Downregulation of aquaporin 9 decreases catabolic factor expression through nuclear factor-κB signaling in chondrocytes

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Abstract. Aquaporins (AQPs) are small integral membrane proteins that are essential for water transport across membranes. AQP9, one of the 13 mammalian AQPs (including AQP0 to 12), has been reported to be highly expressed in hydrarthrosis and synovitis patients. Given that several studies have identified signal transduction as an additional function of AQPs, it is hypothesized that AQP9 may modulate inflammatory signal transduction in chondrocytes. Therefore, the present study used a model of interleukin (IL)-1β-induced inflammation to determine the mechanisms associated with AQP9 functions in chondrocytes. Osteoarthritis (OA) and normal cartilage samples were subjected to immunohistological analysis. In addition, matrix metalloproteinase (MMP)3, MMP13 and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) mRNA and protein analysis was conducted in normal human articular chondrocytes from the knee (NHAC-Kn) stimulated with IL-1β by reverse transcription-polymerase chain reaction (RT-qPCR) and western blotting, respectively. AQP9 knockdown was also performed by transfection of *AQP9*-specific small interfering RNA using Lipofectamine. *AQP1*, *3*, *7*, *9* and *11* mRNA expression levels were detected in OA human chondrocytes and in IL-1β-treated normal human chondrocytes. The levels of *AQP9*, *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA were increased by treatment with 10 ng/ml IL-1β in a time-dependent manner, while knockdown of AQP9 expression significantly decreased the mRNA levels of the *MMP3*, *MMP13* and *ADAMTS-5* genes, as well as the phosphorylation of IκB kinase (IKK). Treatment with a specific IKK inhibitor also significantly decreased the expression levels of *MMP-3*, *MMP-13* and *ADAMTS-5* in response to IL-1β stimulation. Furthermore, immunohistochemical analysis demonstrated that AQP9 and inflammatory markers were highly expressed in OA cartilage. In addition, the downregulation of AQP9 in cultured chondrocytes decreased the catabolic gene expression in response to IL-1β stimulation through nuclear factor-κB signaling. Therefore, AQP9 may be a promising target for the treatment of OA.

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Introduction

Osteoarthritis (OA) is characterized by the progressive destruction and loss of the matrix of articular cartilage due to an imbalance between the anabolic and catabolic activities of chondrocytes (1). Several studies have reported that inflammation serves an important role in OA progression (2-5), and it is widely accepted that the activation of inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6, is critical in this process (6). OA patients display elevated IL-1β levels in the synovial fluid, synovial membrane, cartilage and subchondral bone layer (7). Similar to other inflammatory cytokines, IL-1β blocks the production of cartilage structural proteins, including type II collagen and aggrecan (8,9). Furthermore, IL-1β was reported to upregulate the production of matrix metalloproteinases (MMPs) in chondrocytes, specifically MMP-13, leading to increased destruction of cartilage components (10). A later study revealed that IL-1β stimulation of human chondrocytes significantly upregulated the mRNA levels of both *MMP-13* and a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS-5*) (11), which have been identified as the major enzymes responsible for cartilage degradation during OA progression (12,13).

Aquaporins (AQPs) are small integral membrane proteins that constitute a family with 13 members thus far identified in mammals (including AQP0 to 12), and are essential for water transport across membranes (14). Certain AQPs are also known as aquaglyceroporins since they participate in the transport of other small molecules, such as glycerol, urea or ammonia. AQP9, which was first identified in leukocytes (14), is an aquaglyceroporin that is expressed in numerous organs, with high expression levels detected in the liver, epididymis, testis, spleen and brain (15). There have been several studies on the functions of AQPs in certain organs. Early death has been observed in AQP11 knockout (KO) mice due to malfunction of

the kidney (16), while renal dysfunction has also been reported in AQP1, 3 and 7 KO mice (17,18).

