability upon hyperosmotic challenge.

Primers for three *nkcc* isoforms were designed from the medaka database because brackish medaka is closely related to Japanese medaka (Table 1). The sequences of *Odnkcc1a*, *Odnkcc1b*, and *Odnkcc2* were identified in this study. The expression pattern of *Odnkcc1a* was similar to that in eel (Cutler and Cramb, 2002) and tilapia (Hiroi et al., 2008) and ubiquitous in various organs (Fig. 1). Expression of *Odnkcc1b* was found mainly in the nervous system and gonad. The *nkcc1b* gene was also found in the brains of eel and tilapia (Cutler and Cramb, 2002; Hiroi et al., 2008). On the other hand, the absorptive form *Odnkcc2* was mainly expressed in kidney, intestine, ovary and testis of medaka (Fig. 1). Previous studies reported that *nkcc2* was expressed in intestine and kidney of eel and tilapia for chloride absorption (Cutler and Cramb, 2008; Hiroi et al., 2008). Our results therefore indicate that gills of brackish medaka prominently express *Odnkcc1a* (Fig. 1). As detected by Northern blotting and RT-PCR, gills of tilapia and eel were shown to express *nkcc1a* prominently (Cutler and Cramb, 2002; Hiroi et al., 2008). Twelve conserved transmembrane domains, similar to the orthologs (Gamba, 2005), were identified in the deduced protein sequence of NKCC1a from brackish medaka by hydropathy analysis (Fig. 2). Phylogenetic analysis revealed that NKCC1a of teleosts belongs to a different branch from NKCC1b and NKCC2 (Fig. 3). The deduced amino acid sequence of NKCC1a from brackish medaka showed the highest similarity to NKCC1a sequences of other teleosts, when compared to NKCC1b from other teleosts as well as to NKCC1, NKCC2 and NCC sequences from other vertebrates. The presence of several NKCC1 isoforms in brackish medaka might reflect a whole-genome duplication event in its ancestor, which has also been proposed by comparing human and teleost genomes (Kasahara et al., 2007).

To our knowledge, this is the first study to use the sections from whole mount *in situ* hybridization-treated filaments counterstained with a $Na^+$, $K^+$–ATPase (NKA) α-subunit antibody (α5) to realize the localization of *Odnkcc1a* in gills of the brackish medaka. This protocol has been used to localize other ion transporters such as the $Na^+$, $H^+$ exchanger, the V-type $H^+$–ATPase and SLC10.2 in ionocytes of zebrafish larvae (Lin et al., 2006a; Yan et al., 2007; Wang et al., 2009). *Odnkcc1a* was found prominently in epithelial NKA-immunoreactive cells of the afferent region of gill filaments, and was stronger in SW fish when compared to FW individuals (Figs. 4 and 5). Tse et al. (2006) also reported that isolated epithelial MR cells from eels express more *nkcc1* than pavements cells. Based on these results, epithelial MR cells (indicated by NKA immunoreactivity) in SW-acclimated medaka expressed higher levels of *Odnkcc1a* than other cell types in gills.

The monoclonal anti-human NKCC antibody T4 (Lytle et al., 1995) has been widely used in studies on protein expression and distribution of branchial NKCC in euryhaline teleosts. Compared with antigen sequences for the monoclonal T4 antibody, the corresponding region of the brackish medaka NKCC1a sequence showed 75% similarity (Fig. 2). Colocalization of NKCC1a-like protein and NKA in gills from BW- and SW-acclimated brackish medaka (Fig. 8) revealed that NKCC1a-like protein is located at the basolateral membrane of MR cells. Similar results were also found in MR cells from other euryhaline teleosts such as killifish, salmon and tilapia (Pelis et al., 2001; Wu et al., 2003; Hiroi and McCormick, 2007; Katoh et al., 2008) when exposed to hyperosmotic environments. The distribution of NKCC1a agreed with the currently accepted model of $Cl^-$ secretion via branchial MR cells in SW fish (Evans et al., 2005).

