Membrane channel gene expression in human costal and articular chondrocytes

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ABSTRACT. Chondrocytes are the uniquely resident cells found in all types of cartilage and key to their function is the ability to respond to mechanical loads with changes of metabolic activity. This mechanotransduction property is, in part, mediated through the activity of a range of expressed transmembrane channels; ion channels, gap junction proteins, and porins. Appropriate expression of ion channels has been shown essential for production of extracellular matrix and differential expression of transmembrane channels is correlated to musculoskeletal diseases such as osteoarthritis and Albers-Schönberg. In this study we analyzed the consistency of gene expression between channelomes of chondrocytes from human articular and costal (teenage and fetal origin) cartilages. Notably, we found 14 ion channel genes commonly expressed between articular and both types of costal cartilage chondrocytes. There were several other ion channel genes expressed only in articular (6 genes) or costal chondrocytes (5 genes). Significant differences in expression of *BEST1* and *KCNJ2* (Kir2.1) were observed between fetal and teenage costal cartilage. Interestingly, the large Ca^{2+} activated potassium channel ($BK\alpha$, or *KCNMA1*) was very highly expressed in all chondrocytes examined. Expression of the gap junction genes for Panx1, *GJA1* (Cx43) and *GJC1* (Cx45) was also observed in chondrocytes from all cartilage samples. Together, this data highlights similarities between chondrocyte membrane channel gene expressions in cells derived from different anatomical sites, and may imply that common electrophysiological signaling pathways underlie cellular control. The high expression of a range of mechanically and metabolically sensitive membrane channels suggest that chondrocyte mechanotransduction may be more complex than previously thought.

KEYWORDS. cartilage, chondrocytes, connexin, gap junctions, ion channels, pannexin

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INTRODUCTION

The biology of cartilage is complex and includes tissue-dependent remodeling in response to environmental stimuli.[49] Mechanical signals cause changes in cartilage biosynthetic activity, including changes in matrix production[40] and gene expression.[2] The cartilage repair process is impacted by a lack of blood supply to the tissue, resulting in a relatively hypoxic and acidic environment for cells. The fundamental processes that underlie mechanotransduction in cartilage could potentially be harnessed to enhance tissue regeneration. A promising example of this approach recently used an agonist to the mechanosensitive TRPV4 channel to induce matrix synthesis.[52] Appropriate expression of ion channels is essential for production of extracellular matrix in trachea[39] and in chondrocytes grown *in vitro*.[69,48] Differential expression of transmembrane channels is also correlated to musculoskeletal diseases such as osteoarthritis and Albers-Schönberg.[18,51] While the role of cartilage varies between anatomical sites, it seems likely that key fundamental tissue specific processes will be conserved between different sites.

The biology of cartilage is driven by osmotic and ionic gradients created by the fixed charge density of proteoglycans which draw sodium and water into the tissue.[66] This osmotic gradient is maintained and resisted by matrix proteins secreted by chondrocytes resulting in a hydrostatic pressure contributing to the ability of cartilage to withstand biomechanical stresses and strains.[49] Largely understudied is the response of cells to ionic, osmotic, and pH gradients that are generated as cartilage is exposed to continually changing biomechanical forces generated during movement. Under these conditions, cells attempt to maintain homeostasis by moving ions back and forth across the cell membrane, effectively achieved by the expression of a range of transporters and ion channels. There is interest in the number and diversity of ion channels expressed by chondrocytes since many have unknown functions, but appear conserved between animal species.[6]

Compromised cell-volume regulation has been associated with cartilage degeneration,[12] and a number of ion channels, including TRPV4[15] and transporters[58] are clearly involved with the homeostatic control of chondrocyte volume through modulation of the cell membrane potential.[34,35] The resting membrane potential of articular chondrocytes is more positive compared to many other cell types.[8] This is intriguing because cells with a more positive resting membrane potential tend to have greater regenerative capabilities.[33] The identity of ion channels present in chondrocytes is therefore of biological significance in organogenesis, tissue repair, and regeneration. Such knowledge will provide clues as to which ion channels are important for universal cartilage functions, while others may be involved with site-specific functions like cell proliferation, migration, and differentiation.[1,32]

In addition to ion channels, the membrane channel superfamily includes the so called gap junction proteins comprising both connexins and pannexins.[25] Gap junction proteins form intercellular pores that are permeable to small inorganic ions and larger organic molecules, such as ATP. Gap junction proteins also form "hemi-channels," where the proteins form a transmembrane pore, like an ordinary, but very large ion channel.[61] Unpaired hemichannels are capable of activation by pH, voltage, and intracellular calcium stimuli.[22] Gap junctions have many roles, including mechanotransduction in cartilage,[29] and can be activated through numerous mechanisms, including stretch[57] or voltage-dependent conformational changes.[5] Gap junctions, through their ability to directly connect cells, are able to create networks of cells, an important component of successful tissue regeneration.[31] Expression of gap junction genes is also tissue specific. This is significant in cartilage regeneration because recent reports[42,43] have described chondrocyte networks mediated by gap junctions. Overall, this suggests that transmembrane transport by membrane channel proteins may play an as yet unknown role in human cartilage tissue regeneration and we hypothesize that a diverse array of cell membrane ion transporters will be present in primary human chondrocytes.