In humans, AQPs have been implicated in several inflammatory diseases. For instance, overexpression of AQP1 has been detected in autoimmune and alcoholic pancreatitis (19), Alzheimer disease (20), and cases of rheumatoid and psoriatic arthritis (21). AQP9 has also been connected to a number of diseases (22). Rojek *et al* (22) demonstrated that AQP9 is important for hepatic glycerol metabolism and suggested that it may serve a role in glycerol and glucose metabolism in diabetes mellitus. More recently, Spegel *et al* (23) confirmed the importance of AQP9 in maintaining appropriate blood glucose levels. Furthermore, AQP9 downregulation was reported to result in glaucomatous optic neuropathy (24).

Notably, several studies have demonstrated that AQPs also participate in signal transduction (25-27). Specifically, AQPs are implicated in the phagocytic functions and the activation and migration of immune cells (25,26), as well as in proinflammatory cytokine secretion during inflammation (27). Based on these previous findings on the signal transduction function of AQPs, as well as the reported high expression of AQP9 in patients with hydrarthrosis and synovitis (28), it can be hypothesized that AQP9 may alter the inflammatory signal transduction in chondrocytes and that it may serve an important role in OA progression. Therefore, focusing on IL-1β-induced inflammation, the present study attempted to determine the mechanisms associated with AQP9 functions in chondrocytes.

Materials and methods

Cell culture. Normal human articular chondrocytes isolated from the knee (NHAC-kn; Lonza Group, Ltd., Walkersville, MD, USA) were cultured in 15 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (BioWhittaker; Lonza Group, Ltd., Basel, Switzerland) and 100 units/ml of penicillin-streptomycin at 37˚C in a 5% CO_2 atmosphere. Cells at passages 3-5 were used in the experiments of the present study and considered as 'normal human chondrocytes'.

Human cartilage samples. OA cartilage samples were obtained from the femoral condyle of patients undergoing total knee arthroplasty for primary OA (n=5). Normal cartilage samples were also obtained from patients with femoral neck fractures who were undergoing femoral head replacement surgeries (n=5). The mean age of the OA patients was 77.2 years, while the average age of patients with femoral neck fractures was 83.9 years; however, there was no significant difference in the age between the two groups (Table I). All samples were obtained in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (29).

Normal and OA cartilage samples were fixed in 4% paraformaldehyde, decalcified, embedded in paraffin wax and cut into 10-μm slices. Cartilage destruction was evaluated by safranin O (cat. no. S0145; Tokyo Chemical Industry, Tokyo, Japan) and fastgreen (cat. no. 10720; Chroma-Gesellschaft; Thermo Fisher Scientific, Inc.) staining. Histological evaluation was performed using the grading system described by Mankin *et al* (30).

Table I. Clinical characteristics of samples.

Parameter	Total	Osteoarthritis	Femoral neck fracture
Number of patients	10	5 (50%)	5 (50%)
Mean age (years)	80.2±5.6	77.2±6.1	83.9±4.8
Sex			
Male	2	1	1
Female	8	4	4

Detection of AQP expression by standard reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was conducted to detect the expression of *AQP* mRNA in human chondrocytes (n=5). Briefly, chondrocytes were plated into 6-well plates at a density of $1.0x10^5$ cells/cm^2 and cultured at 37˚C for 24 h. Cells were treated with 10 ng/ml recombinant human IL-1β (R&D Systems, Inc., Minneapolis, MN, USA) for 24 h, followed by RNA extraction using a QIAshredder and RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, 1 μg of total RNA was reverse-transcribed into first-strand cDNA using 1.25 μM oligo-dT primers in 40 μl PCR buffer II containing 2.5 mM $MgCl_2$, 0.5 mM dNTP Mix, 0.5 units RNase inhibitor, and 1.25 units MuLV reverse transcriptase (PerkinElmer, Inc., Foster City, CA, USA) at 42˚C for 60 min. Subsequently, the cDNA solutions were used in PCR analysis for the detection of human *AQP1-12* expression. The cDNA amplification was performed under the following PCR conditions: 94˚C for 5 min; followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec; and a final extension at 72˚C for 2 min using AmpliTaq Gold DNA Polymerase (cat. no. N8080241; Thermo Fisher Scientific, Inc.) and specific primers (Table II). The PCR products were electrophoresed on 3% agarose gels at 100 V for 30 min, and the gels were visualized under ultraviolet transillumination following the electrophoresis. The primer sequences used for the detection of human *AQP1-12* are presented in Table I. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene.