**Fig. 2.** Amino acid sequence alignment of brackish medaka NKCC1a compared to NKCC1 from tilapia (AAR97731), shark (P550113), frog (NP_001116071) and human (NP_001037). Gaps (dashed) were introduced to optimize alignment. All amino acids identical or similar to those of brackish medaka NKCC1a are shaded. Twelve putative transmembrane segments (TM1–TM12) are indicated (red underline). The binding motifs of Sterile 20 protein-related proline alanine-rich kinase (SPAK) and oxidative stress response 1 kinase (OSR1) are represented by a yellow box. Potential N-linked glycosylation sites are marked by the green circle and potential phosphorylation sites are marked by the purple square. The antigen sequence (human) of T4 antibody is marked by the blue underline and the first amino acid (Methionine-902) is marked by a blue circle.

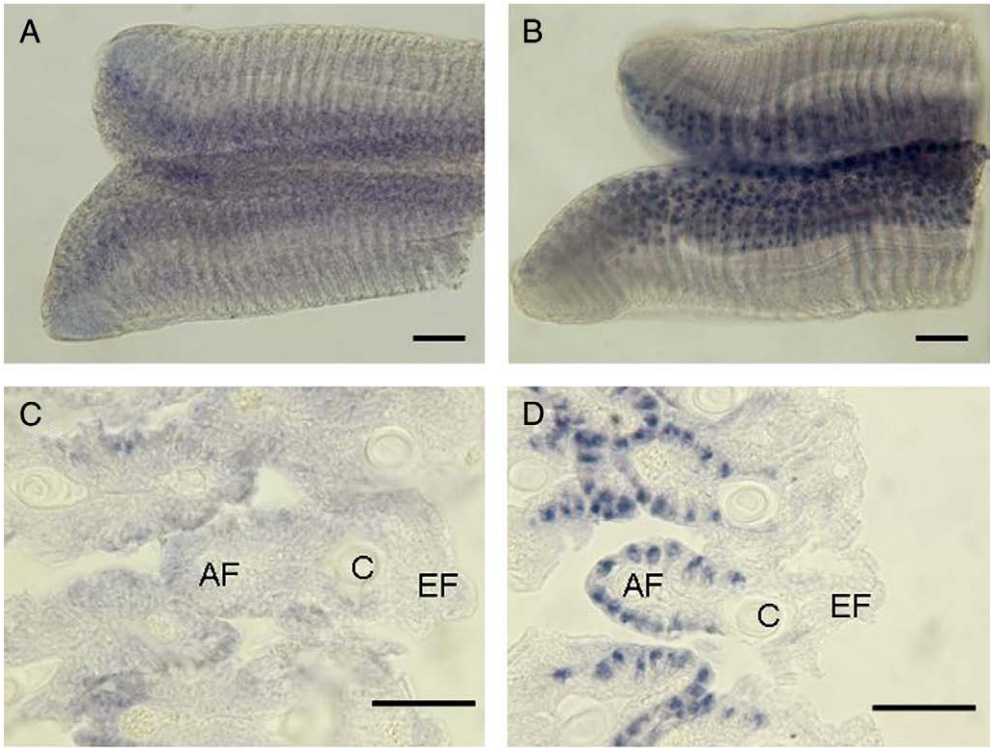



**Fig. 4.** Whole-mount *in situ* hybridization with an antisense *Odnkcc1a* RNA probe on gill filaments of (A) FW- and (B) SW-acclimated brackish medaka. Sections of these hybridized filaments (FW: C, SW: D) showed the cross-staining with *Odnkcc1a* probes. In the filaments from SW fish, the *Odnkcc1a* signals (purple) were found on the epithelial cells of the afferent region. Scale bar = 50 μm. FW, fresh water; SW, seawater.; AF, afferent region; C, cartilage; EF, efferent region.