In this paper, we define a complex and diverse array of ion channels present in a variety of chondrocytes, identify common gap junction genes expressed in human chondrocytes, and suggest a means of communication that is presently under exploited in the regeneration of cartilage.

MATERIALS AND METHODS

Cartilage samples

Cartilage samples were obtained from 9 human subjects. Normal costal cartilage was obtained from a 15-year-old and 2 17 y old males (CON8, CON9 and CON10 respectively) and processed within 24 hours. Femoral head articular chondrocytes from 54 and 57-year-old males and a 59-year-old female were purchased from PromoCell (Heidelberg, Germany). Cells were isolated from apparently normal cartilage of patients undergoing knee/hip replacement surgeries. All cells were cultured in Chondrocyte growth medium (PromoCell). Three fetal samples of costal cartilage were obtained; F1, a 37 weeks gestation female with chorioamnionitis, F2, a 21 weeks gestation female with intrauterine demise due to severe chorioamnionitis and congenital pneumonia, and F3, a 15 week gestational male with severe congenital cardiac disease. Fetal cells were grown in DMEM/F12 (HyClone, Logan, Utah, USA)[14] supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA, USA), 2 mM L-glutamine (InVitrogen, Carlsbad, CA, USA), 50 IU/mL penicillin, and 50 mg/mL streptomycin (InVitrogen) at 37°C with 5% CO_2 in humidified air. For experiments, chondrocytes were maintained in suspension culture[10,65] to maintain their differentiated phenotype, and briefly expanded in tissue culture flasks. All experiments used cells that had been passaged less than 4 times.

Reverse transcription and real-time PCR analysis

RNA was directly isolated from cells in tissue culture dishes, and genomic DNA eliminated using a Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA). Complimentary DNA (cDNA) was generated using an RT-First Strand Kit (Qiagen, Valencia, CA, USA). Polymerase chain reactions (PCRs) were performed using SYBR green detection (Qiagen) and customized ion channel array plates (Qiagen) in a BioRad CFX96 system (BioRad, Hercules, CA, USA). These customized plates provide gene expression data on 84 different ion channel-related genes. Manufacturer guidelines were used for PCR reaction volumes and cycle parameters. The cycling parameters were 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Reaction specificities were assessed with a melt curve of 65°C to 95°C in 0.2°C increments. The data was standardized to 5 (*ACTB, B2M, GAPDH, HPRT1* and *RPLP0*) reference gene expression values for all samples using the ΔCq method. Gap junction gene expression was determined using a connexin and pannexin gene array (Qiagen CA, USA), with data standardized to the 5 reference genes described above. Aggrecan and COMP gene expression was by RT-PCR using commercially available primers (Qiagen) and data standardized against 2 reference genes (*GAPDH* and 18srRNA). Gene expression was calculated as $2^{-\left(Cq_{GOI} - Cq_{Ref}\right)}$, where Cq_{GOI} is the Cq value of the gene of interest and Cq_{Ref}, is the Cq value for the averaged reference genes. Although the assay range using the RT^2 profiler array (Qiagen) is 6.8–35 Cq, in order to minimize the possibility of false-positives and account for variability, a constant concentration of total cDNA was used in all reactions between all samples and a raw Cq cutoff of 30 was used.[13,3]

Immunocytochemistry

Confirmation of *KCNMA1* ($K_{Ca}1.1\alpha$), *KCNA2* ($K_V1.2$), *SCN9A* ($Na_V1.7$), and *GJA1* (Cx43) gene expression were made by immunocytochemistry. Cells were grown on cover slips and fixed in 4% paraformaldehyde for 20 minutes then permeabilized with PBS + 0.5% TRITON-X100 for 10 minutes. Washes with PBS were performed after both steps and cells were then blocked in 10% boiled goat serum (BGS) for 1 hour. Incubation with primary antibodies was performed in 5% BGS at 4°C overnight then washed 3 times in PBS + 0.1% Tween-20 (PBS-T) for 5 minutes each. Rabbit polyclonal primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for $K_{Ca}1.1\alpha$ (sc-25686), $K_V1.2$ (sc-

292447), $Na_V1.7$ (sc-130096), and Cx43 (sc-9059). Negative controls were produced using normal rabbit IgG (Santa Cruz). Secondary staining was with goat anti-rabbit Alexa Fluor 488 (Life Technologies, Carlsbad, CA, USA) in 5% BGS for 45 minutes at room temperature then washed 3 times in PBS-T for 5 minutes each. The nuclei were counterstained using 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole; Sigma) in PBS-T for 5 minutes followed by 3 PBS-T washes for 5 minutes each. After a brief rinse in PBS, the coverslips were mounted on slides using VECTASHIELD antifade mounting medium (Vector Labs, Burlingame, CA, USA). Electronic fluorescent images were captured using an Olympus DP70 CCD camera through an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA, USA).