Immunohistochemical assay. Deparaffinized sections were digested with proteinase K (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 10 min and then treated with 3% hydrogen peroxide (Wako Pure Chemical Industries, Inc., Osaka, Japan) to block any endogenous peroxidase activity. Next, the sections were incubated overnight at 4˚C with the following primary antibodies at a 1:50 dilution: Anti-IL-1β (sc-7884) and anti-AQP9 (sc-74409) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), anti-phosphorylated-IκB kinase (p-IKK) (#2697) was from Cell Signaling Technology (Danvers, MA, USA), while anti-MMP-3 (ab53015), anti-MMP-13 (ab39012) and anti-ADAMTS-5 (ab41037) antibodies were obtained from Abcam (Cambridge, UK). Sections were subsequently incubated with horseradish

Table II. Specific primers for reverse transcription-polymerase chain reaction amplifications.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
GAPDH	GTTCGACAGTCAGCCGCATC	GGAATTTGCATGGGTGGA
AQP1	TGGACACCTCCTGGCTATTG	GGGCCAGGATGAAGTCGTAG
AQP2	CACCCCTGCTCTCTCCATA	GAAGACCCAGTGGTCATCAAAT
AQP3	GCTGTATTATGATGCAATCTGGC	TAAGGGAGGCTGTGCCTATG
AQP4	GAAGGCATGAGTGACAGACC	ATTCCGCTGTGACTGCTTTC
AQP5	GCCACCTTGTCGGAATCTAC	TAAAGCATGGCAGCCAGGAC
AQP6	CACCTCATTGGGATCCACTT	GTTGTAGATCAGTGAGGCCA
AQP7	ATCTCTGGAGCCCACATGAA	GAAGGAGCCCAGGAACTG
AQP8	GTGCCTGTCGGTCATTGAG	CAGGGTTGAAGTGTCCACC
AQP9	TCTCTGAGTTCTTGGGCACG	GGTTGATGTGACCACCAGAG
AQP10	GATAGCCATCTACGTGGGTG	CACAGAAAGCAGACAGCAAC
AQP11	TCCGAACCAAGCTTCGTATC	TAGCGAAAGTGCCAAAGCTG
AQP12	ACTTGTTCTTCTGGCCGTAG	CTTACTGGAGTACGTGCAGG
MMP-3	ATTCCATGGAGCCAGGCTTTC	CATTTGGGTCAAACTCCAACTGTG
MMP-13	TGCTGCATTCTCCTTCAGGA	ATGCATCCAGGGGTCCTGGC
ADAMTS5	TATGACAAGTGCGGACTATG	TTCAGGGCTAAATAGGCAGT

AQP, aquaporin; MMP, matrix metalloproteinase; ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs 5.

peroxidase-labeled mouse anti-rabbit immunoglobulin G (Histofine Simple Stain MAX PO (R); Nichirei Biosciences, Inc., Tokyo, Japan) at room temperature for 60 min. The peroxidase substrate 3,3'-diaminobenzidine (Histofine Simple Stain DAB Solution; Nichirei Biosciences, Inc.) was added and the peroxidase activity was detected by the production of a brown reaction product. The sections were counterstained with hematoxylin and then examined microscopically. Stained cells were independently counted by three blinded observers in three areas of high magnification fields, at both the superficial and deep zones of the cartilage tissues.