On the other hand, previous studies using immunohistochemical staining with this antibody in gills of FW-acclimated euryhaline teleosts revealed three patterns: (1) no signal (lake trout, Hiroi and McCormick, 2007); (2) immunoreactivity in the apical region of MR cells (killifish, Katoh et al., 2008; sea bass, Lorin-Nebel et al., 2006; and tilapia; Wu et al., 2003; Inokuchi et al., 2009); and (3) immunoreactive signals localized to the basolateral membrane of MR cells (goby, McCormick et al., 2003; pufferfish, Prodocimo and Freire, 2006; and sturgeon, Sardella and Kültz, 2009). In FW-acclimated brackish medaka, the immunostaining pattern showing positive signals on the apical membrane of MR cells was similar to the second group as described above (Fig. 8). According to previous studies, two candidate proteins were proposed to be the apically distributed signals. First, Wu et al. (2003) suggested that the apical signals were NKCC2 for ion absorption in FW, because the T4 antibody recognizes both secretory NKCC1 and absorptive NKCC2 in mammals. Gills of brackish medaka, however, do not exhibit *Odnkcc2* expression (Fig. 1). Second, Hiroi

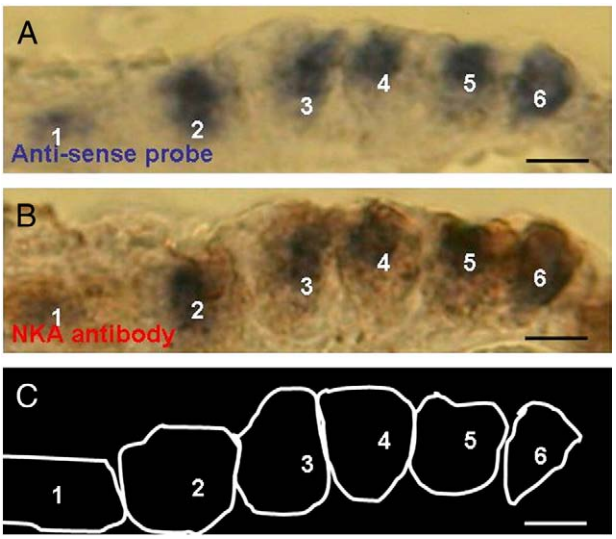



**Fig. 5.** Immunocytochemical staining with $Na^+$, $K^+$–ATPase (NKA) α-subunit antibody on sections of gill filaments from SW-acclimated brackish medaka hybridized with an antisense *Odnkcc1a* probe. (A) The *Odnkcc1a* signals were localized to epithelial cells of the filament. (B) The NKA immunoreactive (NKA-IR) cells (dark brown) are identical to epithelial cells with *nkcc1a* signals (purple) in the same cryosection. (C) The diagram illustrated the outline of each NKA-IR cell. Numbers indicate different epithelial cells. Scale bar = 10 μm.

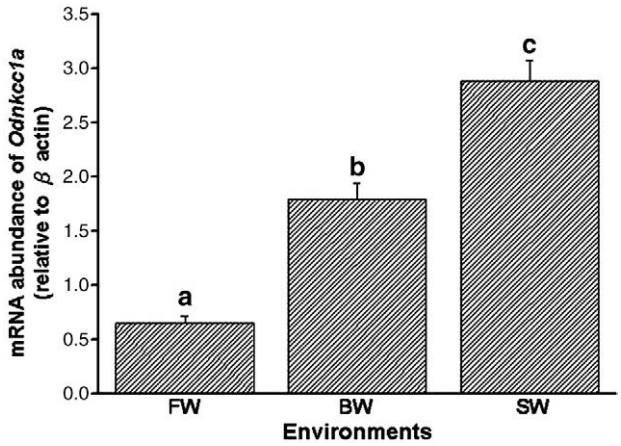



**Fig. 6.** The mRNA levels of *Odnkcc1a* in gills from brackish medaka acclimated to FW, BW or SW ($n = 5$ for all groups). Branchial mRNA abundance for brackish medaka increased significantly with environmental salinity. Different letters indicated significant differences ($p < 0.05$) using Tukey's multiple comparison test following a one-way ANOVA. Values are means ± S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