RESULTS

Chondrocytes express ion channel and gap junction genes

Ion channels

Eighty four genes commonly expressed in excitable tissues were examined for gene expression in each of the 9 human chondrocyte samples. The ΔCq was calculated for each sample using 5 different reference genes. A threshold for detection was conservatively set at a Cq of 30. Expression values of all genes examined can be found in Supplement Table S1. Table 1 summarizes expression of those genes that were considered to be uniformly expressed in the 3 types of cartilage examined. We classified channel genes ontologically under the following 4 groups:

Calcium permeable ion channels

Of the 12 voltage-gated Ca^{2+} channel genes examined and involved in calcium release from intracellular stores, 3 (*CACNA1A* (Cav2.1), *CACNB1* (Cavβ1), *CACNB3* (Cavβ3) show expression in all samples tested (Table 1). Expression of *RYR3* was observed in all fetal samples. *CACNA1C* ($Ca_V1.2$) is the α-subunit

of a voltage-dependent calcium channel mediating Ca^{2+} entry during depolarization. Here we show for the first time its consistent expression in human chondrocytes derived from 3 independent samples of articular cartilage. Expression of this gene was found in 2/3 fetal and 1/3 costal samples which suggest either age related or functional related changes in expression.

Sodium selective ion channels

Expression of 6 sodium ion channels was detected. The voltage-gated sodium ion channel *SCN8A* ($Na_V1.6\alpha$) was expressed across all samples. Of the 2 β-subunits investigated, *SCN1B* ($Na_V1.1\beta$) was also expressed across all samples, whereas *SCN2B* ($Na_V1.2\beta$) is expressed in all 3 articular samples, as well as 2/3 costal and fetal samples (Table 1). The roles of the sodium channel β-subunits are not known, but they may have a role in connecting sodium channels to particular cellular membrane domains or modulation of the behavior of the ion permeable α subunit itself. *SCN2A* ($Na_V1.2\alpha$) was expressed in a single articular sample (ART2) and, along with *SCN3A* ($Na_V1.3\alpha$), were both expressed in 2 of the 3 control costal chondrocytes (CON8 and CON10) and one fetal sample each (F1 and F2 respectively). This is the first time that expression of these 6 genes has been shown in chondrocytes.

Potassium transport

Seven potassium transport genes are shown in Table 1. *KCNMA1* (KCa1.1 BKα subunit) is expressed at very high levels in all samples of chondrocytes, whereas *KCNN2* (KCa2.2) was expressed only in costal cartilage (CON8, 9, and 10, and F1. Table S1). Due to the nature of samples collected, articular samples come from older individuals whereas costal cartilages are from teenagers. A larger age-matched population will help answer questions arising due to age or site related changes in expression. Both genes have previously been described in chondrocytes involved in mechanical stretch and cell volume regulation.[41,46,37] *KCNJ15* (Kir4.2), *KCNAB2* (Kvβ2), and *KCNK1* (TWIK1) are also expressed in all samples. Kir4.2 has not been described in chondrocytes but has a role in

Table 1. Expression of 26 ion channel genes (ΔCq) observed in human costal and articular cartilage. Bolded values are genes expressed with Cq≤30 (n = number of exps). (CON8, CON9, and CON10 are 3 apparently normal teenage costal cartilage samples; ART1, ART2, and ART3 are 3 apparently normal samples of articular chondrocytes; and F1, F2, and F3 are 3 fetal samples of costal cartilage)

