Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

The ClC-7 chloride channel is downregulated by hypoosmotic stress in human chondrocytes

Takashi Kurita, Hisao Yamamura, Yoshiaki Suzuki,

Wayne R Giles, and Yuji Imaizumi

Department of Molecular & Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan (T.K., H.Y., Y.S., Y.I.);

Faculty of Kinesiology, University of Calgary, Calgary, Alberta T2N 1N4, Canada (W.R.G)

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Pathological function of ClC-7 in chondrocytes

Corresponding author:

Yuji Imaizumi, Ph.D.

Department of Molecular & Cellular Pharmacology

Graduate School of Pharmaceutical Sciences

Nagoya City University

3-1 Tanabedori, Mizuhoku, Nagoya 467-8603, Japan

Tel. and Fax: +81-52-836-3431

E-mail: yimaizum@phar.nagoya-cu.ac.jp

Number of text page: 33

Number of tables: 0

Number of figures: 7

Number of references: 38

Number of words in the Abstract: 247

Number of words in the Introduction: 535

Number of words in the Discussion: 1215

Abbreviations: BK_{Ca} channel, large-conductance Ca^{2+}-activated K^+ channel; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; EGTA, *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid; fura-2/AM, fura-2 acetoxymethyl ester; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

bromide; OA, osteoarthritis; RT, reverse transcription; TEA, tetraethylammonium; TRP, transient receptor potential.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Articular chondrocytes in osteoarthritis (OA) patients are exposed to hypoosmotic stress because the osmolality of this synovial fluid is significantly decreased. Hypoosmotic stress can cause an efflux of Cl^-, and an associated decrease of cell volume. We have previously reported that a Cl^- conductance contributes to the regulation of resting membrane potential and thus can alter intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human chondrocytes. The molecular identity and pathological function of these Cl^- channels, however, remained to be determined. Here, we show that the ClC-7 Cl^- channel is strongly expressed in a human chondrocyte cell line (OUMS-27), and that it is responsible for Cl^- currents that are activated by extracellular acidification (pH 5.0). These acid-sensitive currents are inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, IC_{50} = 13 μM) and are markedly reduced by siRNA-induced knockdown of ClC-7. DIDS hyperpolarized these chondrocytes and this was followed by an increase in $[Ca^{2+}]_i$. ClC-7 knockdown caused a similar hyperpolarization of the membrane potential. Short-term culture (48 h) in hypoosmotic medium (270 mOsm) reduced the expression of ClC-7 and decreased the acid-sensitive currents. Interestingly, these hypoosmotic culture conditions, or ClC-7 knockdown, resulted in enhanced cell death. Taken together, our results show that the significant hyperpolarization due to ClC-7 impairment in chondrocytes can significantly increase $[Ca^{2+}]_i$ and cell death. Thus, downregulation of ClC-7 channels during the hypoosmotic stress that accompanies OA progression is one important concept of the complex etiology of OA. These findings suggest novel targets for therapeutic intervention(s) and drug development for OA.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Articular cartilage consists mainly of chondrocytes and the associated extracellular matrix containing collagens and proteoglycans that protect the diarthrodial joints. These articular chondrocytes play pivotal roles of synthesis and degradation of the extracellular matrix. Osteoarthritis (OA) is caused by mechanical damage to the articular cartilage and is associated with chronic inflammation of joints. Progress of OA is characterized by an increased degradation of articular cartilage. Although, many aspects of the pathogenesis are still unclear (Goldring, 2006; Martel-Pelletier et al., 2008); the osmolality of synovial fluid from OA patients is known to be decreased (249~277 mOsm) with respect to that in normal subjects (295~340 mOsm) (Bertram and Krawetz, 2012). In addition, articular chondrocytes are reported to be sensitive to changes in osmolarity in their extracellular environment (Bush and Hall, 2001; Kerrigan et al., 2006). When chondrocytes are exposed to hypoosmotic environment, they swell and then subsequently regain their original cell volume following efflux of osmolytes such as ions, sugars, and amino acids. This process is referred as the regulatory volume decrease. In contrast, a hyperosmotic environment leads to chondrocyte shrinking: in this setting cell, its volume recovers by influx of osmolytes, which is known as the regulatory volume increase (Hoffmann et al., 2009).

The regulation of cell volume due to regulatory volume decrease and increase mechanisms is mediated, in part, by ion channels and transporters in many types of cells, including chondrocytes (Hoffmann et al., 2009). Various types of ion channels have been identified on the plasma membrane in mammalian chondrocytes: voltage-dependent Na^+ channels, epithelial Na^+ channels, voltage-dependent Ca^{2+} channels, voltage-dependent K^+ channels, Ca^{2+}-dependent K^+ channels, ATP-sensitive K^+ channels, transient receptor potential (TRP) channels, aquaporin water channels, *N*-methyl-D-aspartate receptor channels, and Cl^-

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

channels (Barrett-Jolley et al., 2010; Mobasheri et al., 2012). In articular chondrocytes, voltage-dependent K^+ channels and two-pore domain K^+ channels contribute to the maintenance of the resting membrane potential (Clark et al., 2011; Mobasheri et al., 2012; Wilson et al., 2004). In addition, in human chondrocytes, we have shown (Funabashi et al., 2010a) that blockage of Cl^- channels causes a significant hyperpolarization that is followed by an enhanced Ca^{2+} influx through non-selective cation channels. In chondrocytes, an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) facilitates the syntheses of aggrecan and type X collagen (Alford et al., 2003; Bonen and Schmid, 1991; Wu and Chen, 2000). Accordingly, the Cl^- channel conductance in chondrocytes has been suggested to be involved in the synthesis of the extracellular matrix in addition to the regulation of resting membrane potential.

The OUMS-27 cell line was originally derived from human chondrosarcoma. Based on showing that these OUMS-27 cells can exhibit virtually all major functions of chondrocytes, these cells are widely used as a model of chondrocyte physiology and pathophysiology (Demircan et al., 2005; Kunisada et al., 1998). However, the molecular component(s) and pathological roles of the Cl^- channels that respond to a variety of stimuli in chondrocytes are not fully understood. Important new results from our study include: (i) that ClC-7 (also known as CLCN7) is responsible for the Cl^- conductance in OUMS-27 cells, and (ii) that downregulation of ClC-7 during hypoosmotic stress alters $[Ca^{2+}]_i$ and promotes cell death. Thus the Cl^- channel ClC-7 and $[Ca^{2+}]_i$ are important variable in OA patients.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Cell culture and human tissue

A human chondrocyte cell line, OUMS-27, was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). OUMS-27 cells were cultured at 37℃ in 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin (Wako, Osaka, Japan), and 0.1 mg/ml streptomycin (Meiji Seika, Tokyo, Japan). Culture medium for isotonic and hypoosmotic conditions were prepared as follows: 350 or 270 mOsm medium was a 1:1 mixture of solution (72 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, 10 mM HEPES, and 160 or 0 mM sucrose, respectively, pH 7.4) and Dulbecco's modified Eagle's medium (347 mOsm). Articular cartilage tissues from normal subjects and OA patients were obtained from Articular Engineering (Northbrook, IL).

RT-PCR and quantitative real-time PCR

The extraction of total RNA from homogenates of OUMS-27 cells and the reverse transcription (RT) were performed as reported previously (Funabashi et al., 2010b). RT-PCR was performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). PCR amplification was carried out for 30 or 35 cycles in OUMS-27 cells or articular cartilage tissues, respectively. Quantitative real-time PCR analysis was performed using the SYBR Green assay (Power SYBR Green PCR Master Mix; Applied Biosystems) on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Each cDNA sample was tested in triplet. Values for each unknown samples relative to the standard curve for specific primers were calculated, yielding transcriptional quantitation of gene products relative to the endogenous standard (β-actin or GAPDH). Specific primers for human genes were designed

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

as follows: ClC-1 (GenBank Accession Number, NM_000083, 1567-1667), ClC-2 (NM_004366, 884-1000), ClC-3 (NM_001243372, 2816-2920), ClC-4 (NM_001830, 361-469), ClC-5 (NM_001127899, 5190-5297), ClC-6 (NM_001286, 500-600), ClC-7 (NM_001287, 793-893), ClC-Ka (NM_004070, 2390-2490), ClC-Kb (NM_000085, 862-993), cystic fibrosis transmembrane conductance regulator (CFTR; NM_000492, 623-739), β-actin (NM_001101, 411-511), and GAPDH (NM_002046, 418-518).

