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# Distribution and dynamics of branchial ionocytes in houndshark reared in full-strength and diluted seawater environments



## Souichirou Takabe<sup>a,\*</sup>, Mayu Inokuchi<sup>a</sup>, Yoko Yamaguchi<sup>a,b</sup>, Susumu Hyodo<sup>a</sup>

<sup>a</sup> Laboratory of Physiology, Atmosphere and Ocean Research Institute, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan <sup>b</sup> Hawai'i Institute of Marine Biology, University of Hawai'i, 46-007 Lilipuna Road, Kaneohe, HI 96744, USA

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## ABSTRACT

In teleost fishes, it is well-established that the gill serves as an important ionoregulatory organ in addition to its primary function of respiratory gas exchange. In elasmobranchs, however, the ionoregulatory function of the gills is still incompletely understood. Although two types of ionocytes,  $Na^+/K^+$ -ATPase (NKA)-rich (type-A) cell and vacuolar-type H<sup>+</sup>-ATPase (V-ATPase)-rich (type-B) cell, have been found in elasmobranch fishes, these cells were considered to function primarily in acid–base regulation. In the present study, we examined ion-transporting proteins expressed in ionocytes of Japanese-banded houndshark, *Triakis scyllium*, reared in full-strength seawater (SW) and transferred to diluted (30%) SW. In addition to the upregulation of NKA and Na<sup>+</sup>/H<sup>+</sup> exchanger type 3 (NHE3) mRNAs in the type-A ionocytes, we found that Na<sup>+</sup>, Cl<sup>-</sup> cotransporter (NCC, Slc12a3) is expressed in a subpopulation of the type-B ionocytes, and that the expression level of NCC mRNA was enhanced in houndsharks transferred to a low-salinity environment. These results suggest that elasmobranch gill ionocytes contribute to NaCl uptake in addition to the already described function of acid–base regulation, and that NCC is most probably one of the key molecules for hyper-osmoregulatory function of elasmobranch gills. The existence of two types of ionocytes (NHE3- and NCC-expressing cells) that are responsible for NaCl absorption seems to be a common feature in both teleosts and elasmobranchs for adaptation to a low salinity environment. A possible driving mechanism for NCC in type-B ionocytes is discussed.

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## 1. Introduction

Ion regulation is one of the most important issues in the maintenance of body-fluid homeostasis. This is particularly vital for fishes in aquatic environments where they are surrounded by waters where the concentrations of ions can change. It is well-established that the teleost fish gill contributes importantly to ion regulation, in addition to the primary function of respiratory gas exchange. In teleost fish, ionocytes, also known as chloride cells or mitochondrion-rich cells, in the branchial epithelium are responsible for ionoregulation (see review, Evans et al., 2005; Kaneko et al., 2008). Ionocytes of seawater (SW) teleost fish have a well-developed tubular system with numerous mitochondria and form a multicellular complex with an accessory cell to provide a paracellular pathway through which Na<sup>+</sup> is thought to be secreted. A set of ion-transporting proteins, namely basolaterally-located  $Na^+/K^+$ -ATPase (NKA) and Na<sup>+</sup>, K<sup>+</sup>, 2Cl<sup>-</sup> cotransporter isoform 1 (NKCC1) and apically-located cystic fibrosis transmembrane conductance regulator (CFTR), are expressed in ionocytes of SW teleost fish to secrete Cl<sup>-</sup> transcellularly (Marshall, 2002). Contributions of multiple membrane proteins, including NKA, vacuolar-type H<sup>+</sup>-ATPase (V-ATPase), Na<sup>+</sup>/ H<sup>+</sup> exchangers (NHEs) and Na<sup>+</sup>, Cl<sup>-</sup> cotransporter (NCC, slc12a10), to ion uptake have also been described in freshwater (FW) teleosts; however, the molecular mechanisms for ion uptake seem to vary among species (Hwang and Lee, 2007; Hwang et al., 2011, Dymowska et al., 2012). In Mozambique tilapia and zebrafish, multiple types of cells (cell types I to IV for tilapia; and NCC, NaR and HR cells for zebrafish) have been identified according to the membrane transporters expressed in those cells, and on their possible function (Hiroi et al., 2008; Inokuchi et al., 2009; Hwang et al., 2011).