Reverse transcription-quantitative (q)PCR analysis. Chondrocytes (n=5) were plated into 6-well plates at a density of $1.0x10^5$ cells/cm^2, cultured at 37˚C for 24 h and then transfected with the following: Non-specific small interfering RNA (siRNA), serving as the negative control (NC) siRNA (cat. no. AM4613; Thermo Fisher Scientific, Inc.); 10 nM AQP9-1 specific siRNA (siAQP9-1; cat. no. 4392420; Invitrogen; Thermo Fisher Scientific, Inc.); or 10 nM AQP9-2 specific siRNA (siAQP9-2; cat. no. 4392422; Invitrogen; Thermo Fisher Scientific, Inc.). Transfection was performed for 24 h using the Lipofectamine RMAiMAX transfection reagent (cat. no. 13778150; Invitrogen; Thermo Fisher Scientific, Inc.). A control group which was treated only with DMEM medium was used. Subsequent to changing medium, 10 ng/ml recombinant human IL-1β was added for 24-h incubation. As previously reported, anti-inflammatory drugs can affect OA progression (31). Thus, a group of cultures was subjected to 30-min pretreatment with 10 μM BMS-345541 (cat. no. 095M4739V; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), a p-IKK inhibitor, prior to the addition of IL-1β (32).

RNA extraction and RT were performed as described earlier in this study. The relative mRNA levels of *AQP9*, *MMP-3*, *MMP-13* and *ADAMTS-5* were analyzed by SYBR Green Real-Time PCR Master Mix on an ABI Prism 7500 sequence-detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The results were quantified using the quantification cycle (Cq) method (33), with *GAPDH* serving as an internal control gene. The difference between the mean Cq values of the gene of interest and the internal control gene was denoted as ΔCq, and the difference between ΔCq and the Cq value of the calibrated sample was denoted as ΔΔCq. The $\log_2(\Delta\Delta Cq)$ provides the relative value of gene expression. Primer sequences used for the detection of the aforementioned genes are presented in Table II.

Western blot analysis. Chondrocytes (n=5) were plated into 6-well plates at a density of $1.0x10^5$ cells/cm^2, cultured at 37˚C, and transfected with non-specific siRNA or AQP9-specific siRNA (10 nM) for 24 h using the Lipofectamine RMAiMAX transfection reagent. Following, incubation with fresh medium for another 24 h at 37˚C, chondrocytes were cultured with or without 10 ng/ml recombinant human IL-1β for 30 min, washed with Tris-buffered saline with Tween-20 (TBST) and then lysed in a buffer containing 25 mM Tris, 1% Nonidet P-40, 150 mM NaCl, 1.5 mM EGTA and a protease/phosphatase inhibitor mix (Roche Diagnostics, Basel, Switzerland). Lysates were centrifuged at 4˚C at 15,000 x g for 10 min to remove cellular debris. Next, the supernatants were collected, mixed with 4X electrophoresis sample buffer, electrophoresed on a 7.5-15% SDS-polyacrylamide gradient gel (Biocraft, Tokyo, Japan) and electrically transferred onto a polyvinylidene difluoride blotting membrane (GE Healthcare Life