Immunocytochemistry and Western blotting

Immunocytochemical and Western blot experiments were performed as described previously (Funabashi et al., 2010b). In brief, OUMS-27 cells were incubated with ClC-7 antibody (1:50 dilution; ab86196, Abcam, MA) for 12 h at 4°C, and then were labeled with Alexa Fluor 488 anti-rabbit IgG (1:200 dilution; A11008, Invitrogen/Molecular Probes, Eugene, OR) for 1 h at room temperature (24±1°C). Immunocytochemical images were obtained using a laser scanning confocal fluorescent microscope system (A1R; Nikon, Tokyo, Japan). The protein fraction of plasma membrane was extracted from OUMS-27 cells, and 50 μg/lane of protein was subjected to 10% SDS-PAGE. Resulting blots were incubated with ClC-7 antibody (1:200 dilution) for 12 h at 4°C, and then treated with anti-rabbit horseradish peroxidase-conjugated IgG (Chemicon International, Temecula, CA) for 1 h at 4°C, and finally exposed to an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The luminescence images were analyzed using a LAS-1000 system (Fujifilm, Tokyo, Japan).

Electrophysiological recordings

Electrophysiological studies were performed as described previously (Funabashi et al., 2010b). In brief, OUMS-27 cells were incubated in phosphate-buffered saline containing 1%

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

collagenase (Amano, Nagoya, Japan) for 5~10 min at 37°C to remove their extracellular matrix. Whole-cell patch-clamp was then applied to single OUMS-27 cells using CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), Digidata 1440A, and pClamp software (version 10, Molecular Devices/Axon Instruments, Foster City, CA). A 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-buffered solution was used as the standard extracellular solution: 137 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, and 10 mM HEPES. Solutions having acidic pH values were prepared by removal of HEPES and addition to equivalent 2-morpholinoethanesulfonic acid (monohydrate, MES). These pH values were adjusted to 7.4, 6.0 or 5.0 with 10 N NaOH. The pipette solution for Cl^- current recordings had an ionic composition of: 120 mM CsCl, 20 mM tetraethylammonium (TEA)-Cl, 4 mM $MgCl_2$, 2 mM $ATPNa_2$, 5 mM EGTA, and 10 mM HEPES. The pH was adjusted to 7.2 with 1 N CsOH. The pipette solution for membrane potential recordings contained 140 mM KCl, 4 mM $MgCl_2$, 2 mM $ATPNa_2$, 0.05 mM EGTA, and 10 mM HEPES. The pH was adjusted to 7.2 with 1 N KOH. All electrophysiological experiments were carried out at room temperature (24±1°C).

siRNA knockdown

Knockdown of ClC-7 was performed using the siRNA method. Control (Medium GC Duplex #3) and ClC-7 siRNAs (Stealth RNAi, CLCN7HSS101979) were obtained from Invitrogen. OUMS-27 cells were electroporated with 200 nM of siRNA construct using the Amaxa Human Chondrocyte Nucleofector Kit (Lonza, Köln, Germany). Experiments were performed 48 h after electroporation.

$[Ca^{2+}]_i$ measurements

Measurements of $[Ca^{2+}]_i$ were performed using the ARGUS/HiSCA system (Hamamatsu

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Photonics, Hamamatsu, Japan), as described previously (Funabashi et al., 2010b). In brief, OUMS-27 cells were incubated with 10 μM fura-2 acetoxymethyl ester (fura-2/AM; Invitrogen/Molecular Probes) in HEPES-buffered solution at room temperature (24±1°C) for 30 min. These cells were alternatively illuminated at 340- and 380-nm wavelengths, and the fluorescence emissions (>520 nm) were captured. These $[Ca^{2+}]_i$ images were acquired every 2.3 or 4.5 s. *In vitro* calibration of the fura-2 signal was performed by a method reported previously (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = K_d(R_{min} - R)/(R - R_{max})$, where K_d is the dissociation constant of fura-2 (224 nM), R is the fluorescence ratio (F_{340}/F_{380}), and R_{min} and R_{max} are the fluorescence ratios in the absence of and with saturation of Ca^{2+}, respectively. The HEPES-buffered solution was used as an extracellular solution. Extracellular 0 mM Ca^{2+} solution was prepared by containing 1 mM *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA) instead of 2.2 mM $CaCl_2$ in normal HEPES-buffered solution. $[Ca^{2+}]_i$ measurements were carried out at room temperature (24±1°C).

MTT assays

After OUMS-27 cells were electroporated with control or ClC-7 siRNA, these cells were cultured onto 96-well plates (approximately 1,500 cells per well) for 12 h at 37°C in 5% CO_2. Then cells were exposed to either 350 or 270 mOsm medium for 48 h at 37°C. Cell viability was monitored by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Cells were treated with MTT solution (10 μl per 100 μl medium) at 37°C for 4 h and then exposed to 20% w/v SDS at 37°C for 6 h. Cell viability was evaluated as absorbance of 595-nm wavelength using a Multiscan JX (version 1.1, Thermo Labsystems, Franklin, MA).

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Drugs

Pharmacological reagents were obtained from Sigma-Aldrich except for EGTA, HEPES, and MES (Dojin, Kumamoto, Japan). Stock solutions (100 mM) of niflumic acid and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were made using dimethyl sulfoxide.

Statistics

All Pooled data are shown as mean±SE. Statistical significance between two groups was determined by Student's *t*-test. Statistical significance among groups was determined by Scheffé's test after one-way analysis of variance (ANOVA). Significant differences are denoted as *$p<0.05$ or **, ##$p<0.01$. Data for the concentration-response relationships were fitted by the following equation after normalization (Fig. 2E): relative current = $1-(1-C)/\{1+(K_d/[DIDS])^n\}$, where C is the component resistant to DIDS, K_d is the apparent dissociation constant of DIDS, [DIDS] is the concentration of DIDS, and n is the Hill coefficient.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Expression of ClC-7 channels in OUMS-27 cells

In our initial experiments, the mRNA expression pattern for ClC Cl^- channels was obtained from OUMS-27 cells using a RT-PCR technique. This analysis (30 cycles) showed detectable transcripts of all known ClC subtypes except for ClC-1 and ClC-5 (N=3). Therefore, the mRNA expression levels for ClC channels (excluding ClC-1 and ClC-5) were examined in OUMS-27 cells using a quantitative real-time PCR technique. This analysis showed that the expression of ClC-7 mRNA was abundant in OUMS-27 cells (0.039±0.006, N=4; Fig. 1A). ClC-3 and ClC-4 were expressed at low levels (N=4). Other types of Cl^- channel genes, ClC-2, ClC-6, ClC-Ka, ClC-Kb, and CFTR, gave mRNA signals that were near the threshold for detection (N=4). Subsequent Western blot analysis of the plasma membrane fraction revealed the expression of ClC-7 protein in OUMS-27 cells (N=3; Fig. 1B). Immunocytochemical staining also demonstrated the expression of ClC-7 protein in the plasma membrane (N=3; Fig. 1C).

Acid-sensitive Cl^- currents in OUMS-27 cells

To confirm these findings and determine physiological roles of ClC-7 channels, Cl^- currents were recorded in OUMS-27 cells using a whole-cell patch-clamp technique. In these experiments, K^+ currents were blocked by 120 mM Cs^+ and 20 mM TEA in the pipette solution and Ca^{2+}-activated currents were blocked by 5 mM EGTA in the pipette solution. The cell capacitance of OUMS-27 cells was 41.7±2.7 pF (n=17). Each chondrocyte was voltage-clamped at a holding potential of -40 mV and then activated by a series of step pulse (-100~+100 mV in 20-mV increment) for 500 ms every 15 s. In normal HEPES-buffered solution (pH 7.4), OUMS-27 cells exhibited small inward currents at negative potentials (-2.4±0.6 pA/pF at -100 mV, n=4) and relatively large outwardly rectifying currents at

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

positive potentials (4.7±0.9 pA/pF at +100 mV, n=4; Fig. 2A and B). These inward and outward currents were significantly inhibited by 100 μM DIDS, a blocker of Cl^- channels (41 and 42% decreases, respectively, n=3 for each, $p<0.05$ vs. control).