Gene	Name	CON8(3)	CON9(1)	CON10(1)	ART1(3)	ART2(1)	ART3(1)	F1(1)	F2(1)	F3(1)
KCNMA1	KCa1.1α (BKα)	**1.6E-01**	**1.9E-01**	**1.6E-01**	**1.3E-01**	**2.3E-01**	**2.3E-01**	**6.4E-02**	**5.0E-02**	**9.1E-02**
KCNJ2	Kir2.1	**1.3E-02**	**1.5E-02**	**2.0E-02**	**2.0E-03**	**2.4E-03**	**8.0E-04**	**1.5E-03**	**2.9E-03**	4.0E-04
KCNJ14	Kir2.4	**2.0E-03**	4.9E-04	3.4E-04	1.0E-03	**1.3E-03**	**2.2E-03**	**1.3E-03**	**2.3E-03**	**1.2E-03**
KCNJ15	Kir4.2	**1.6E-03**	**1.4E-03**	**8.7E-03**	**7.5E-03**	**1.5E-02**	**9.2E-03**	**5.3E-04**	**2.1E-03**	**1.8E-03**
KCNAB2	KV β2	**4.4E-03**	**8.5E-03**	**5.0E-03**	**4.4E-03**	**5.8E-03**	**5.3E-03**	**5.2E-03**	**4.6E-03**	**9.0E-03**
KCNB1	KV2.1	**3.1E-03**	2.2E-04	**2.9E-03**	**1.6E-03**	**2.4E-02**	**6.1E-03**	**8.9E-04**	1.1E-06	1.7E-04
KCNK1	TWIK1	**3.5E-02**	**3.2E-02**	**1.5E-02**	**1.8E-02**	**4.2E-02**	**3.7E-02**	**4.5E-02**	**1.9E-02**	**2.9E-02**
Gene	Name	CON8(3)	CON9(1)	CON10(1)	ART1(3)	ART2(1)	ART3(1)	F1(1)	F2(1)	F3(1)
CACNB1	CaV β1	**3.8E-03**	**3.3E-03**	**4.4E-03**	**3.5E-03**	**8.8E-03**	**1.5E-02**	**7.7E-03**	**6.3E-03**	**1.2E-02**
CACNB3	CaV β3	**7.4E-03**	**7.3E-03**	**5.0E-03**	**5.8E-03**	**6.4E-03**	**7.3E-03**	**6.7E-03**	**1.1E-02**	**6.7E-03**
CACNA1C	CaV1.2	**4.0E-03**	7.6E-04	**1.3E-03**	**1.6E-03**	**9.2E-03**	**4.4E-03**	**2.8E-03**	4.1E-04	**1.0E-03**
CACNA1A	CaV2.1	**4.3E-03**	**1.3E-03**	**2.8E-03**	**2.5E-03**	**8.9E-03**	**1.3E-02**	**2.4E-03**	**3.1E-03**	**1.8E-03**
RYR3	Ryanodine receptor 3	5.8E-04	**1.4E-03**	7.9E-04	6.3E-04	**1.4E-03**	**2.5E-03**	6.9E-04	**2.9E-03**	**1.5E-03**
Gene	Name	CON8(3)	CON9(1)	CON10(1)	ART1(3)	ART2(1)	ART3(1)	F1(1)	F2(1)	F3(1)
CLCN2	CLC2	**6.2E-04**	1.0E-03	5.0E-04	6.1E-04	**2.0E-03**	**2.4E-03**	**6.7E-04**	**2.9E-03**	**1.4E-03**
CLCN3	CLC3	**3.1E-02**	**4.4E-02**	**2.1E-02**	**2.6E-02**	**4.3E-02**	**4.9E-02**	**4.7E-02**	**5.1E-02**	**3.9E-02**
CLCN7	CLC7	**1.5E-02**	**3.1E-02**	**8.2E-03**	**3.7E-02**	**2.2E-02**	**1.8E-02**	**2.2E-02**	**1.6E-02**	**1.6E-02**
Gene	Name	CON8(3)	CON9(1)	CON10(1)	ART1(3)	ART2(1)	ART3(1)	F1(1)	F2(1)	F3(1)
SCN1B	NaV1.1β	**1.2E-02**	**1.7E-02**	**9.9E-03**	**4.1E-02**	**3.4E-02**	**3.4E-02**	**1.1E-02**	**8.4E-03**	**1.3E-02**
SCN2B	NaV1.2β	**5.0E-03**	**1.4E-03**	**1.3E-03**	**1.6E-03**	**3.8E-03**	**2.3E-03**	**1.2E-02**	7.9E-04	**1.6E-03**
SCN8A	NaV1.6α	**6.2E-03**	**3.5E-03**	**4.2E-03**	**8.6E-03**	**2.4E-02**	**3.8E-02**	**6.6E-03**	**5.6E-03**	**4.1E-03**
SCN9A	NaV1.7α	**2.8E-03**	9.8E-04	**5.1E-03**	1.7E-04	**8.6E-04**	2.8E-04	**9.4E-03**	**4.0E-03**	4.3E-04
Gene	Name	CON8(3)	CON9(1)	CON10(1)	ART1(3)	ART2(1)	ART3(1)	F1(1)	F2(1)	F3(1)
ACCN2	ASIC1	**4.3E-03**	**1.3E-03**	1.4E-03	**1.9E-03**	**5.0E-03**	**4.0E-03**	**3.1E-03**	**3.7E-03**	**7.0E-03**
HCN2	BCNG2	**2.1E-03**	**2.0E-03**	3.1E-04	**1.9E-03**	3.6E-04	**1.3E-03**	**1.1E-03**	2.2E-04	4.9E-04
BEST1	BEST1	**1.3E-03**	1.7E-04	**1.4E-03**	**2.0E-03**	**3.0E-03**	**6.8E-03**	**1.7E-03**	**4.4E-03**	**4.9E-03**
TRPC1	TRPC1	**1.4E-02**	**1.6E-02**	**9.7E-03**	**9.8E-03**	**2.3E-02**	**3.2E-02**	**1.2E-02**	**1.0E-02**	**1.5E-02**
TRPV1	TRPV1	**1.4E-03**	5.5E-04	**1.4E-03**	6.4E-04	**4.0E-03**	**4.8E-03**	**1.3E-03**	**2.8E-03**	**2.5E-03**
TRPV2	TRPV2	**2.2E-02**	**3.0E-02**	**2.4E-02**	**2.6E-02**	**6.2E-03**	**2.7E-02**	**1.0E-02**	**1.7E-02**	**1.2E-02**
TRPV3	TRPV3	**1.6E-03**	**1.5E-03**	5.3E-04	7.0E-04	**3.0E-03**	**2.1E-03**	3.0E-04	5.4E-04	**1.8E-03**
TRPV4	TRPV4	**5.1E-03**	**5.8E-03**	**3.0E-03**	**6.0E-03**	**6.2E-03**	**1.1E-02**	**2.0E-03**	**3.9E-03**	**3.7E-02**