In contrast to teleost fish, the iono-regulatory function of the elasmobranch gill is less well understood. Although ionocytes have also been found in elasmobranch gill epithelia, their function has been considered to be different from that of teleost ionocytes (Wilson and Laurent, 2002; Evans et al., 2005). Marine elasmobranchs have a specialized salt-secreting gland, the rectal gland (Burger, 1965), and the gill ionocytes have consequently been proposed to be involved in acidbase regulation (Edwards et al., 2002; Tresguerres et al., 2006; Choe et al., 2007; Tresguerres et al., 2007). Molecular and histochemical investigations of ion-transporting proteins have revealed that there are two types of ionocytes in elasmobranch gills: type-A ionocytes (NKArich ionocytes) and type-B ionocytes (V-ATPase-rich ionocytes) in spiny dogfish *Squalus acanthias* and Atlantic stingray *Dasyatis sabina* (Choe et al., 2005; Choe et al., 2007; Piermarini and Evans, 2001). The

<sup>\*</sup> Corresponding author. E-mail address: takabe@aori.u-tokyo.ac.jp (S. Takabe).

type-A ionocytes express NHE isoform 3 (NHE3) on the apical membrane, suggesting that low intracellular Na<sup>+</sup> created by the basolaterally-located NKA promotes H<sup>+</sup> excretion to the environment concomitantly with Na<sup>+</sup> uptake (Choe et al., 2005). On the other hand,  $HCO_3^-$  excretion is proposed in the type-B ionocytes, since the pendrin-like Cl<sup>-</sup>/HCO<sub>3</sub> exchanger (PDN, slc26a4) is located on the apical membrane (Piermarini et al., 2002). These molecular investigations supported the idea that elasmobranch gill ionocytes are involved in acid-base regulation rather than NaCl excretion in the SW environment.

Recently, we discovered a novel aggregate structure made up of cells with basolaterally-expressed NKA in the inter-filamental space of the gill septum (Takabe et al., 2012). The cell aggregates, named follicularlyarranged NKA-rich cells, express NHE3 and Ca<sup>2+</sup> transporter 1 (CaT), and thus are most likely involved in Ca<sup>2+</sup> homeostasis. During the course of this investigation, we also found expression of CaT mRNA in a small number of ionocytes in the branchial filament. These observations imply that elasmobranch ionocytes still have unidentified functions for ion homeostasis. In the present study, to expose the roles of ionocytes of elasmobranchs, we examined expression of ion-transporting proteins in ionocytes of Japanese-banded houndshark Triakis scyllium. We found that, in addition to NKA, NHE3, V-ATPase and PDN already found in the gills of spiny dogfish and Atlantic stingray, CaT and NCC (Slc12a3) were expressed in a certain portion of type-A and type-B cells, respectively. Acclimation to a low-salinity environment induced increases in the numbers of NKA- and NHE3-expressing type-A cells and of V-ATPase-expressing type-B cells. After transfer, the proportion of NCC-expressing type-B cells (type-B-II cell) to total type-B cells (V-ATPase expressing cell) rose, implying that elasmobranch branchial ionocytes contribute importantly to hyper-osmoregulatory ability.

#### 2. Materials and methods

## 2.1. Fish

All animal experiments were conducted according to the Guidelines for Care and Use of Animals approved by the committee of the University of Tokyo. Japanese banded houndshark, T. scyllium, (750–1550 g) were collected in Koajiro Bay, Kanagawa, Japan. They were transported to the Atmosphere and Ocean Research Institute and kept in  $2 \times 10^{3}$ L holding tanks (20-22 °C, aerated) under a constant photoperiod (12 h:12 h, L:D). The fish were fed on squid for at least 2 weeks before experiments. For sampling, houndsharks were anaesthetized with 0.02% (*w*/*v*) 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MO, USA).

### 2.2. Transfer experiments

The transfer experiments on houndsharks were performed as described in detail by Yamaguchi et al. (2009). In brief, houndsharks were kept separately in two tanks  $(1 - 2 \times 10^{3} \text{L})$  filled with fullstrength SW. On day 1, the salinity of the water of the experimental group was reduced by adding FW to achieve a salinity of 80% SW. On days 2 and 3, salinity was further reduced to achieve a salinity of 60% SW and 40% SW, respectively. And FW was again added on day 4 to produce a final salinity of 30% SW (10 ppt, n = 4). Control fish (n = 5) were kept in full-strength SW (34 ppt) during the transfer protocol. Fish were maintained for 1 week in each salinity condition, and then euthanized for sampling.