ClC-7 currents are known to be activated by extracellular acidic pH (Diewald et al., 2002; Kajiya et al., 2009; Ohgi et al., 2011). In our experiments, the Cl^- currents were not affected by pH 6.0 (2.8±0.4 pA/pF at +100 mV, n=4, $p>0.05$ vs. pH 7.4). However, although pH 5.0 did not change the inward current (-3.3±0.6 pA/pF at -100 mV, n=4, $p>0.05$), the outward current was significantly enhanced (13.1±0.9 pA/pF at +100 mV, n=4, $p<0.01$). These pH-induced outward currents were completely abolished by 100 μM DIDS (4.1±0.4 pA/pF at +100 mV, n=4, $p<0.01$ vs. pH 5.0). The DIDS-sensitive inward and outward currents in pH 5.0 solution were -0.9±0.4 pA/pF at -100 mV and 9.9±0.6 pA/pF at +100 mV, respectively (n=4; Fig. 2C). Note that the reversal potential was -4.9±0.8 mV (n=4), a value that is very close to a theoretical reversal potential of Cl^- (+0.28 mV) under these experimental conditions. The concentration effect of relationship for DIDS on these pH 5.0-induced Cl^- currents was examined in detail. As shown in Fig. 2D and E, the currents clearly inhibited by DIDS at concentration range of 10 μM and more (n=3, $p<0.05$ vs. control). The IC_{50} of DIDS on acidification-induced Cl^- currents was 13.4±2.2 μM and the Hill coefficient was 0.73±0.13 (n=3).

Attenuation of acid-sensitive Cl^- currents by ClC-7 knockdown in OUMS-27 cells

siRNA knockdown of ClC-7 was performed to obtain a direct evidence that these acid-sensitive Cl^- currents were mediated by ClC-7 channels in OUMS-27 cells. First, the selectivity of 200 nM of ClC-7 siRNA was confirmed by a quantitative real-time PCR method (62% decrease, N=3, $p<0.05$ vs. control siRNA; Fig. 3A). There were no significant changes

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

in mRNA expressions of other Cl^- channels between control and ClC-7 siRNA-treated cells (N=3, p>0.05). In addition, Western blot analysis showed that siRNA knockdown of ClC-7 reduced the protein expression (82% decrease, N=3, p<0.01 vs. control siRNA; Fig. 3B). Furthermore, in OUMS-27 cells treated with control siRNA, Cl^- currents were strongly activated by extracellular pH 5.0 (20.7±3.0 pA/pF at +100 mV, n=4, p<0.01 vs. pH 7.4 of 4.1±1.3 pA/pF, n=4; Fig. 3C and D). In contrast, pH 5.0 superfusate did not elicit an increase in Cl^- current amplitude in OUMS-27 cells pre-treated with ClC-7 siRNA (7.0±1.1 pA/pF at +100 mV, n=3, p>0.05 vs. pH 7.4 of 5.4±0.7 pA/pF, n=3). In summary, the DIDS-sensitive currents in pH 5.0 solution in ClC-7 siRNA-treated cells were significantly smaller than that in control siRNA-treated cells (5.9±0.8 pA/pF at +100 mV, n=3, p<0.05 vs. control siRNA of 20.2±2.8 pA/pF, n=4; Fig. 3E).

Involvement of ClC-7 channels on resting membrane potential in OUMS-27 cells

Previously, we reported that a Cl^- conductance regulated the resting membrane potential in these OUMS-27 cells. However, the molecular identity and biophysical property were not identified in our initial study (Funabashi et al., 2010a). To obtain this data, the effect of siRNA knockdown of ClC-7 on the resting membrane potential in OUMS-27 cells was examined using whole-cell current-clamp recordings. In control siRNA-treated OUMS-27 cells, 100 μM DIDS caused a hyperpolarization from -25.9±4.7 to -39.1±7.1 mV (n=4, p<0.05; Fig. 4A and B), or a difference of 13.2±2.4 mV (n=4; Fig. 4C). In contrast, when ClC-7 siRNA-treated cells were analyzed, membrane potential hyperpolarized (from -45.5±3.0 to -50.8±3.6 mV, or a difference of 5.3±1.5 mV, n=3, p<0.05) much less than in control siRNA-treated cells. Similar results were obtained when 100 μM niflumic acid (another Cl^- channel blocker) was used to evaluate the resting membrane potential in OUMS-27 cells. The membrane hyperpolarizations in niflumic acid were 22.7±3.4 or

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

10.3±1.4 mV in OUMS-27 cells treated with control or ClC-7 siRNA, respectively (n=5, $p<0.05$).

$[Ca^{2+}]_i$ increase by blockage of Cl^- channels in OUMS-27 cells

We have also examined whether the Cl^- current mediated-membrane hyperpolarization could induce an increase in $[Ca^{2+}]_i$ in OUMS-27 cells. Single chondrocytes were isolated with 1% collagenase, and then were loaded with a cell-permeable Ca^{2+} fluorescent indicator, 10 μM fura-2/AM. The resting $[Ca^{2+}]_i$ in control siRNA-treated OUMS-27 cells was 54.7±4.2 nM (n=51; Fig. 4D and E). Application of 100 μM DIDS caused a significant increase in $[Ca^{2+}]_i$ by 94.7±22.3 nM (n=51, $p<0.01$ vs. control; Fig. 4F). This DIDS-induced $[Ca^{2+}]_i$ increase disappeared after the removal of DIDS in all cells examined (n=51). In contrast, in ClC-7 siRNA-treated cells, the resting $[Ca^{2+}]_i$ was 84.0±5.8 nM (n=45) and significantly higher than that in control siRNA-treated cells ($p<0.01$). Correspondingly, the $[Ca^{2+}]_i$ in the presence of DIDS in ClC-7 siRNA-treated cells was as low as 42.0±7.0 nM (n=45, $p<0.05$ vs. control siRNA).

Downregulation of ClC-7 channels during hypoosmotic stress in OUMS-27 cells

It is known that the osmolality of synovial fluid in OA patients (249~277 mOsm) is lower than physiological levels (295~340 mOsm) (Bertram and Krawetz, 2012). Therefore, chondrocytes in OA patients are continuously exposed to hypoosmotic environment. Here, we examined whether the expression of ClC-7 channels was affected in OUMS-27 cells under hypoosmotic conditions. Short-term treatment with hypoosmotic medium (270 mOsm) to downregulate the mRNA expression of ClC-7 in OUMS-27 cells revealed a 46% decrease at 24 h and a 43% decrease at 48 h (N=4, $p<0.05$ vs. 350 mOsm; Fig. 5A). When OUMS-27 cells were cultured in isotonic medium (350 mOsm) for 48 h, the peak amplitudes of Cl^-

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

currents at -100 and +100 mV in normal bath solution (pH 7.4) were -2.5±0.3 and 6.0±0.9 pA/pF, respectively (n=3; Fig. 5B and C). Extracellular acidification (pH 5.0) markedly the enhanced Cl^- currents (12.6±0.4 pA/pF at +100 mV, n=3, $p<0.01$ vs. pH 7.4). On the other hand, after hypoosmotic stress (270 mOsm) for 48 h, the amplitudes of Cl^- currents in normal HEPES-buffered solution (-2.3±1.0 pA/pF at -100 mV and 5.2±2.3 pA/pF at +100 mV, n=3, $p>0.05$ vs. 350 mOsm medium) were similar to these in iso-osmotic medium. Furthermore, under these conditions, external acidification (pH 5.0) did not enhance the Cl^- currents (n=3, $p>0.05$). All of these Cl^- currents in pH 5.0 solution were blocked by 100 μM DIDS regardless of the osmolarity of culture medium (n=3). However, there was significant difference in the expression level (density of DIDS-sensitive currents) between isotonic and hypoosmotic media (2.6±0.7 pA/pF at +100 mV after 270 mOsm stress, n=3, $p<0.05$ vs. 350 mOsm of 8.4±1.7 pA/pF, n=3; Fig. 5D).

Membrane hyperpolarization followed by augmented $[Ca^{2+}]_i$ following hypoosmotic stress in OUMS-27 cells

Effects of the culture under hypoosmotic stress on the resting membrane potential and $[Ca^{2+}]_i$ in chondrocytes were analyzed using whole-cell current-clamp recordings and $[Ca^{2+}]_i$ imaging. When OUMS-27 cells were cultured in hypoosmotic medium (270 mOsm) for 48 h, the resting membrane potential shifted in hyperpolarizing direction in normal HEPES-buffered solution (-35.4±2.0 mV, n=3, $p<0.05$ vs. normal 350 mOsm medium of -21.6±2.7 mV, n=3; Fig. 6A and B). DIDS (100 μM)-induced hyperpolarization in OUMS-27 cells under isotonic or hypoosmotic conditions was 28.1±1.9 or 13.3±3.6 mV, respectively (n=3, $p<0.05$; Fig. 6C). Thereby, the resting $[Ca^{2+}]_i$ in hypoosmotic-treated OUMS-27 cells was higher (85.3±8.0 nM, n=52) than that in control OUMS-27 cells (52.0±3.1 nM, n=55, $p<0.01$; Fig. 6D and E).