insulin secretion in diabetes. Kvβ2 is a β-sub-unit that associates with active α-subunits forming a voltage-gated channel and whose function in chondrocytes is not known. TWIK1 has a role in modulating chondrocyte membrane potential.[17] *KCNJ2* (Kir2.1) is expressed in all samples except F3. This gene has not previously been described in chondrocytes and functions as an inwardly rectifying K$^+$ channel in other tissue types with likely functions in membrane potential stabilization. The voltage-gated potassium channel gene, *KCNB1* (K$_V$2.1) has previously shown to have a role in maintenance of membrane potential in mouse chondrocytes.[16] We observed expression in all articular samples as well as CON8, CON10, and F1. *KCNJ14* (Kir2.4), expressed in all fetal samples as well as CON8, ART2 and ART3, has not been previously described in chondrocytes.

Chloride transport

CLCN3 (CLC3) and *CLCN7* (CLC7) genes are both highly expressed in all types of chondrocytes examined (Table 1). Previous studies

have shown that both *CLCN3* and *CLCN7* are expressed in the human chondrosarcoma cell line OUMS-27,[30] and that it plays a role in cell volume regulation and cell death. *CLCN2* (CLC2) expression was observed in 2 of the 3 articular samples (ART2 and ART3), in all fetal costal cartilage samples, and in one of the costal cartilage samples (CON8), indicative of a temporal or site-specific role in chondrocyte development.

Non-selective ion channels

Eight non-selective ion channels were detected. The gene *BEST1*, showed high expression in all 3 articular and fetal samples but was only expressed in 1/3 teenage costal cartilages (CON8). In other tissue types (cardiomyocytes) its product functions as a chloride ion channel. *TRPC1, TRPV2,* and *TRPV4* are expressed in all samples (Table 1) and known to all be expressed in chondrocytes. *TRPC1* is a non-selective calcium permeant cation channel,[20] and *TRPV4* a Ca^{2+} permeable osmomechano-sensitive-TRP channel contributing to

early stages of hypo-osmotic stress.[21,52] *TRPV2* is activated at high temperatures (T $>59°C$), its role in chondrocyte biology is unknown. *HCN2* (BCNG2) is a hyperpolarization-activated channel whose activity is modulated by intracellular chloride ions and pH, where an acidic pH shifts the activation to more negative voltages. BCNG2 was expressed in 2 of the costal cartilage samples (CON8 and CON9), 2 of the articular cartilage samples (ART1 and ART3) and 1 fetal sample (F1). *ACCN2* (ASIC1) is an acid sensing channel, activated by a drop in extracellular pH and showed expression in all articular and fetal samples and 2/3 costal cartilage samples (CON8 and CON9). These are all conditions that chondrocytes are exposed to in cartilage and may be expected to have a role in their biology. *TRPV1* and *TRPV3* have been identified in chondrocytes. *TRPV1* show enhanced expression in freshly isolated chondrocytes from older patients with osteoarthritis.[20] We show expression in ART2, and ART3 for both *TRPV1* and *TRPV3*.

Expression results summarized from Table 1 are shown in Figure 1 and demonstrate the number and diversity of ion channels expressed in