## 2.3. cDNA cloning

The gills of houndshark were dissected out, quickly frozen in liquid nitrogen and kept at -80 °C. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan), and cDNAs were then synthesized using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). We already cloned partial sequences of cDNAs encoding NKA α1-subunit, NKCC1, CFTR, NHE3 and CaT (Takabe et al., 2012). In addition, in the present study, degenerate primer pairs were designed to amplify conserved regions of Na<sup>+</sup>, K<sup>+</sup>, 2Cl<sup>-</sup> cotransporter isoform 2 (NKCC2), NCC, V-ATPase, PDN, and chloride channel type-3 (CLC3), and their sequences are shown in Table 1. PCR was performed with Ex-Tag DNA polymerase (TaKaRa, Shiga, Japan). The amplified products were electrophoresed on agarose gels, excised and ligated into pGEM-T Easy Vector (Promega Corp., Madison, WI). The nucleotide sequences were determined by an automated DNA sequencer (PRISM 3130, Applied Biosystems, Foster City, CA).

### 2.4. Molecular phylogenetic analysis

The deduced amino acid sequences of the obtained cDNAs from houndshark were aligned with those from other animals using ClustalX software (http://www.clustal.org/); sequences were obtained from the DDBJ and Ensembl databases. Molecular phylogenetic trees were constructed using ClustalX software by the neighbor-joining method with bootstrap analysis for 1000 cycles. The rat K<sup>+</sup>-Cl<sup>-</sup> cotransporter 1 (KCC1) and human CLCKa were used as outgroups for the phylogenetic trees of the NKCC/NCC and CLC protein families, respectively.

#### 2.5. Real-time quantitative PCR assay and RT-PCR

The expression levels of mRNAs were determined by real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection

Table 1				
Primer sets used	in	the	present	studv.