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Cell death induced by hypoosmolarity or ClC-7 impairment in OUMS-27 cells

To begin to identify the pathological role(s) of ClC-7 channels in the chondrocytes, the viability of OUMS-27 cells was examined using a standard MTT assay. The cell viability was significantly reduced by the treatment with 100 μM DIDS for 48 h (to 1.09±0.01, n=18, $p<0.01$ vs. control of 1.18±0.02, n=18). As shown in Fig. 7, the cell viability was significantly reduced by the hypoosmotic stress (270 mOsm) for 24 h (1.19±0.02, n=12, $p<0.01$ vs. 350 mOsm of 1.36±0.07, n=12) and 48 h (1.32±0.02, n=12, $p<0.01$ vs. 350 mOsm of 1.61±0.03, n=12). In addition, siRNA knockdown of ClC-7 in 350 mOsm medium for 48 h also reduced the cell viability (1.30±0.01, n=12, $p<0.01$ vs. control siRNA). Finally, the combination of hypoosmotic stress and siRNA knockdown of ClC-7 for 48 h reduced the cell viability in an additive fashion (1.10±0.01, n=12, $p<0.01$ vs. control siRNA in 270 mOsm medium).

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Our previous work showed that Cl^- current(s) can contribute significantly to the regulation of resting membrane potential and modulate $[Ca^{2+}]_i$ in a human chondrocyte cell line, OUMS-27 (Funabashi et al., 2010a). However, the molecular component(s) and pathological significance of Cl^- current(s) in chondrocytes remained to be identified. The present data show that ClC-7 channels are the predominant Cl^- channels subtype and that this Cl^- conductance accounts for physiologically important Cl^- fluxes in OUMS-27 cells. Importantly, the ClC-7 channels are downregulated during hypoosmotic stress, resulting in membrane hyperpolarization followed by $[Ca^{2+}]_i$ increase, and finally cell death.

In addition to voltage-dependent cation currents (Barrett-Jolley et al., 2010; Mobasheri et al., 2012), voltage-dependent Cl^- currents are expressed in articular chondrocytes (Sugimoto et al., 1996; Tsuga et al., 2002). ClC gene family contains nine members coding voltage-dependent Cl^- channels (Jentsch et al., 2002; Li and Weinman, 2002; Parkerson and Sontheimer, 2004). ClC-2 currents exhibit an inward rectification, whereas ClC-4, ClC-5, and ClC-7 currents show strong outward rectification. ClC-3 currents are activated by membrane hyperpolarization, and inactivated in a time-dependent fashion at strongly depolarizing voltages (Li and Weinman, 2002; Parkerson and Sontheimer, 2004). ClC-2 and ClC-7 channels are activated by acidic pH, whereas ClC-5 currents are inhibited by acidic pH. However, the pH sensitivities of ClC-3 and ClC-4 vary with the type of cells (Diewald et al., 2002; Kawasaki et al., 1999; Matsuda et al., 2010; Matsuda et al., 2008; Parkerson and Sontheimer, 2004). Among ClC channels, ClC-2 and ClC-3 channels are activated by hypoosmotic stimuli. In addition, ClC-3, ClC-4, and ClC-7 currents are sensitive to DIDS, whereas ClC-2 and ClC-5 currents are resistant to DIDS (Li and Weinman, 2002; Parkerson and Sontheimer, 2004). In rat articular cartilages, there is mRNA expression of ClC-3 (Ponce

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 2012). Two human (ClC-3 and ClC-7) and three rodent (ClC-3, ClC-4, and ClC-6) ClC transcripts have been detected using chondrocyte Affymetrix DNA microarrays (Lewis et al., 2013). Our data indicate that acid- and DIDS-sensitive ClC-7 channels are functionally expressed in OUMS-27 cells. The IC_{50} value of DIDS on the acid pH-induced Cl^- currents was 13 μM, which is close to that of ClC-7 (39 μM) in reconstituted cells (Schulz et al., 2010). In addition, siRNA knockdown of ClC-7 attenuated Cl^- currents elicited by acidic pH solution, strongly supporting our conclusion that ClC-7 is the molecular basis for Cl^- current in OUMS-27 cells.

Recently, it has been reported that TMEM16A is expressed in rat articular chondrocytes (Ponce et al., 2012). TMEM16A has been suggested to be the molecular correlate for Ca^{2+}-activated Cl^- channels in airway epithelial cells, vascular smooth muscle cells, and interstitial cells of Cajal in the gastrointestinal tract (Huang et al., 2012). Previously, we have studied Ca^{2+}-activated Cl^- channels in OUMS-27 cells, under conditions where $[Ca^{2+}]_i \geq 1$ μM (Funabashi et al., 2010a). However, the kinetics of the Cl^- currents in this paper differ substantially from these generated by TMEM16A channels (for example, the distinct tail currents). However, the $[Ca^{2+}]_i$ increase induced by some stimuli may trigger the activation of TMEM16A channels in addition to the ClC-7 channel activity.

In articular chondrocytes, the resting membrane potential is regulated by Cl^- conductances (Funabashi et al., 2010a; Sugimoto et al., 1996; Tsuga et al., 2002), in addition to K^+ conductances (Clark et al., 2011; Mobasheri et al., 2012; Wilson et al., 2004). In many types of cells, Cl^- efflux counterbalances K^+ thus maintaining the electroneutrality. Our previous work suggested a stronger dependence of resting membrane potential on Cl^- than K^+ conductance (Funabashi et al., 2010a). In this study, blockage of DIDS- and niflumic

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

acid-sensitive Cl^- channels caused a hyperpolarization of ~13-23 mV in OUMS-27 cells. In addition, the resting membrane potential shifted in hyperpolarizing direction (by ~20 mV) in OUMS-27 cells treated with ClC-7 siRNA. These results clearly indicate that much of the Cl^- flux is due to ClC-7 channels. In non-excitable cells such as chondrocytes, membrane hyperpolarization facilitates Ca^{2+} entry through non-selective cation channels (Funabashi et al., 2010b; Gavenis et al., 2009; Hdud et al., 2012). Blockage of Cl^- channels by DIDS caused a significant hyperpolarization followed by an increase in $[Ca^{2+}]_i$.

As OA progresses, there is degradation and loss of articular cartilage, as well as enhanced inflammation of the synovial membrane (Goldring, 2006; Martel-Pelletier et al., 2008). In OA patients, the osmolality of the synovial fluid (249~277 mOsm) is lower than normal (295~340 mOsm) (Bertram and Krawetz, 2012). For these reasons, we have studied the expression levels of ClC-7 channels in the settings of both isotonic (350 mOsm) and hypotonic (270 mOsm) cell culture. Importantly, the expression of ClC-7 was downregulated by approximately 45% in OUMS-27 cells when these cells were cultured in hypoosmotic medium for 48 h. The acid-sensitive Cl^- currents mediated by ClC-7 channels were also attenuated by short-term hypo-osmotic stress. Recently, microarray analysis has suggested that large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, intermediate-conductance Ca^{2+}-activated K^+ channels, Ca^{2+}-activated Cl^- channels, and aquaporin water channels are upregulated, whereas TASK-2 channels, $K_{Ca}4.2$ subunit, and epithelial Na^+ channels was downregulated at the transcript levels in tissues from OA patients (Lewis et al., 2013). A recent report describes that the hypoosmotic stress increases the expression levels of BK_{Ca} and TRPV4 channels in equine articular chondrocytes (Hdud et al., 2014). In addition, we found ClC-7 channels downregulated by hypoosmotic stress that mimics an external OA environment. The expression of ClC-7 mRNA was detected in articular cartilages isolated

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

from normal subjects and OA patients by RT-PCR (unpublished observation). Unfortunately, in our study, the amount of tissue was too small to compare quantitatively the expression level between these two groups. Further experiments are necessary for elucidating the mechanism underlying the downregulation of ClC-7 channels during hypoosmotic stress in chondrocytes.

An increase in $[Ca^{2+}]_i$ can initiate and modulate important chondrocyte functions such as cell proliferation/differentiation and matrix formation (Alford et al., 2003; Bonen and Schmid, 1991; Wu and Chen, 2000). The contributions of ion channels to cell proliferation of chondrocytes has been discussed (Barrett-Jolley et al., 2010; Mobasheri et al., 2012; Wohlrab et al., 2001; Wu and Chen, 2000). A corresponding moderate increase in $[Ca^{2+}]_i$ promotes cell proliferation, while larger, maintained $[Ca^{2+}]_i$ increases cause cell death. Our cell viability data reveals that short-term hypoosmotic stress causes enhanced cell death in OUMS-27 cells. This increase in cell death was mimicked by siRNA knockdown of ClC-7. Taken together, these results strongly indicate that ClC-7 channels substantially regulate chondrocyte resting membrane potential and $[Ca^{2+}]_i$, and can modulate cell survival. Specifically, ClC-7 impairment due to hypoosmotic stress causes membrane hyperpolarization of the resting potential and this is followed by $[Ca^{2+}]_i$ increase leading to eventually cell death in these chondrocytes. The number of apoptotic chondrocytes is increased in articular cartilage from OA patients and hence the cartilage degradation is facilitated in OA patients (Hashimoto et al., 1998).