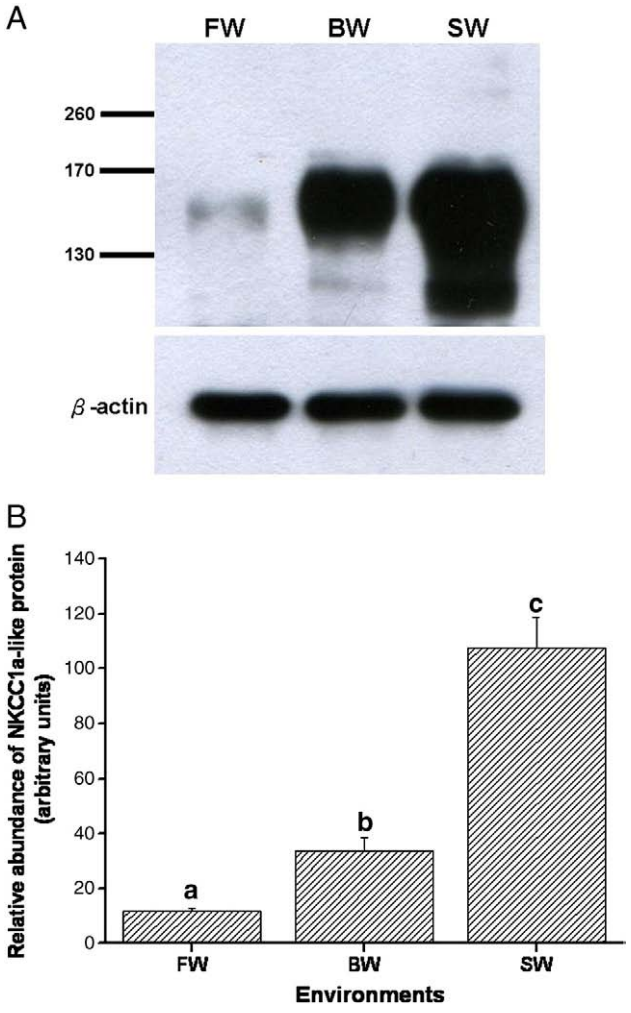



**Fig. 7.** (A) Representative immunoblots of NKCC1a-like protein in gills from brackish medaka detected with monoclonal antibody T4. The immunoreactive bands indicate a molecular mass from 120 to 170 kDa, centered at 150 kDa (A). β-actin was used as the loading control. (B) Relative intensities of immunoreactive bands of NKCC1a-like protein in gills from different salinity groups ($n=6$ for all groups) were compared to show that the levels of NKCC1a-like protein increased with environmental salinity. Different letters indicated significant differences ($p<0.05$, Tukey's multiple comparison test following one-way ANOVA). The values are means ± S.E.M. FW, fresh water; BW, brackish water; SW, seawater.

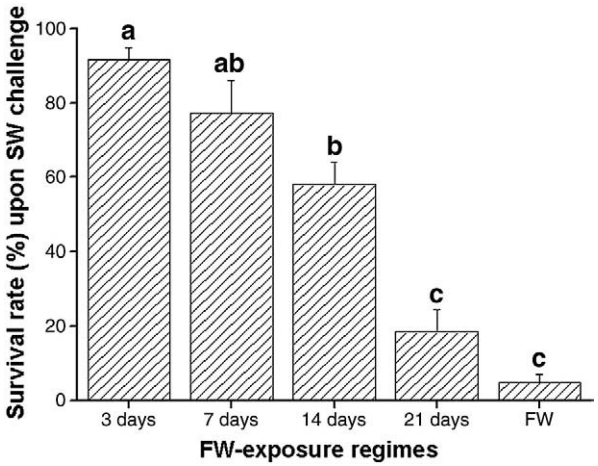



**Fig. 9.** Upon SW challenge, the survival rates of the control group (i.e. FW-acclimated medaka) and the four transfer groups (i.e. SW-acclimated medaka under various FW exposure regimes) were compared ($n=6$ for all groups). When transferred back to SW, the survival rates were significantly reduced in the 14 and 21 day FW exposure groups. Different letters indicated significant differences ($p<0.05$, Tukey's multiple comparison test following one-way ANOVA). FW, fresh water; SW, seawater.

et al. (2008) reported that apical signals in absorptive MR cells of the FW tilapia are due to the $Na^+$, $Cl^-$ cotransporter (NCC; SLC12A3), the other member of SLC12A family. The sequences of an NCC-like protein (NCCa, ENSORLP00000000509; NCCb, ENSORLP00000023616; and NCCc, ENSORLP00000025284) were also found in the Japanese medaka database, but they displayed only 37–46% similarity to the antigenic sequence of the T4 antibody. It is clear that brackish medaka requires expression of the NKCC/NCC-like protein on the apical membrane of MR cells to take up $Cl^-$ for acclimating to hyposmotic environments. In summary, these results indicate that MR cells of brackish medaka exhibited higher gene and protein expression of NKCC1a than other cell types in gills.