FIGURE 1. Distribution of ion channel gene expression between articular, teenage costal, and fetal costal chondrocytes.
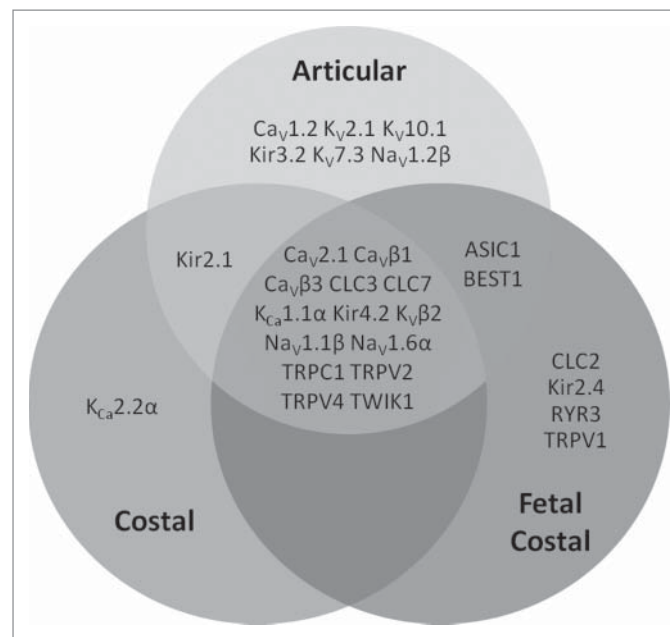
chondrocytes and how they are distributed. Expression of 14 genes is shared between all samples. One gene is uniquely expressed in teenage costal cartilage and 6 in articular cartilage, with one gene (*KCNJ2*/Kir2.1) shared between them. Four genes are uniquely expressed in fetal costal cartilage, with none shared between only fetal and teenage costal cartilage. Expression of *ACCN2*/ASIC1 and *BEST1* are shared between fetal and articular chondrocytes. During tissue development changes in gene expression may be expected. Comparing gene expression between fetal and teenage costal cartilage, of note was the 4-fold higher expression of *BEST1* in fetal costal cartilage and 12-fold higher expression of Kir2.1 in teenage cartilage. Articular and costal chondrocytes share expression of many membrane channel genes; however there are a number that are specific to either costal or articular chondrocytes that may be reflective of different functional properties of the tissues they were derived from. Taken together, there is a surprising number and diversity of expression in human chondrocytes where, however, channel function is largely unknown.

Gap junctions

RT-PCR from chondrocytes derived from 2 different human sources (normal teenage costal cartilage (CON8) and femoral head articular cartilage (ART1)), was performed on 17 connexin and 3 pannexin genes. All genes showed relative fold differences in expression lower than the mean of reference genes, however, 3 genes, *GJA1* (Cx43), *GJC1* (Cx45), and Panx1 showed much higher expression than all other genes (Table S2) in both samples suggestive of tissue specificity. Confirmation of only Cx43, Cx45, and Panx1 gene expression was determined in 2 additional costal cartilage and 2 additional articular cartilage samples (Table 2).

Ion channel and gap junction immunocytochemistry

Although it was not possible to confirm the presence of all expressed ion channel genes, we confirmed the presence of KCa1.1α expressed from gene *KCNMA1* (Fig. 2A) and Kv1.2 from gene *KCNA2* (Fig. 2B), but with little presence of Nav1.7 from gene *SCN9A* (Fig. 2C), albeit that it does show some expression in CON8. The gap junction protein Cx43 was also confirmed to be abundantly present (Fig. 2D). All immunocytochemistry was performed on CON8.

Determination of chondrocyte phenotype

Chondrocyte phenotype was determined by expression of aggrecan (*ACAN*) and *COMP*, genes normally expressed by differentiated chondrocytes. Both genes were expressed in all samples with relative ΔCq values given in Table S3 relative to 2 reference genes.

DISCUSSION

In this work we analyzed the expression of a panel of membrane channels from a range of cartilage phenotypes. We identify channels which are conservatively expressed across phenotypes and others which are differentially expressed. Several of the channels we identified have not previously been described/identified in cartilage. These channels belong to several different classes and families. Some groups of channels are expected based on

Table 2. Expression (ΔCq relative to 2 reference genes) of Cx43, Cx45, and Panx1 genes in human costal (CON8, 9, and 10) chondrocytes, articular (ART1, 2, and 3) chondrocytes, and fetal (F1, 2, and 3) costal chondrocytes. Bolding represents raw Cq values $\leq$30.

Gene	CON8	CON9	CON10	ART1	ART2	ART3	F1	F2	F3
Cx43	5.80E-02	1.40E-01	3.60E-02	1.60E-01	2.80E-01	2.10E-01	7.20E-02	4.80E-02	3.70E-02
Cx45	6.50E-03	5.80E-03	5.80E-03	1.00E-02	8.60E-03	8.00E-03	3.90E-03	5.30E-03	6.30E-03
Px1	7.10E-03	6.60E-03	3.30E-03	5.60E-03	7.00E-03	9.30E-03	3.90E-03	3.60E-03	2.20E-03