In conclusion, ClC-7 channels are functionally expressed in OUMS-27 cells. Their expression levels and functional properties were reduced by hypoosmotic stress that mimics the external environment of OA patient articular joint. Downregulation of ClC-7 channels causes a hyperpolarization of the resting membrane potential and this produces an increase in $[Ca^{2+}]_i$,

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

and leads to enhanced cell death. Thus, in combination, our results identify the molecule correlate of chondrocyte Cl^- channels and demonstrate modulation of these treatment by OA. These findings provide important new insights for the drug discovery.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

None.

Authorship contributions

Participated in research design: Yamamura and Imaizumi.

Conducted experiments: Kurita, Yamamura, and Suzuki.

Performed data analysis: Kurita, Yamamura, Suzuki, Giles, and Imaizumi.

Wrote or contributed to the writing of the manuscript: Yamamura, Giles, and Imaizumi.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

References

Alford AI, Yellowley CE, Jacobs CR and Donahue HJ (2003) Increases in cytosolic calcium, but not fluid flow, affect aggrecan mRNA levels in articular chondrocytes. *J Cell Biochem* **90**(5): 938-944.

Barrett-Jolley R, Lewis R, Fallman R and Mobasheri A (2010) The emerging chondrocyte channelome. *Front Physiol* **1**: 135.

Bertram KL and Krawetz RJ (2012) Osmolarity regulates chondrogenic differentiation potential of synovial fluid derived mesenchymal progenitor cells. *Biochem Biophys Res Commun* **422**(3): 455-461.

Bonen DK and Schmid TM (1991) Elevated extracellular calcium concentrations induce type X collagen synthesis in chondrocyte cultures. *J Cell Biol* **115**(4): 1171-1178.

Bush PG and Hall AC (2001) The osmotic sensitivity of isolated and in situ bovine articular chondrocytes. *J Orthop Res* **19**(5): 768-778.

Clark RB, Kondo C, Belke DD and Giles WR (2011) Two-pore domain K^+ channels regulate membrane potential of isolated human articular chondrocytes. *J Physiol* **589**(Pt 21): 5071-5089.

Demircan K, Hirohata S, Nishida K, Hatipoglu OF, Oohashi T, Yonezawa T, Apte SS and Ninomiya Y (2005) ADAMTS-9 is synergistically induced by interleukin-1β and tumor necrosis factor α in OUMS-27 chondrosarcoma cells and in human chondrocytes. *Arthritis Rheum* **52**(5): 1451-1460.

Diewald L, Rupp J, Dreger M, Hucho F, Gillen C and Nawrath H (2002) Activation by acidic pH of CLC-7 expressed in oocytes from *Xenopus laevis*. *Biochem Biophys Res Commun* **291**(2): 421-424.

Funabashi K, Fujii M, Yamamura H, Ohya S and Imaizumi Y (2010a) Contribution of chloride channel conductance to the regulation of resting membrane potential in

chondrocytes. *J Pharmacol Sci* **113**(1): 94-99.

Funabashi K, Ohya S, Yamamura H, Hatano N, Muraki K, Giles W and Imaizumi Y (2010b) Accelerated Ca^{2+} entry by membrane hyperpolarization due to Ca^{2+}-activated K^+ channel activation in response to histamine in chondrocytes. *Am J Physiol Cell Physiol* **298**(4): C786-797.

Gavenis K, Schumacher C, Schneider U, Eisfeld J, Mollenhauer J and Schmidt-Rohlfing B (2009) Expression of ion channels of the TRP family in articular chondrocytes from osteoarthritic patients: changes between native and in vitro propagated chondrocytes. *Mol Cell Biochem* **321**(1-2): 135-143.

Goldring MB (2006) Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best Pract Res Clin Rheumatol* **20**(5): 1003-1025.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**(6): 3440-3450.

Hashimoto S, Ochs RL, Komiya S and Lotz M (1998) Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. *Arthritis Rheum* **41**(9): 1632-1638.

Hdud IM, El-Shafei AA, Loughna P, Barrett-Jolley R and Mobasheri A (2012) Expression of transient receptor potential vanilloid (TRPV) channels in different passages of articular chondrocytes. *Int J Mol Sci* **13**(4): 4433-4445.

Hdud IM, Mobasheri A and Loughna PT (2014) Effect of osmotic stress on the expression of TRPV4 and BK_{Ca} channels and possible interaction with ERK1/2 and p38 in cultured equine chondrocytes. *Am J Physiol Cell Physiol* **306**(11): C1050-1057.

Hoffmann EK, Lambert IH and Pedersen SF (2009) Physiology of cell volume regulation in vertebrates. *Physiol Rev* **89**(1): 193-277.

Huang F, Wong X and Jan LY (2012) International Union of Basic and Clinical Pharmacology. LXXXV: calcium-activated chloride channels. *Pharmacol Rev* **64**(1): 1-15.

Jentsch TJ, Stein V, Weinreich F and Zdebik AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* **82**(2): 503-568.

Kajiya H, Okamoto F, Ohgi K, Nakao A, Fukushima H and Okabe K (2009) Characteristics of ClC7 Cl^- channels and their inhibition in mutant (G215R) associated with autosomal dominant osteopetrosis type II in native osteoclasts and h*Clcn7* gene-expressing cells. *Pflügers Arch* **458**(6): 1049-1059.

Kawasaki M, Fukuma T, Yamauchi K, Sakamoto H, Marumo F and Sasaki S (1999) Identification of an acid-activated Cl^- channel from human skeletal muscles. *Am J Physiol* **277**(5 Pt 1): C948-954.

Kerrigan MJ, Hook CS, Qusous A and Hall AC (2006) Regulatory volume increase (RVI) by in situ and isolated bovine articular chondrocytes. *J Cell Physiol* **209**(2): 481-492.

Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H and Namba M (1998) A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. *Int J Cancer* **77**(6): 854-859.

Lewis R, May H, Mobasheri A and Barrett-Jolley R (2013) Chondrocyte channel transcriptomics: do microarray data fit with expression and functional data? *Channels (Austin)* **7**(6): 459-467.

Li X and Weinman SA (2002) Chloride channels and hepatocellular function: prospects for molecular identification. *Annu Rev Physiol* **64**: 609-633.

Martel-Pelletier J, Boileau C, Pelletier JP and Roughley PJ (2008) Cartilage in normal and osteoarthritis conditions. *Best Pract Res Clin Rheumatol* **22**(2): 351-384.

Matsuda JJ, Filali MS, Collins MM, Volk KA and Lamb FS (2010) The ClC-3 Cl^-/H^+ antiporter becomes uncoupled at low extracellular pH. *J Biol Chem* **285**(4): 2569-2579.

Matsuda JJ, Filali MS, Volk KA, Collins MM, Moreland JG and Lamb FS (2008)

Overexpression of CLC-3 in HEK293T cells yields novel currents that are pH dependent. *Am J Physiol Cell Physiol* **294**(1): C251-262.

Mobasheri A, Lewis R, Ferreira-Mendes A, Rufino A, Dart C and Barrett-Jolley R (2012) Potassium channels in articular chondrocytes. *Channels (Austin)* **6**(6): 416-425.

Ohgi K, Okamoto F, Kajiya H, Sakagami R and Okabe K (2011) Antibodies against ClC7 inhibit extracellular acidification-induced Cl^- currents and bone resorption activity in mouse osteoclasts. *Naunyn Schmiedebergs Arch Pharmacol* **383**(1): 79-90.

Parkerson KA and Sontheimer H (2004) Biophysical and pharmacological characterization of hypotonically activated chloride currents in cortical astrocytes. *Glia* **46**(4): 419-436.

Ponce A, Jimenez-Peña L and Tejeda-Guzman C (2012) The role of swelling-activated chloride currents ($I_{CL,swell}$) in the regulatory volume decrease response of freshly dissociated rat articular chondrocytes. *Cell Physiol Biochem* **30**(5): 1254-1270.

Schulz P, Werner J, Stauber T, Henriksen K and Fendler K (2010) The G215R mutation in the Cl^-/H^+-antiporter ClC-7 found in ADO II osteopetrosis does not abolish function but causes a severe trafficking defect. *PLoS One* **5**(9): e12585.