Gills of euryhaline teleosts normally secrete excess $Cl^-$ to maintain constant plasma $Cl^-$ concentrations in various saline environments (Evans et al., 2005). Upon hyperosmotic challenge, eel (Cutler and Cramb, 2002; Tse et al., 2006), killifish (Scott et al., 2004, 2005, 2008), sea bass (Lorin-Nebel et al., 2006), salmon (Tipsmark et al., 2002; Nilsen et al., 2007) and tilapia (Hiroi et al., 2008) all increase their transcription of *nkcc1* (*nkcc1a*) in gills. In brackish medaka, mRNA levels of branchial *Odnkcc1a* also increase with environmental salinity

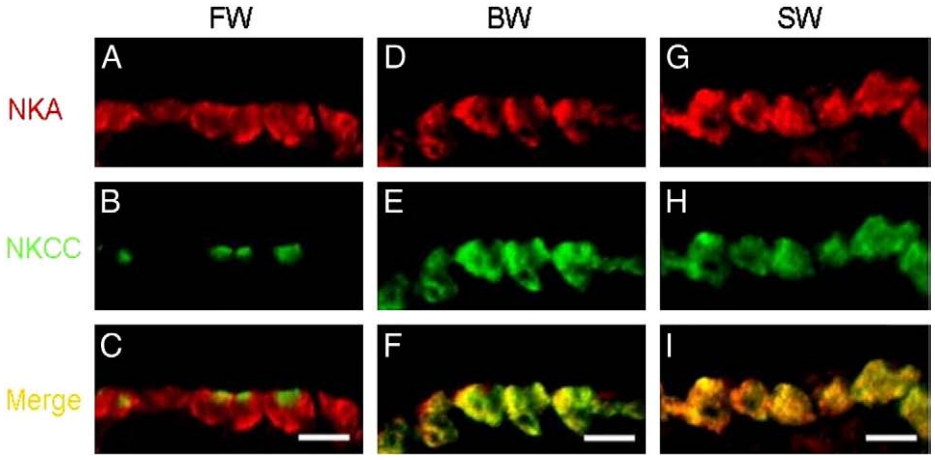



**Fig. 8.** Double immunofluorescence staining with anti-NKA (red; A, D and G) and anti-NKCC (green; B, E and H) antibodies on the cross-sections of gills from brackish medaka acclimated to FW, BW and SW. The merged images (C, F and I) reveal that NKCC1a-like protein is colocalized at the basolateral membrane of NKA immunoreactive (NKA-IR) cells in the BW and SW groups. The NKCC1a-like signals of FW fish, however, are found on the apical region of NKA-IR cells. Bar = 10 μm. FW, fresh water; BW, brackish water; SW, seawater.