FIGURE 2. Immunocytochemistry detection of 4 membrane channel genes expressed in chondrocytes derived from CON8. Scale bar is 50 μm.
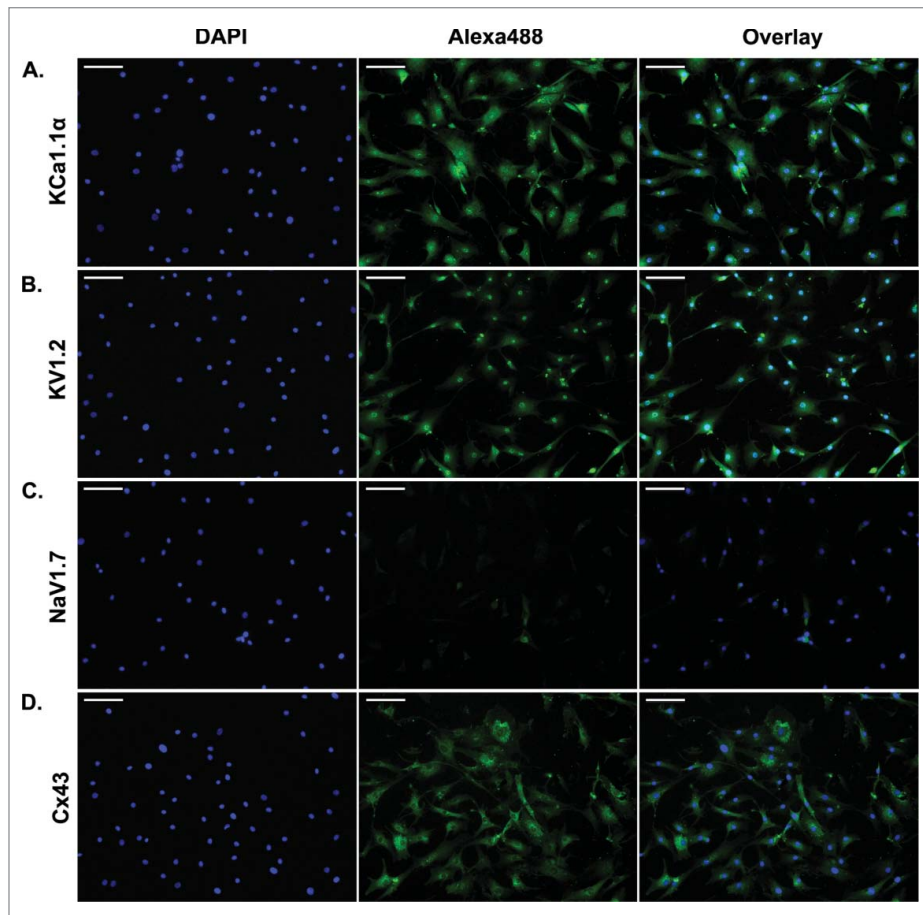
electrophysiological studies, but the specific members have not been identified and characterized. One such group is the potassium transport channels. There was preexisting evidence that members of the voltage-gated Kv and inwardly rectifying potassium channels (Kir) subgroups exist at the RNA and protein level.[45,16,64] However, our results show expression of 5 additional Kv and Kir channels (Kvβ2, Kv2.1, Kir2.1, Kir2.4, and Kir4.2). The function of these channels in chondrocytes is thought to be involved in differentiation, maintenance of the membrane potential, and regulation of calcium signaling. Previous reports[68,67,16,35,64] have shown that the resting membrane potential (RMP) of chondrocytes is significantly more depolarized than excitable tissues (-40 mV to -10 mV in chondrocytes compared to -60 to -95 mV in excitable cells.[24,23] This membrane potential is significantly more positive than the equilibrium potential for K$^+$ ions and therefore allows rapid efflux from the cells when channels open. The higher expression of Kir2.1 in teenage costal cartilage compared to fetal indicates an increasingly important role in membrane potential homeostasis in a tissue outside of its embryonic environment.

When cartilage is compressed, water is exuded and osmolality increases within the tissue leaving chondrocytes susceptible to shrinkage. Cellular homeostasis is maintained by entry of cations and water into the cells.[35] The two primary candidate proteins mediating this

cation influx are the Na^+, K^+, $2Cl(-)$ (NKCC1) co-transporter[55,28] and the epithelial sodium channel.[36] Under relaxation, water reenters cartilage and the osmolality decreases. Hypo-osmolality cause's chondrocytes to swell, however, the relatively positive RMP allows K^+ to leave the cell along with excess water, reducing swelling and maintaining homeostasis. Associated with any loss of cations there must be an equivalent loss of anions, such as chloride ions. Chloride channels are known to be involved in RMP maintenance and cell volume regulation in chondrocytes. The ion channels CLC1, CLC2, and CLC3 were thought to be present in chondrocytes based on electrophysiological and pharmacological studies,[62,26] with both CLC2 and CLC3, and additionally CLC4, CLC6, and CLC7, expression recently observed in human-derived chondrosarcoma cells.[30] Our own analysis confirms the expression of CLC3 and CLC7 in all cartilage types, while CLC2 and BEST1were expressed primarily in fetal costal and articular chondrocytes. When chloride channels are blocked using NPPB (5-nitro-2-(3-phenylpropyl-amino) benzoic acid) chicken mandibular mesenchymal stem cells (MSCs) undergoing chondrogenesis showed a decrease both in rate of proliferation and production of extracellular matrix proteins, including inhibition of terminal differentiation measured by collagen X expression.[63] This suggests further functional roles for chloride channels in chondrocyte biology.