Sugimoto T, Yoshino M, Nagao M, Ishii S and Yabu H (1996) Voltage-gated ionic channels in cultured rabbit articular chondrocytes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **115**(3): 223-232.

Tsuga K, Tohse N, Yoshino M, Sugimoto T, Yamashita T, Ishii S and Yabu H (2002) Chloride conductance determining membrane potential of rabbit articular chondrocytes. *J Membr Biol* **185**(1): 75-81.

Wilson JR, Duncan NA, Giles WR and Clark RB (2004) A voltage-dependent K^+ current contributes to membrane potential of acutely isolated canine articular chondrocytes. *J Physiol* **557**(Pt 1): 93-104.

Wohlrab D, Wohlrab J, Reichel H and Hein W (2001) Is the proliferation of human

chondrocytes regulated by ionic channels? *J Orthop Sci* **6**(2): 155-159.

Wu QQ and Chen Q (2000) Mechanoregulation of chondrocyte proliferation, maturation, and hypertrophy: ion-channel dependent transduction of matrix deformation signals. *Exp Cell Res* **256**(2): 383-391.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This investigation was supported by Grant-in-Aids for Scientific Research (B) [26293021] and Scientific Research (C) [25460104] from the Japan Society for the Promotion of Science. This work was also supported by a Grant-in-Aid from the Japan Research Foundation for Clinical Pharmacology.

Reprint requests

Dr. Yuji Imaizumi, Department of Molecular & Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuhoku, Nagoya 467-8603, Japan; yimaizum@phar.nagoya-cu.ac.jp

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. Molecular identification of ClC channels in human chondrocytes (OUMS-27)

The expression pattern for ClC Cl^- channels was obtained from OUMS-27 cells using quantitative real-time PCR, Western blot, and immunocytochemical techniques. **A:** mRNA expression of ClC and CFTR Cl^- channels profiled in OUMS-27 cells using quantitative real-time PCR analysis. mRNA expression of Cl^- channels was normalized to that of endogenous β-actin. **B:** Western blot analysis performed using a protein fraction of plasma membrane in OUMS-27 cells (50 μg/lane) and a ClC-7 specific antibody. The size of ClC-7 is estimated to be approximately 89 kDa. **C:** Confocal fluorescent image of OUMS-27 cells immunestained with a specific ClC-7 antibody.

Fig. 2. Activation of Cl^- currents by acidification in OUMS-27 cells

Effect of acidic pH on Cl^- currents in OUMS-27 cells recorded using whole-cell voltage-clamp methods. To prevent contamination of intrinsic K^+ and Ca^{2+}-activated ion currents, a pipette solution containing 120 mM Cs^+, 20 mM TEA, and 5 mM EGTA was used. **A:** Chondrocytes were voltage-clamped at a holding potential of -40 mV and then hyperpolarized or depolarized to -100~+100 mV in 20-mV increments for 500 ms voltage-clamp steps were repeated every 15 s. Representative traces of Cl^- currents in control conditions (pH 7.4), as well as pH 6.0 and pH 5.0 bath solutions are shown both in the absence and presence of 100 μM DIDS, a blocker of Cl^- channels. Dotted lines indicate zero current level. **B:** Current-voltage relationships in pH 7.4, pH 6.0, and pH 5.0 solutions. The current amplitudes were measured at the end of the voltage-clamp pulses. **C:** DIDS-sensitive current density in pH 5.0 solution. Note that the reversal potential is -4.9 mV. **D:** pH 5.0-induced Cl^- currents in the absence and presence of DIDS (0.1~1000 μM). Each chondrocyte was depolarized from a holding potential of -40 mV to +60 mV for 500 ms.

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

Dotted lines indicate zero current level. **E:** Dose-response curve for inhibition by DIDS on acidification-induced Cl^- currents in OUMS-27 cells. The IC_{50} value was 13 μM.

Fig. 3. Attenuation of acid-sensitive Cl^- currents by ClC-7 knockdown in OUMS-27 cells

To reveal the molecular identity of the Cl^- channels in OUMS-27 cells, the effect of siRNA knockdown of ClC-7 on the acid pH-induced Cl^- currents was examined using whole-cell voltage-clamp recordings. **A & B:** The selectivity of 200 nM ClC-7 siRNA (siClC-7) was evaluated using quantitative real-time PCR (**A**) and Western blot (**B**). Statistical significance is indicated as *p<0.05 or **p<0.01 vs. control siRNA. **C:** Each chondrocyte was voltage-clamped at a holding potential of -40 mV and the membrane potential was displaced in the range shown. Representative traces of Cl^- currents in normal (pH 7.4) and pH 5.0 bath solutions in the absence and presence of 100 μM DIDS in chondrocytes previously electroporated with control (*upper*) or ClC-7 (*bottom*) siRNA. Dotted lines indicate zero current level. **D:** Current-voltage relationships of pH 5.0-sensitive currents in control (*left*) and ClC-7 (*right*) siRNA-treated cells. **E:** DIDS-sensitive current densities in pH 5.0 solution in control and ClC-7 siRNA-treated cells.

Fig. 4. Membrane hyperpolarization and $[Ca^{2+}]_i$ increase by inhibition of Cl^- channels in OUMS-27 cells

Effect of siRNA knockdown of ClC-7 on the resting membrane potential and $[Ca^{2+}]_i$ in OUMS-27 cells. **A:** Effect of 100 μM DIDS on the membrane potential (MP) in chondrocytes treated with control (*left*) or ClC-7 (siClC-7; *right*) siRNA. **B:** The resting membrane potential (RMP) in OUMS-27 cells treated with control or ClC-7 siRNA. **C:** Membrane hyperpolarization induced by DIDS in control or ClC-7 siRNA-treated cells. **D:** Effects of 100 μM DIDS on $[Ca^{2+}]_i$ in OUMS-27 cells treated with control (*black*) or ClC-7 (*glay*)

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

siRNA. OUMS-27 cells were loaded with a cell-permeable Ca^{2+} indicator, 10 μM fura-2/AM. **E:** The resting $[Ca^{2+}]_i$s in OUMS-27 cells treated with control or ClC-7 siRNA. **F:** The $[Ca^{2+}]_i$s in control and ClC-7 siRNA-treated cells in the presence of DIDS. Statistical significance is indicated as *p<0.05 or **p<0.01 vs. control siRNA.

Fig. 5. Downregulation of ClC-7 channels due to hypoosmotic stress in OUMS-27 cells

The expression and function of ClC-7 in OUMS-27 cells as a consequence of hypoosmotic stress in chondrocytes from OA patients. **A:** Expression levels of ClC-7 transcripts due to short-term hypoosmotic stress (270 mOsm) in OUMS-27 cells, analyzed by quantitative real-time PCR. **B:** Chondrocytes were voltage-clamped at a holding potential of -40 mV, and then depolarized or hyperpolarized in the range of -100~+100 mV (in 20-mV increments) for 500 ms every 15 s in normal HEPES-buffered solution. Representative traces of Cl^- currents in normal (pH 7.4; *left*) and acidic pH (5.0) solutions, in the absence (*middle*) and presence (*right*) of 100 μM DIDS after 48 h culture media having isotonic (350 mOsm; *upper*) or hypoosmotic (270 mOsm; *bottom*) condition are shown. Dotted lines indicate zero current level. **C:** Current-voltage relationships for the acid-sensitive current changes due to short-term culture in isotonic (*left*) and hypoosmotic (*right*) media. **D:** DIDS-sensitive current densities in pH 5.0 solution in isotonic and hypoosmotic media.

Fig. 6. Membrane hyperpolarization and $[Ca^{2+}]_i$ increase by hypoosmotic stress in OUMS-27 cells

Effects of the culture under hypoosmotic stress on the resting membrane potential and $[Ca^{2+}]_i$ in normal HEPES-buffered solution in OUMS-27 cells. **A:** Effect of 100 μM DIDS on the membrane potential (MP) in chondrocytes cultured for 48 h in isotonic (350 mOsm; *left*) or hypoosmotic (270 mOsm; *right*) medium. **B:** The resting membrane potential (RMP) in

Molecular Pharmacology Fast Forward. Published on May 5, 2015 as DOI: 10.1124/mol.115.098160
This article has not been copyedited and formatted. The final version may differ from this version.