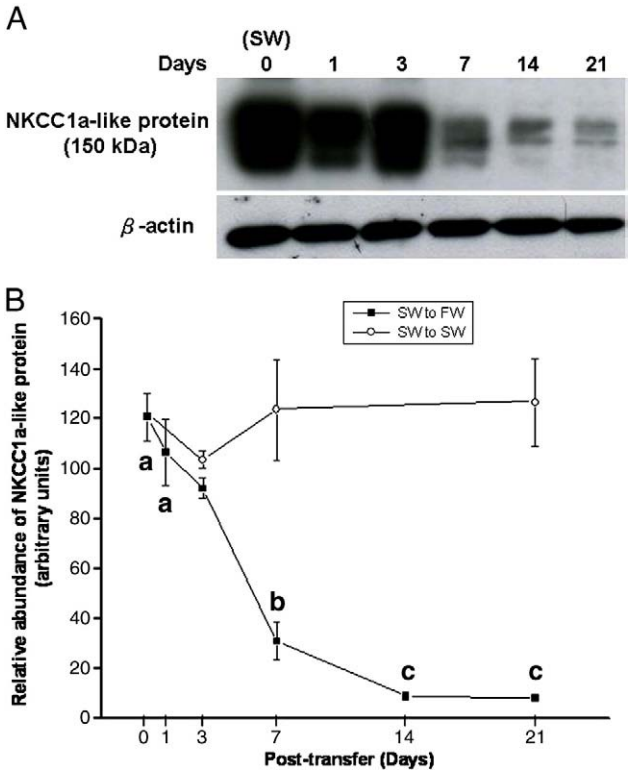



**Fig. 10.** (A) Representative immunoblot of NKCC1a-like protein in gills from brackish medaka after transfer from SW to FW detected by monoclonal antibody T4. β-actin was used as the loading control. (B) Relative abundance of NKCC1a-like protein in gills of brackish medaka following transfer from SW to FW. A black square (■) indicates that NKCC1a-like protein levels declined gradually during the 7 days post-transfer (4-fold) and decreased to 14-fold at 14 and 21 days after transfer. Values are means ± S.E.M ($n=8$). The fish in the control group are indicated by a white circle (○) and were transferred from SW to SW ($n=3$). Different letters indicated significant differences ($p<0.05$) among various sampling time points using Tukey's multiple comparison test following one-way ANOVA. FW, fresh water; SW, seawater.

(Fig. 6). Whole-mount *in situ* hybridization further confirmed that the trend of NKCC1a gene expression in gills of brackish medaka was salinity-dependent (Fig. 4).

Based on the deduced protein sequence of NKCC1a from brackish medaka (Fig. 2), its molecular weight is predicted to be 125 kDa (Gasteiger et al., 2005). The immunoreactive bands detected from gill lysates of species including brackish medaka, however, range from 120 to 170 kDa. The molecular weights of immunoreactive bands in brackish medaka extend over a wide range, similar to the results for pufferfish (Tang and Lee, 2007), salmon (Pelis et al., 2001; Hiroi and McCormick, 2007) and tilapia (Hiroi et al., 2008). Since two potential N-linked glycosylation sites are predicted in the NKCC1a sequence between transmembrane domains 7 and 8 (Fig. 2), different degrees of glycosylation might lead to variable molecular weights of the NKCC1a-like protein (Tipsmark et al., 2002; Wu et al., 2003). According to previous studies, an increased abundance of NKCC1-like protein in response to hyperosmotic challenge was reported in gills of eel (Tse et al., 2006), flounder (Tipsmark et al., 2008b), goby (Chew et al., 2009), killifish (Scott et al., 2004), pufferfish (Tang and Lee, 2007), salmon (Pelis et al., 2001; Tipsmark et al., 2002; Hiroi and McCormick, 2007; Nilsen et al., 2007), sea bass (Lorin-Nebel et al., 2006), striped bass (Tipsmark et al., 2004), sturgeon (Sardella and Kültz, 2009) and tilapia (Wu et al., 2003; Tipsmark et al., 2008a). In the present study, higher levels of NKCC1a-like protein were also found in gills of brackish medaka acclimated to hyperosmotic environments (i.e. BW and SW; Fig. 7B). The results of WISH, qPCR and immunoblotting, however, reveal that relative levels of gene and protein for gill NKCC1a in FW-acclimated medaka was less but not completely gone, compared to those in SW individuals (Figs. 6 and 7). The *nkcc1* gene was also found in isolated pavement cells and MR cells from gills of the FW eel (Tse et al., 2006). Therefore, basal expression of NKCC1a protein in epithelial cells of gills was suggested to participate in cellular volume regulation (Gamba, 2005). The above results together with the results of WISH and immunofluorescence staining (Figs. 4 and 8) demonstrate similar salinity-dependent patterns for both the NKCC1a-like protein and *Odnkcc1a* gene in MR cells. Prominent increases in NKCC 1a expression in MR cells of gills at the gene and protein levels could promote the efficiency of $Cl^-$ exclusion from the plasma of brackish medaka when acclimated to hyperosmotic environments.