Primer sets for real-time PCR   Gense   TGCTTACACTTTAACCAGCAATATCC     NKA   Antisense   GGCTGTCTCTTCATTATATCACTTTC     NHE3   Sense   CGACAGGCGATTATTTCTCAGTAC     NHE3   Antisense   ATCGTCAGCCCTGGAAGAT     V-ATPase   Sense   CGCACTGATCCATCACTATCACT     PDN   Sense   CGCTGTTTCCAAGCCTGTGCTTC     NCC   Sense   CGGCAACTGTCACCACAGAAACCAC     NCC   Sense   GGGAACTGTCACCACAGAAAACCAC     β-Actin   Sense   CACATCGGCCCATCTACGACACATG     β-Actin   Sense   CACATCGTGCCCATCTACGAA     NKC2   Sense   CACATCGTGCCCATCTACGAA     NKC2   Sense   CACATCTGCCACTAGGAAACCAT     NKC2   Sense   CACATCTGCCACTTACGAAACCAT     NCC   Sense   CACACTGTGCCCATCTACGAA     NKC2   Sense   CACACTGTGCCCATCTACGAA     NCC   Sense   Antisense     NKC2   Sense   CGCCACCARTTDATNACRAACAT     NCC   Sense   GGNTAYGGNAARAAYAAYGARCC     Antisense   GCCACCCCCCARTTDATNACRAACAT     NCC   Sense   CACNCTNGCNGCNCARATHTGC     Antisense <td< th=""><th>Gene name</th><th colspan="3">Primer sequence 5' to 3'</th></td<>	Gene name	Primer sequence 5' to 3'				
NKASenseTGCTTACACTTTAACCAGCATATCCNKAAntisenseGGCTGTCTTTATATCACTTTCNHE3SenseCGACAGGCGATTATTTCTCACTACAntisenseATCGTCAGCCCTGGAAGATV-ATPaseAntisenseATCGTCAGCCCTGGAAGATPDNSenseCGCTGTTTCTCAAGCCTTTTGNCCSenseGGCAACTGTCACCCAAGGCATATCCAGCLC3SenseCGCAACTGTGCCACTGGCATATCGGAβ-ActinSenseCACATCGGCCCATCACCAACAGAPrimer sets for molecular cloningSenseCACACTGTGCCCACTAGGAACAANKC2SenseACNGTNGCNGGNATGGARTGGGARNCCSenseGCCAAATCCAGACGCAAAACCACβ-ActinSenseCACACTGTGCCCATCTACGAANKC2SenseCACACTGTGCCCATCTACGAAANKC2SenseCACACTGTGCCACTTAGAACATNCCSenseGGNTAYGGNAARAAYAAYGARCCNCCSenseGGNTAYGGNAARAAYAAYGARCCNCCSenseGCCACACATTDATNACRAACATNCCSenseCACACTGCCCNCNCKRTTV-ATPaseSenseCAYAAYGARATHCCCNCACATHTGV-ATPaseSenseCGRTCNCTDATTTCACTCATCGCATCT	Primer sets for real-time PCR					
$\begin{tabular}{ c c c c c c } \hline NKA & Antisense & GGCTGTCTTCATTATATCACTTTC \\ \hline NHE3 & Sense & CGAGAGGCGATTATTTGTCAGTAC \\ \hline NHE3 & Antisense & ATCGTCAGCCCCTGGAAGAT \\ \hline V-ATPase & Sense & ACCATCGAACGCATCATCATC \\ \hline Antisense & TCTCACATTGATACGCCAGGAA \\ \hline PDN & Antisense & GAGTTTTGCCACTGGCATTC \\ \hline NCC & Sense & GGGAACTGTCACCAAAAACCAC \\ \hline Antisense & TGATGTGTCCACTAGGGCTTAG \\ \hline CLC3 & Sense & CACATCGTAGGCCATAGGCATATCTCAG \\ \hline G-Actin & Sense & CACATCGACGCAACATG \\ \hline Antisense & GAGAAAATATTACCGCAGCAACATG \\ \hline Antisense & GACATCATGGCCACTAGGACACATG \\ \hline B-Actin & Sense & CACACTGTGCCCATCAGAAA \\ \hline Primer sets for molecular cloning \\ \hline NKCC2 & Sense & ACNGTNGCNGGNATGGARTGGGAR \\ \hline NCC & Sense & GGNTAYGGNAARAAYAAYGARCC \\ \hline NCC & Sense & TINCKYTCYTCRTCCATNCKRTT \\ \hline V-ATPase & Sense & CAYAAYGARATHCGCNCARATHTG \\ \hline V-ATPase & GGRTAYGGNAATHCGTCACARATHTG \\ \hline CGRTCNCTDATTRACCGCACCAATHTG \\ \hline CACATGTCACCARTTDATNACRAACAT \\ \hline NCC & Sense & CACACTCNCCNCRATHTG \\ \hline V-ATPase & Sense & CAYAAYGARATHCCOCCAT \\ \hline \ DTTCKYTCYTCRTCCANCCRATHTG \\ \hline \ CGRTCNCTDATTRTCPTCPTTCPTCPTCPTCPTCPTCPTCPTCPTCPTCPTCP$	NIZA	Sense	TGCTTACACTTTAACCAGCAATATCC			