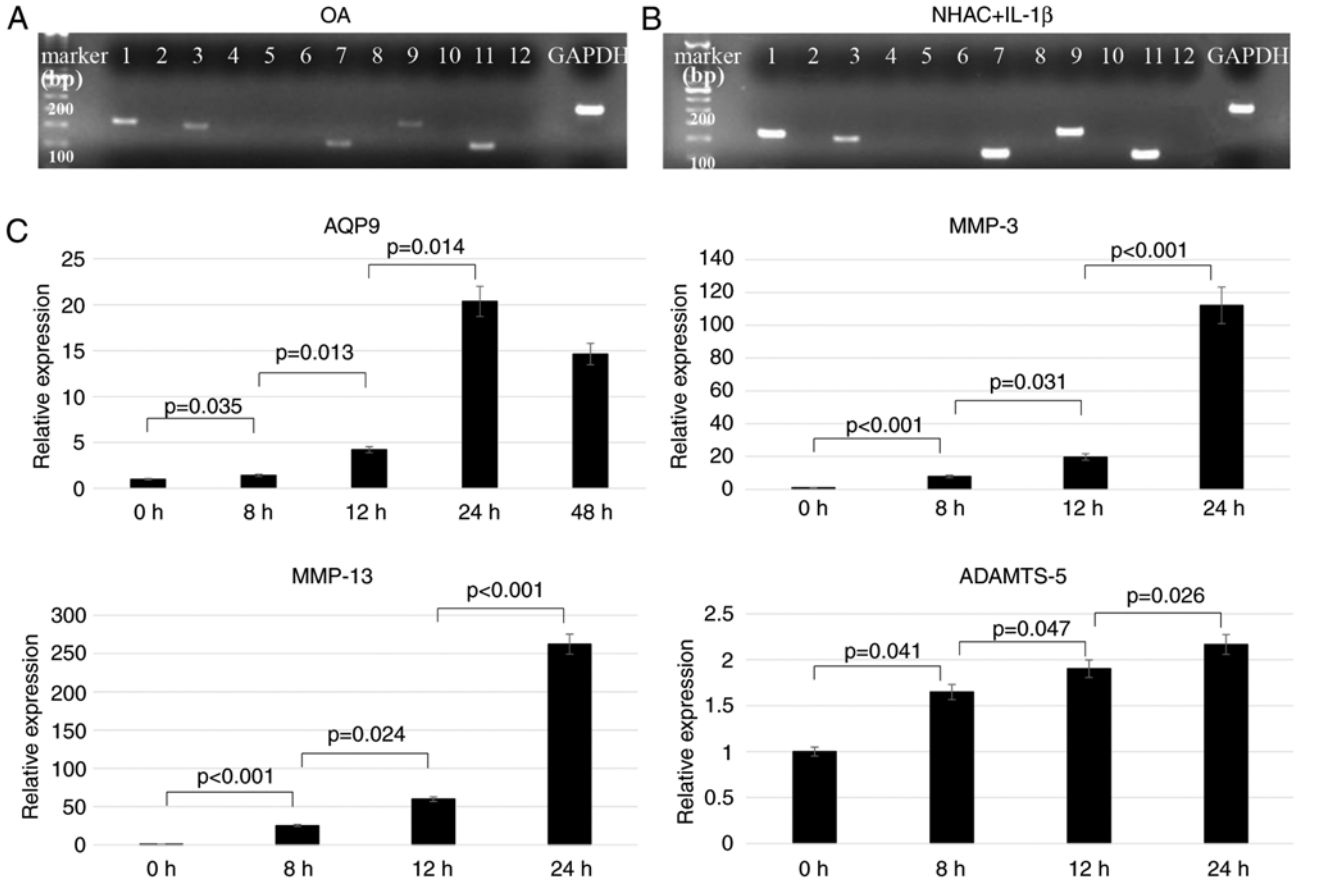

Figure 1. Expression of different AQPs and catabolic genes in chondrocytes. Agarose gel electrophoresis of PCR products obtained from (A) human osteoarthritis chondrocytes and (B) IL-1β-treated normal human chondrocytes, using RT-PCR and primers specific for each of the 12 human AQPs, namely *AQP1-12*. (C) Chondrocytes were cultured with IL-1β for 8, 12, 24 or 48 h, and the levels of *AQP9*, *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA were quantified by qPCR (n=5). AQP, aquaporin; MMP, matrix metalloproteinase; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; IL-1β, interleukin-1β; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Sciences, Little Chalfont, UK). Membranes were blocked with 5% skimmed milk in TBST for 30 min at 25˚C. Membranes were incubated with antibodies against p38 mitogen-activated protein kinase (MAPK) (#9212), phosphorylated (p)-p38 MAPK (#9211), extracellular signal-regulated kinase (ERK) (#4695), p-ERK (#4370), c-Jun N-terminal kinase (JNK) (#9252), p-JNK (#9251), IKK (#2682) or p-IKK (#2697), all of which were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody was used as a secondary antibody, and proteins were subsequently visualized using the ECL Plus reagent (GE Healthcare Life Sciences) in a Chemilumino Analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan). Protein expression was determined by semiquantification of digitally captured images using the National Institutes of Health ImageJ software (http://imagej.nih.gov/ij/).