Voltage-gated sodium channels comprise a family of membrane channel genes that are expressed in all of our chondrocyte samples. Previously, $Na_V1.4$ was found to be expressed in chondrogenic MSCs and present at the protein level.[64] However, due to the depolarized nature of chondrocytes, it is possible that they do not function as conventional sodium transporters. In contrast, the β-subunits of Na_V have established non-sodium transport functions including mechanotransduction, and cell adhesion.[11] In our analysis, we have identified expression of several Na_V α-subunits and 2 β-subunits including $Na_V1.6\alpha$ and $Na_V1.1\beta$ that are expressed in all samples and have not previously been described in chondrocytes.

Voltage-gated calcium channels (Ca_V) mediate calcium entry during depolarization in excitable cells. In mouse limb-bud chondrocytes, $Ca_V1.4$ is associated with $\beta1$-integrin suggesting involvement in mechanotransduction.[60] Our analysis showed expression of $Ca_V1.2$, $Ca_V2.1$, $Ca_V\beta1$, and $Ca_V\beta3$, though function of these genes remains unclear, we speculate they may have similar functions.

Gap junction proteins are expressed in a very tissue specific manner, undoubtedly due to functional requirements of any one tissue, and impairment of their function contributes to the pathophysiology of many disorders. Typically, 2–3 gap junction genes are expressed in any one tissue, although compensatory mechanisms exist for functional loss of a gap junction. We observed expression of 3 gap junction genes, *GJA1* (Cx43)*, GJC1* (Cx45), and Panx1, in both human primary costal and articular cartilage. Cx43 has been previously observed in the primary cilium of bovine chondrocytes and involved in ATP release in response to mechanical stimulation.[29] A study on ATP binding receptors in chondrocytes[56] indicates that P2X receptors act as ATP-gated ion channels that quickly increase intracellular calcium. Other studies show interactions between P2X receptors and pannexins, and a considerable body of evidence shows P2X receptors in neurons are important mediators of pain.[50] Cx43 and Cx45 genes identified in human articular cartilage have been suggested to be involved in intercellular signaling,[44] but ours is the first report of Panx1 gene expression. Expression of Cx43 and Cx45 is of interest because of the rectifying properties of Cx43:Cx45 heterotypic channels whereby Cx45 is able to modulate the fast gating properties of Cx43, possibly through docking induced conformational changes in Cx43.[19] In addition to Cx43 and Cx45, Blanco's group[44] also noted expression of the genes for Cx32 and Cx46. Cx32 has been strongly associated with the neurophysiological disorder Charcot-Marie-Tooth disease.[7] Interestingly, Cx46 is expressed in lens tissue, and like cartilage, does not have a blood supply and survives under hypoxic conditions. The promoter region of Cx46 is strongly influenced by hypoxia,[47] and there is a possibility that our observations

did not include Cx46 as our cells were not grown under hypoxic conditions. Of the 3 pannexin genes, *Panx3* has been shown to regulate intracellular ATP/cAMP and promote chondrocyte differentiation in mice.[27] *Panx3* is also expressed in hypertrophic chondrocytes during mouse long bone development, but expression is very strongly linked to osteogenic differentiation.[9] Pannexin 2 expression has been observed in, but not restricted to, the central nervous system[54] and has not been observed in chondrocytes. Pannexin 1 has been reported in many tissues including brain, testis, skeletal muscle and skin.[4] The expression of Panx1 here in cultured cells suggests a role for pannexin genes in chondrocyte differentiation. Pannexins, including Panx1, are mechanosensitive suggesting the presence of Panx1 related channel activity through biophysical stimuli.[59] Their presence could act as a growth regulator (modulating cell cycle progression) with differentiation tied in to growth regulation and mechanical stress.

Overall these results show remarkable consistency in expression of subsets of ion channel genes. The consistency is all the more remarkable because of the different sources and ages of chondrocytes, suggesting that these genes are important in general chondrocyte biology. The roles of each gene briefly described are known in other cell types, and may not have the same function in chondrocytes. There is expression of certain genes (Table 1) where variation may be accounted for by site, disorder, age, or individual differences, however larger numbers of samples need to be analyzed from these sub-groups to confirm this.

Gap junction proteins are crucial mediators of cell communication during embryogenesis, tissue regeneration, and disease.[53] Additionally, an important role of ion channel-driven resting membrane potential is tissue patterning.[38] Mesenchymal stem cells express a variety of ion channels involved in numerous cellular processes, and their use for cell replacement therapy and tissue engineering has gained considerable interest[70,71] suggesting that multiple functional ion transporters may be necessary for regenerative pathways in cartilage that are presently understudied.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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