OUMS-27 cells treated with isotonic or hypoosmotic medium. **C:** Membrane hyperpolarization induced by DIDS in isotonic or hypoosmotic medium-treated cells. **D:** Effects of removal of extracellular Ca^{2+} (0 mM Ca^{2+}) on $[Ca^{2+}]_i$ in isotonic or hypoosmotic medium-treated OUMS-27 cells loaded with 10 μM fura-2/AM. **E:** The resting $[Ca^{2+}]_i$s in OUMS-27 cells cultured in isotonic or hypoosmotic medium. Statistical significance is indicated as *p<0.05 or **p<0.01 vs. normal medium (350 mOsm).

Fig. 7. Cell viability following either hypoosmotic stress or ClC-7 knockdown in OUMS-27 cells

To begin to elucidate the pathological functions of ClC-7 channels, effects of hypoosmolarity and ClC-7 knockdown on chondrocyte viability were examined. Cell viability in control or ClC-7 siRNA-treated OUMS-27 cells cultured in isotonic (350 mOsm) and hypoosmotic (270 mOsm) medium was assayed using the MTT method. Statistical significance is indicated as *p<0.05 or **p<0.01 vs. normal medium (350 mOsm), and $^{\#\#}$p<0.01 vs. control siRNA. Note that short-term hypoosmotic stress causes enhanced cell death in OUMS-27 cells and this increase in cell death was mimicked by siRNA knockdown of ClC-7.

Figure 1

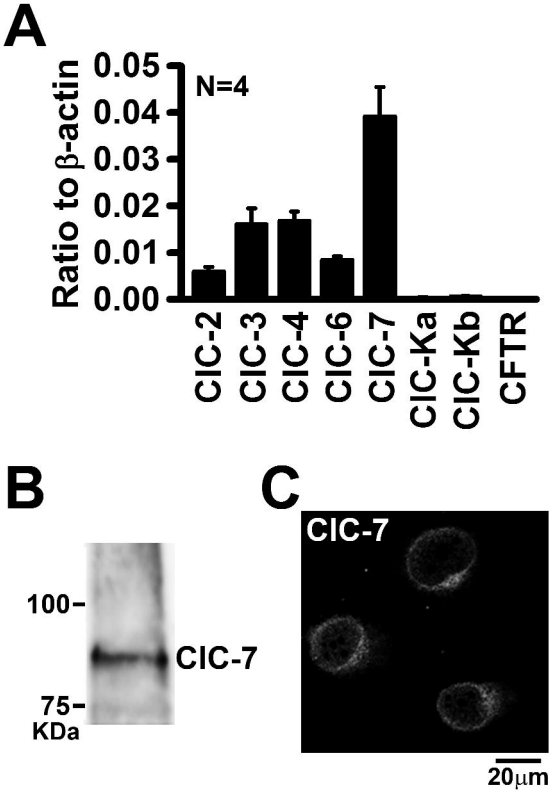

A
Ratio to β-actin
0.05
0.04
0.03
0.02
0.01
0.00
N=4
CIC-2
CIC-3
CIC-4
CIC-6
CIC-7
CIC-Ka
CIC-Kb
CFTR
B
100
75
KDa
CIC-7
C
CIC-7
20μm

Figure 2

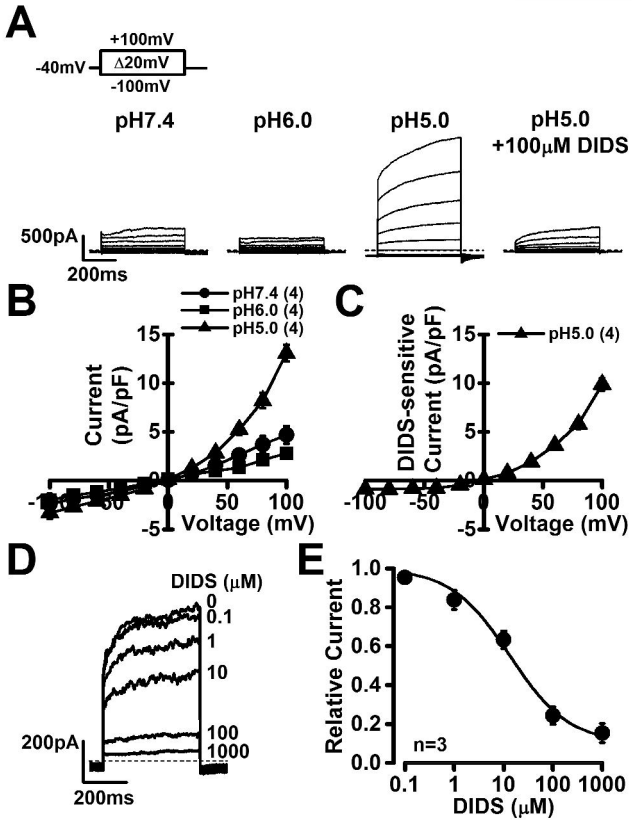

A
+100mV
-40mV
Δ20mV
-100mV
pH7.4
pH6.0
pH5.0
pH5.0
+100μM DIDS
500pA
200ms
B
pH7.4 (4)
pH6.0 (4)
pH5.0 (4)
Current (pA/pF)
Voltage (mV)
C
pH5.0 (4)
DIDS-sensitive Current (pA/pF)
Voltage (mV)
D
DIDS (μM)
0
0.1
1
10
100
1000
200pA
200ms
E
Relative Current
n=3
DIDS (μM)

Figure 3

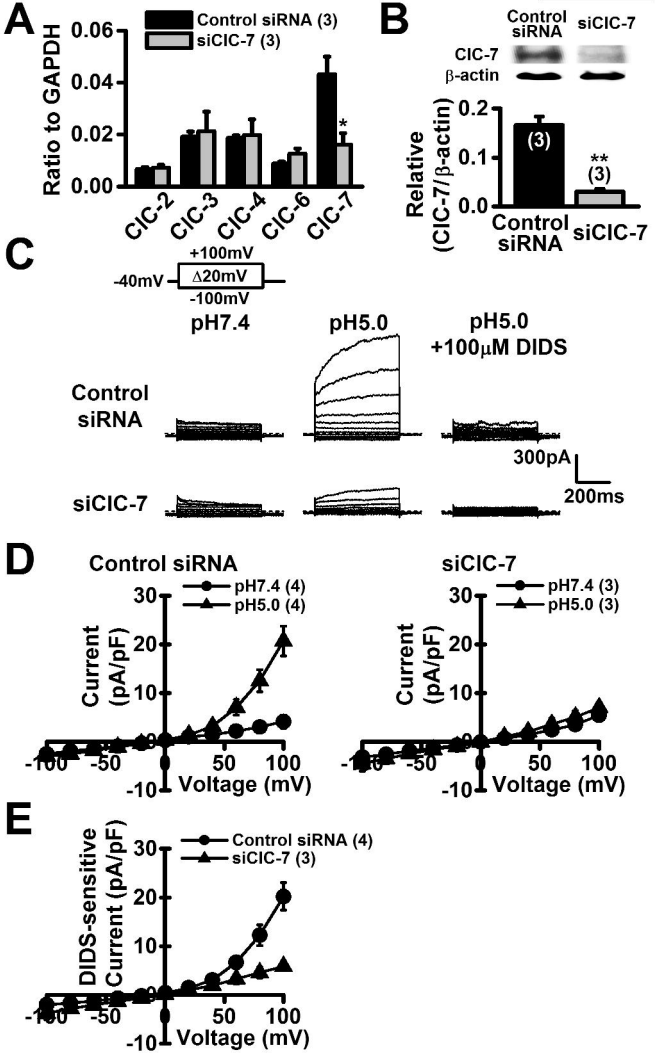

A
Control siRNA (3)
siCIC-7 (3)
Ratio to GAPDH
0.06
0.04
0.02
0.00
*
CIC-2
CIC-3
CIC-4
CIC-6
CIC-7
B
Control siRNA
siCIC-7
CIC-7
β-actin
Relative (CIC-7/β-actin)
0.2
0.1
0.0
(3)
**
(3)
Control siRNA
siCIC-7
C
+100mV
-40mV
Δ20mV
-100mV
pH7.4
pH5.0
pH5.0 +100μM DIDS
Control siRNA
300pA
200ms
siCIC-7
D
Control siRNA
pH7.4 (4)
pH5.0 (4)
Current (pA/pF)
30
20
10
-10
-100
-50
50
100
Voltage (mV)
siCIC-7
pH7.4 (3)
pH5.0 (3)
E
Control siRNA (4)
siCIC-7 (3)
DIDS-sensitive Current (pA/pF)