NHE3     Sense     CGAGAGGCGATTATTTGTCAGTAC       Antisense     ATCGTCAGCCCCTGGAAGAT       V-ATPase     Sense     ACCATCGAACGCATCATCACT       Antisense     TCTCACATTGATACGCCAGGAA       PDN     Sense     CGGCTGTTTCTCAGCTTTTG       NCC     Sense     CGGCTGTTTCTCAGCTTTTG       NCC     Sense     GGGAACTGTCACCAGAAAACCAC       Antisense     GAGTGTTGCCACTAGAGCATATCTCAG       CLC3     Sense     TGATGTGTCCCATGGGCTTAG       β-Actin     Sense     CACACTGTGCCCAGGAACACGG       Primer sets for molecular cloning     KKC2     Sense       NKCC2     Sense     Antisense       NKCC2     Sense     GGNTAYGGNAARAAYAAYGARCC       NCC     Sense     GGNTAYGGNAARAAYAAYGARCC       NCC     Sense     GCNTAYGGNAARAAYAAYGARCC       NKC2     Sense     GGNTAYGGNAARAAYAAYGARCC       NCC     Sense     GNTAYGGNAARAAYAAYGARCC       NCC     Sense     GNTAYGGNAARAAYAAYGARCC       NCC     Sense     GNTAYGGNAARAAYAAYGARCC       NCC     Sense     GNTAYGGNAARAAYAAYGARCC	NKA	Antisense	GGCTGTCTCTTCATTATATCACTTTC			
INTED     Antisense     ATCGTCAGCCCCTGGAAGAT       V-ATPase     Sense     ACCATCGAACGCATCATCACT       V-ATPase     Antisense     TCTCACATTGATACGCCAGGAA       PDN     Sense     CGGCTGTTTCTCAAGCTTTTTG       NCC     Sense     GAGTTTTGCCACCTGGCATTCCAG       NCC     Sense     GGGAACTGTCACCAAAAACCAC       Antisense     TGATGTGTCCACTAGAGCATATCTCAG       CLC3     Sense     TGATGTGTCCACTAGGCGCTTAG       Sense     CACACTGTGCCCACTAGGCACACATG       G-Actin     Sense     CACACTGTGCCCATCAGGAA       Primer sets for molecular cloning        NKCC2     Sense     ACNGTNGCNGGNATGGARTGGGAR       NCC     Sense     GCCACACTTDATNACRAACAT       NCC     Sense     GCCCACCARTTDATNACRAACAT       NCC     Sense     GCCACCARTTDATNACRAACAT       NCC     Sense     GGNTAYGGNAARAAYAAYGARCC       NCC     Sense     GCNTAYGGNAARAAYAAYGARCC       NCC     Sense     GCNTAYGCNAARAAYAAYGARCC       NCC     Sense     CANAYAGARATHCCONCARATHTG       V-ATPase     Sense     CANAYAGARATHCCONCARATHTGC <td>NUE2</td> <td>Sense</td> <td colspan="2">CGAGAGGCGATTATTTGTCAGTAC</td>	NUE2	Sense	CGAGAGGCGATTATTTGTCAGTAC			
$\begin{array}{c} V-ATPase & Sense & ACCATCGAACGCATCATCACT \\ Antisense & TCTCACATTGATACGCCAGGAA \\ PDN & Sense & CGGCTGTTTCTCAAGCTTTTG \\ Antisense & GAGTTTTGCCACCTGTGCTTTC \\ NCC & Sense & GGGAACTGTCACCAAAAACCAC \\ Antisense & TGATGTCTCACTAGAGCATATCTCAG \\ CLC3 & Sense & TAGCTGTTGCCACTAGAGCATATCTCAG \\ Antisense & GAGAAAATATTACCGCAGGAACATG \\ \beta-Actin & Sense & CACACTGTGCCCATCTACGAA \\ Antisense & GCCAAATCCAGACACAC \\ Primer sets for molecular cloning \\ NKCC2 & Sense & ACNGTNGCNGGNATGGARTGGGAR \\ NCC & Sense & GCCACCATTDATNACRAACAT \\ NCC & Sense & GCCAACTGTGCCACTTDATNACRAACAT \\ NCC & Sense & GGNTAYGGNAARAAYAAYGARCC \\ Antisense & TTNCKYTCYTCRTCCATNCKRTT \\ V-ATPase & GCRATCAGAACTGAACATBTCPTCPTCPTCPTCPTCPTCPTCPTCPTCPTCPTCPTCPT$	INTES	Antisense	ATCGTCAGCCCCTGGAAGAT			