Ethics. The study protocol was approved by the Ethics Committee of Kobe University Graduate School of Medicine (Kobe, Japan) on March 19th, 2015 (no. 1721), and all patients provided informed consent prior to participation.

Statistical analysis. Data are expressed as the mean ± standard deviation. A three-way analysis of variance with the Tukey-Kramer post-hoc test was used to assess differences in experimental groups. The Mann-Whitney U test was used to perform comparisons between groups. A value of $P<0.05$ was considered to indicate a difference that was statistically significant. Statistical analyses were performed using BellCurve a software (Social Survey Research Information Co., Ltd., Tokyo, Japan) add-on for Excel (Microsoft Corporation, Redmond, WA, USA).

Results

AQP1, 3, 7, 9 and 11 are expressed in human chondrocytes. Electrophoresis analysis with PCR products demonstrated that *AQP1, 3, 7, 9* and *11* were expressed in chondrocytes obtained from OA patients (Fig. 1A) and in IL-1β-treated normal human chondrocytes (Fig. 1B). By contrast, non-stimulated normal human chondrocytes did not express any of the *AQP* genes (data not shown). Therefore, all subsequent experiments were performed using normal human chondrocytes under IL-1β stimulation.

IL-1β increases AQP9 mRNA expression in chondrocytes. RT-qPCR demonstrated that the level of *AQP9* mRNA was significantly increased with time upon exposure to 10 ng/ml IL-1β. The peak *AQP9* mRNA expression occurred at 24 h after IL-1β treatment. Increases were also observed in the