Figure 4

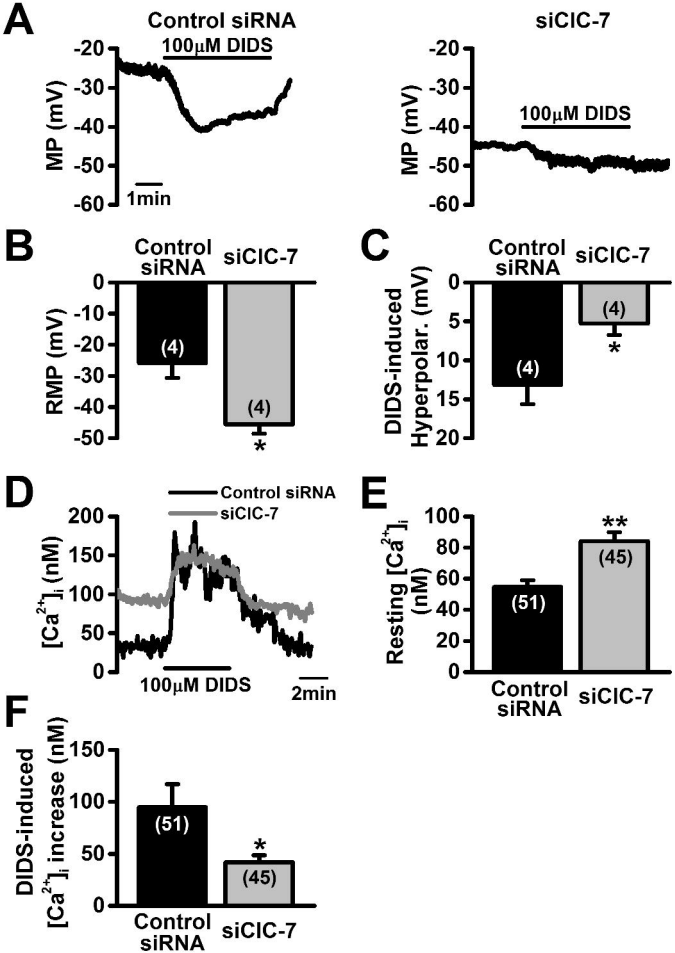

A
Control siRNA
100μM DIDS
MP (mV)
1min
siCIC-7
100μM DIDS
MP (mV)
B
Control siRNA
siCIC-7
RMP (mV)
(4)
(4)
*
C
Control siRNA
siCIC-7
DIDS-induced Hyperpolar. (mV)
(4)
(4)
*
D
Control siRNA
siCIC-7
[Ca2+]i (nM)
100μM DIDS
2min
E
Resting [Ca2+]i (nM)
(51)
(45)
**
Control siRNA
siCIC-7
F
DIDS-induced [Ca2+]i increase (nM)
(51)
(45)
*
Control siRNA
siCIC-7

Figure 5

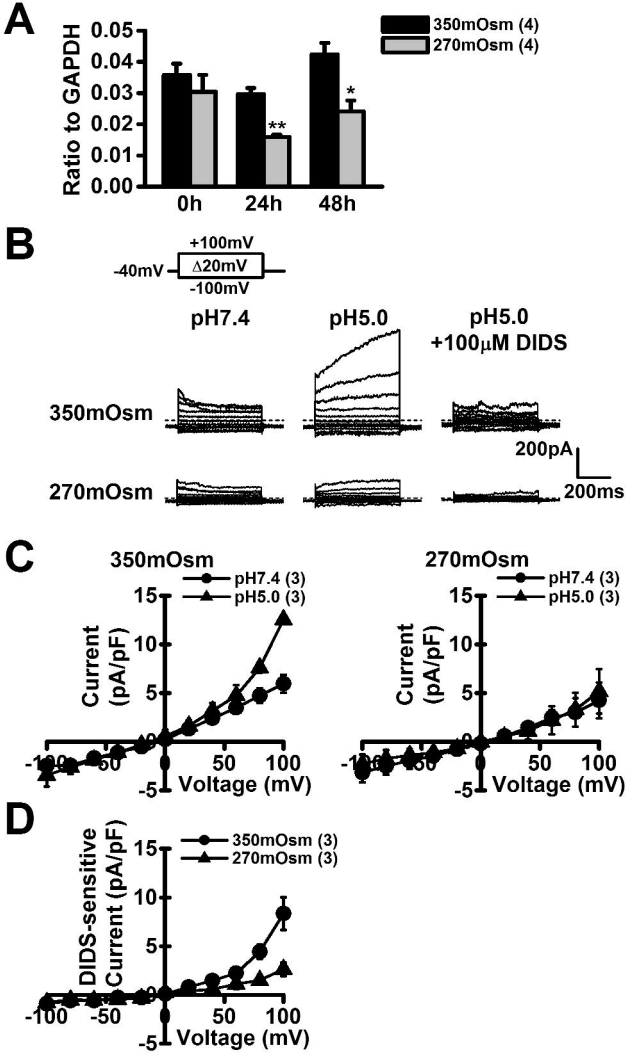

A
Ratio to GAPDH
0.05
0.04
0.03
0.02
0.01
0.00
0h
24h
48h
**
*
350mOsm (4)
270mOsm (4)
B
+100mV
-40mV
Δ20mV
-100mV
pH7.4
pH5.0
pH5.0
+100μM DIDS
350mOsm
270mOsm
200pA
200ms
C
350mOsm
pH7.4 (3)
pH5.0 (3)
Current (pA/pF)
Voltage (mV)
270mOsm
pH7.4 (3)
pH5.0 (3)
Current (pA/pF)
Voltage (mV)
D
DIDS-sensitive Current (pA/pF)
350mOsm (3)
270mOsm (3)
Voltage (mV)

Figure 6

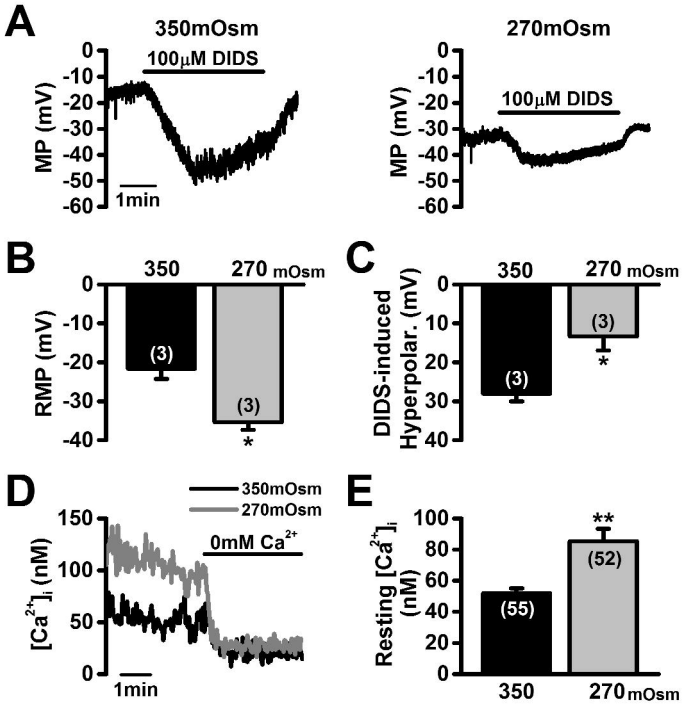
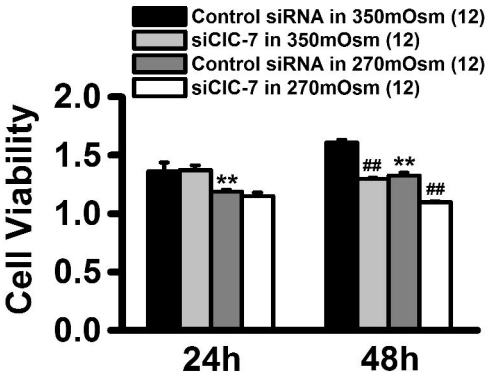

A
350mOsm
100μM DIDS
MP (mV)
0
-10
-20
-30
-40
-50
-60
1min
270mOsm
100μM DIDS
MP (mV)
B
350
270 mOsm
RMP (mV)
(3)
(3)
*
C
350
270 mOsm
DIDS-induced Hyperpolar. (mV)
(3)
(3)
*
D
350mOsm
270mOsm
0mM Ca2+
[Ca2+]i (nM)
150
100
50
0
1min
E
Resting [Ca2+]i (nM)
100
80
60
40
20
0
**
(52)
(55)
350
270 mOsm

Figure 7


Control siRNA in 350mOsm (12)
siCIC-7 in 350mOsm (12)
Control siRNA in 270mOsm (12)
siCIC-7 in 270mOsm (12)
Cell Viability
2.0
1.5
1.0
0.5
0.0
**
##
**
##
24h
48h