V-ATFASE Antisense TCTCACATTGATACGCCAGGAA   PDN Sense CGGCTGTTTCTCAAGCTTTTTG   NCC Sense GGGAACTGTCACCAAGAACCAC   NCC Antisense TGATGTGTCCACCTAGGCATATCTCAG   CLC3 Sense TGATGTGTCCACTAGGCATATCTCAG   β-Actin Sense CACACTGTGCCCATCTACGAA   Primer sets for molecular cloning Sense ANTISENSE   NKCC2 Sense ACNGTNGCNGGNATGGARTGGGAR   NKCC2 Sense GCCACCARTTDATNACRAACAT   NCC Sense GCCAACCARTTDATNACRAACAT   NCC Sense GCNTAYGGNAARAAYAAYGARCC   Antisense GCNTAYGGNAARAAYAAYGARCC   NCC Sense CAYAAYGARATHGCNGCNACTHTG   V-ATPase Sense CAYAAYGARATHGCCGATTGCCATTTG	V-ATPase	Sense	ACCATCGAACGCATCATCACT			
$\begin{array}{c c c c c c c c c c c c c c c c c c c $		Antisense	TCTCACATTGATACGCCAGGAA			
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	ארוע	Sense	CGGCTGTTTCTCAAGCTTTTTG			
$\begin{array}{c} \mbox{NCC} & Sense & GGGAACTGTCACCAAAAACCAC \\ \mbox{Antisense} & TGATGTGTCACCATAGAGCATATCTCAG \\ \mbox{CLC3} & Sense & TAGCTGTTGCCCATGGGGCTAG \\ \mbox{Antisense} & GAGAAAATATTACCGCAGCAACATG \\ \mbox{B-Actin} & Sense & CACACTGTGCCCATCAGAACACT \\ \mbox{B-Actin} & Sense & CACACTGTGCCCATCAGAACAC \\ \mbox{Primer sets for molecular cloning} \\ \mbox{NKC2} & Sense & ACNGTNGCNGGNATGGARTGGGAR \\ \mbox{NKC2} & Sense & ACNGTNGCNGGNATGGARTGGGAR \\ \mbox{NKC2} & Sense & GGNTAYGGNAARAAYAAYGARCC \\ \mbox{NCC} & Sense & GGNTAYGGNAARAAYAAYGARCC \\ \mbox{Antisense} & TTNCKYTCYTCRTCCATNCKRTT \\ \mbox{V-ATPase} & Sense & GAYAATGATHGCNGCNCARATHTG \\ \mbox{Antisense} & GGRTANGGARATHGCCNGCNCARATHTG CCAT \\ \end{tabular}$	PDN	Antisense	GAGTTTTGCCACCTGTGCTTTC			
$\begin{tabular}{lllllllllllllllllllllllllllllllllll$	NCC	Sense	GGGAACTGTCACCAAAAACCAC			
$\begin{array}{c} \text{CLC3} & \begin{array}{c} \text{Sense} & \text{TAGCTGTTGCCTCAGGGCTTAG} \\ \text{Antisense} & \text{GAGAAAATATTACCGCAGGAACATG} \\ \end{array} \\ \begin{array}{c} \text{Actin} & \begin{array}{c} \text{Sense} & \text{CACACTGTGCCCATCTACGAA} \\ \text{Antisense} & \text{GCCAAATCCAGACGCAGAA} \\ \end{array} \\ \begin{array}{c} \text{Primer sets for molecular cloning} \\ \end{array} \\ \begin{array}{c} \text{NKCC2} & \begin{array}{c} \text{Sense} & \text{ACNGTNGCNGGNATGGARTGGGAR} \\ \text{Antisense} & \text{GCCCACCARTTDATNACRAACAT} \\ \end{array} \\ \begin{array}{c} \text{NCC} & \begin{array}{c} \text{Sense} & \text{GCNTAYGGNAARAAYAAYGARCC} \\ \text{Antisense} & \text{TNCKYTCYTCRTCCATNCKRTT} \\ \end{array} \\ \begin{array}{c} \text{V-ATPase} & \begin{array}{c} \text{Sense} & \text{GAYAATHGCNGCNACRAATHTG} \\ \end{array} \end{array} \end{array}$	INCC	Antisense	TGATGTGTCCACTAGAGCATATCTCAG			
CLCS Antisense GAGAAATATTACCGCAGCAACATG   β-Actin Sense CACACTGTGCCCATCTACGAA   β-Actin Sense GCCAAATCCAGACGCAGAA   Primer sets for molecular cloning KCC2 Sense   NKCC2 Sense ACNGTNGCNGGNATGGARTGGGAR   NCC Sense GCCACCARTTDATNACRAACAT   V-ATPase Gense CAYAAYGARATHGCNGCNACTGCARATHTG	CI C2	Sense	TAGCTGTTGCCTCAGGGCTTAG			
β-Actin     Sense Antisense     CACACTGTGCCCATCTACGAA GCCAAATCCAGACGCAGAA       Primer sets for molecular cloning        NKCC2     Sense Antisense     ACNGTNGCNGGNATGGARTGGGAR GCCCACCARTTDATNACRAACAT       NCC     Sense Antisense     GGNTAYGGNAARAAYAAYGARCC Antisense       V-ATPase     Sense GGRTGNGTDATCCATCTCATT	CLCS	Antisense	GAGAAAATATTACCGCAGCAACATG			