Hiroi and McCormick (2007) determined survival rates for three salmonid species following direct or gradual transfer from FW to SW, in order to compare their salinity tolerances. Although Inoue and Takei (2002) reported that *O. dancena* could survive after direct transfer from FW to SW or vice versa, our preliminary data revealed different salinity tolerances (survival rates) for individuals upon salinity challenge between the group of FW-acclimated brackish medaka (over 1 month) and the group of SW-acclimated fish exposed to FW for 1 week. When transferred back to SW, survival rates were compared in this study among groups of SW-acclimated medaka with different (3, 7, 14 and 21) days of FW exposure. The results revealed that the hyposmoregulatory endurance of SW-acclimated medaka after exposure to FW was maintained for at least 14 days (Fig. 9). Hence, we suggest that the hyposmoregulatory ability of SW-acclimated medaka was not eliminated until they had been exposed to FW for 14 days and regulated their internal osmolality in response to abrupt hyperosmotic challenge.

In addition to survival rates, when euryhaline teleosts were transferred from SW to FW dynamic gene or protein levels for branchial NKCC1 were reported. The *nkcc1* gene levels in gills of striped bass and tilapia (Tipsmark et al., 2004; Hiroi et al., 2008) were reduced 12 h after transfer from SW to FW. Moreover, the amounts of branchial NKCC1-like protein in SW-acclimated flounder (Tipsmark et al., 2008b), salmon (Tipsmark et al., 2002), sea bass (Lorin-Nebel et al., 2006), striped bass (Tipsmark et al., 2004) and tilapia (Tipsmark et al., 2008a) all declined significantly after 7 days of FW exposure. Concerning the dynamics of changes in gene expression, NKCC1 protein was maintained for a longer period than the *nkcc1* gene in gills when exposed to hyposmotic environments. Therefore, this study focused on the dynamic expression of NKCC1a-like protein in brackish medaka when transferred from SW to FW. Our results revealed that relative amounts of branchial NKCC1a-like protein 7 days following transfer from SW to FW were similar to those in BW fish, and the lowest expression, similar to FW fish, was found in the groups following 14 and 21 days of exposure (Fig. 10). The changing profile of branchial NKCC1a in medaka transferred from SW to FW conformed to that in other species described above, and indicates that degradation of NKCC1a-like protein takes about 1 week.

Taken together, our data show that decreased expression of NKCC1a-like protein corresponds to a weakening of hyposmoregulatory endurance for brackish medaka (Figs. 9 and 10). Previous studies revealed that the expression of NKCC1 protein in tilapia, striped bass and flounder increased after 48 h upon hyperosmotic challenge (Tipsmark et al., 2004, 2008a,b). The duration of produced NKCC1 protein, however, was longer than the mortality period of medaka upon hyperosmotic challenge (about 5 h). Hoffmann et al. (2007) reported that NKCC expression in epithelial cells was inhibited by dephosphorylation with Ser/Thr protein phosphatases (PP1 and PP2A) and activated through phosphorylation by Sterile 20 protein-related proline alanine-rich kinase (SPAK) and oxidative stress response 1 kinase (OSR1). In addition, the potential phosphorylation sites and interaction sites for SPAK and OSR1 in mammalian NKCC (Piechotta et al., 2002) were found in the deduced protein sequence of

NKCC1a in brackish medaka (Fig. 2). Therefore, when exposed to FW, the branchial NKCC1a of SW-acclimated medaka might be inhibited by PP1A and PP2 until 7 days, but activated rapidly by SPAK and OSR1 to exclude $Cl^-$ because the fish are able to survive when transferred back to SW. After 7 days exposure to FW, the amounts of NKCC1a-like protein are reduced and the hyposmoregulatory ability of brackish medaka is weakened. Although this hyposmoregulatory mechanism is complex, the present study is the first to show that, when exposed to hyposmotic challenge, SW-acclimated medaka retains branchial NKCC1a protein for maintaining its hyposmoregulatory endurance.

In summary, this study identifies salinity-dependent expression of NKCC1a in branchial MR cells of brackish medaka, from genetic to cellular levels. We also performed a time-course transfer experiment to illustrate a critical role for NKCC1a in hyposmoregulatory endurance of medaka. In addition, this study demonstrates that a new experimental animal, brackish medaka, is suitable for studying the hyposmoregulatory mechanism in SW-acclimated euryhaline teleosts. Future studies will focus on functional assays for NKCC1a in brackish medaka using the methods of gene knockdown and gene overexpression, and the regulatory pathways for branchial NKCC1a in medaka will be examined.

## Acknowledgements

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