Primer sets for molecular cloning NKCC2 Sense ACNGTNGCNGGNATGGARTGGGAR NCC Sense GCCCACCARTTDATNACRAACAT NCC Sense GGNTAYGGNAARAAYAAYGARCC Antisense TTNCKYTCYTCRTCCATNCKRTT V-ATPase GGRTENGTDATRTCPTCPTTPTC	Q Actin	Sense	CACACTGTGCCCATCTACGAA			
Primer sets for molecular cloning NKCC2 Sense ACNGTNGCNGGNATGGARTGGGAR NCC Sense GCCCACCARTTDATNACRAACAT NCC Sense GGNTAYGGNAARAAYAAYGARCC Antisense TINCKYTCYTCRTCCATNCKRTT V-ATPase GGRTGNGTDATRTCPTCPTTDTCCCAT	p-Acuii	Antisense	GCCAAATCCAGACGCAGAA			
NKCC2     Sense     ACNGTNGCNGGNATGGARTGGGAR       NKCC2     Antisense     GCCCACCARTTDATNACRAACAT       NCC     Sense     GGNTAYGGNAARAAYAAYGARCC       Antisense     TINCKYTCYTCRTCCATNCKRTT       V-ATPase     Sense     CAYAAYGARATHGCNGCNCARATHTG       Antisense     CAYAAYGARATHGCNGCNCARATHTG	Primer sets for molecul	ar cloning				
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NCC Sense GGNTAYGGNAARAAYAAYGARCC Antisense TTNCKYTCYTCRTCCATNCKRTT V-ATPase Sense CAYAAYGARATHGCNGCNCARATHTG Antisense CGRTCNGTDATRTCPTCPTTNCCCAT	NKCC2	Antisense	GCCCACCARTTDATNACRAACAT			
NCC     Antisense     TTNCKYTCYTCRTCCATNCKRTT       V-ATPase     Sense     CAYAAYGARATHGCNGCNCARATHTG       V-ATPase     Antisense     CGRTCNGTDATRTCPTCPTTNCCCAT	NCC	Sense	GGNTAYGGNAARAAYAAYGARCC			
V-ATPase Sense CAYAAYGARATHGCNGCNCARATHTG	NCC	Antisense	TTNCKYTCYTCRTCCATNCKRTT			
V-AIPase Antisense CCRTCNCTDATRTCPTCPTTNCCCAT	V ATD	Sense	CAYAAYGARATHGCNGCNCARATHTG			
	v-AIPase	Antisense	GGRTGNGTDATRTCRTCRTTNGGCAT			
Sense GGNAAYCARGARTTYATHGCNTTY	DDN	Sense	GGNAAYCARGARTTYATHGCNTTY			
PDN Antisense SWRTTCCARTCNACYTGDATYTC	PDN	Antisense	SWRTTCCARTCNACYTGDATYTC			
Sense ATHGAYTGGGTNMGNGARAARTGY	CLCD	Sense	ATHGAYTGGGTNMGNGARAARTGY			
Antisense RAANARNACNARNCK	CLC3	Antisense	RAANARNACNARNCK			
Primer sets for <i>in situ</i> hybridization						
NIZA Sense GGTGCCATTGTAGCTGTGAC	NIZA	Sense	GGTGCCATTGTAGCTGTGAC			
Antisense TATAAGGGAAGGCGCAGAACCACCA	INKA	Antisense	TATAAGGGAAGGCGCAGAACCACCA			
Sense TTTTCGAGGAGGTTCATGTG	NHE3	Sense	TTTTCGAGGAGGTTCATGTG			
Antisense TGTAATTGTGGCCGATCTGC		Antisense	TGTAATTGTGGCCGATCTGC			
V ATDece Sense CACAACGAAATAGCCGCTCA	V ATDese	Sense	CACAACGAAATAGCCGCTCA			
Antisense GGGTGCGTGATGTCGTCGTT	V-ATPase	Antisense	GGGTGCGTGATGTCGTCGTT			
Sense GGGAACCAGGAGTTTATTGC	DDN	Sense	GGGAACCAGGAGTTTATTGC			
Antisense GTGTTCCAGTCGACTTGGAT	PDN	Antisense	GTGTTCCAGTCGACTTGGAT			
Sense GGGTACGGGAAGAACAACGA	NCC	Sense	GGGTACGGGAAGAACAACGA			
Antisense CTTACGCTCTTCGTCCATCC	NCC	Antisense	CTTACGCTCTTCGTCCATCC			
GUT Sense GGGAACACTGTTATGTTTCA	C-T	Sense	GGGAACACTGTTATGTTTCA			
CATTTGGAAACCCCGAGCGA	Cal	Antisense	CATTTGGAAACCCCGAGCGA			
Sense ATTGATTGGGTGCGGGAGAA	CLC3	Sense	ATTGATTGGGTGCGGGAGAA			
Antisense AAACAGGACCAGACGGCTGT		Antisense	AAACAGGACCAGACGGCTGT			

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