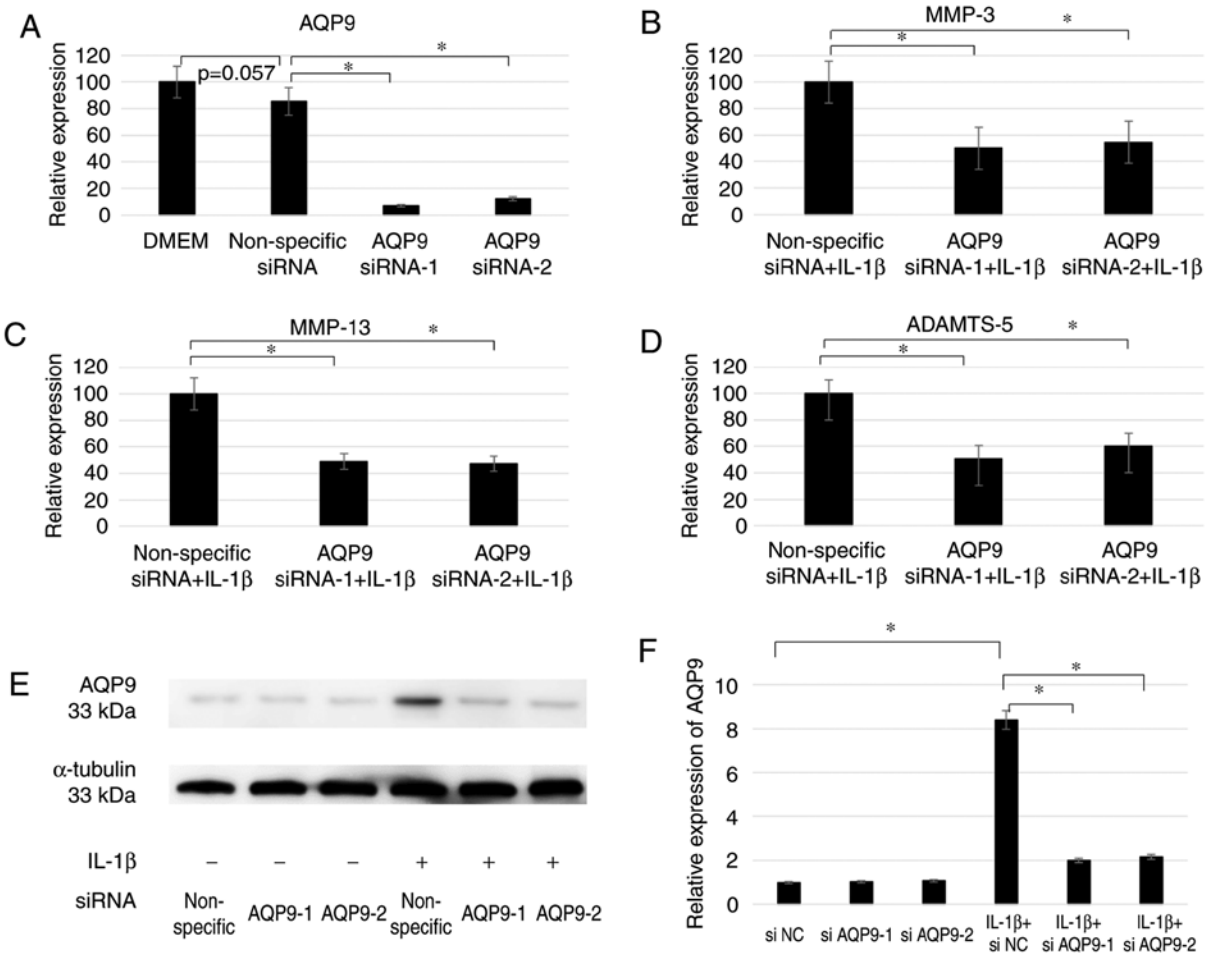

Figure 2. Effects of AQP9 knockdown on the mRNA levels of catabolic genes. Chondrocytes were transfected with two different *AQP9*-specific siRNAs or non-specific control siRNA, and then stimulated with IL-1β. Quantification of the mRNA levels of (A) *AQP9*, (B) *MMP-3*, (C) *MMP-13* and (D) *ADAMTS-5* was performed by quantitative polymerase chain reaction. Chondrocytes were transfected with two different AQP9-specific siRNAs for 12 h and then stimulated without or with IL-1β for 24 h. (E) AQP9 protein expression was assessed by western blotting, and (F) the protein expression levels were semiquantified. α-tubulin was used as a control to estimate the protein loading on the gel. *P<0.001 (n=5). AQP9, aquaporin 9; MMP, matrix metalloproteinase; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; IL-1β, interleukin-1β; siRNA, small interfering RNA.

mRNA levels of *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA levels (Fig. 1C).

Downregulation of AQP9 expression decreases catabolic gene expression in IL-1β-stimulated chondrocytes. To investigate the functions of AQP9 in chondrocytes, cells were transfected with two AQP9-specific siRNAs and then treated with IL-1β. RT-qPCR demonstrated that the knockdown was successfully achieved by siRNA transfection, as the level of *AQP9* mRNA in the siAQP9-1-transfected and siAQP9-2 transfected cells only amounted to 9.7 and 12.2%, respectively, of the corresponding level in the cells transfected with non-specific siRNA (Fig. 2A). The downregulation of AQP9 expression also resulted in decreased *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA levels (Fig. 2B-D). The level of *MMP-3* mRNA was significantly decreased to 49.9 and 54.5% using siAQP9-1 and siAQP9-2, respectively, under IL-1β stimulation (both P<0.001; Fig. 2B). In addition, the level of *MMP-13* mRNA was decreased to 48.9 and 47.2% (both P<0.005; Fig. 2C), and the level of *ADAMTS-5* mRNA was decreased to 50.5 and 60.1% (both P<0.005; Fig. 2D), following transfection with siAQP9-1 and siAQP9-2, respectively. These findings suggest that AQP9 positively regulates the expression levels of *MMP-3*, *MMP-13* and *ADAMTS-5*. Furthermore, since IL-1β was previously demonstrated to upregulate all four genes, it is likely that AQP9 mediates, at least partly, the ability of IL-1β to induce the expression of the other three genes. Western blotting confirmed that siAQP9-1 and siAQP9-2 significantly decreased AQP9 expression at the protein level in chondrocytes with IL-1β stimulation in comparison with the non-specific siRNA (Fig. 2E and F).

Downregulation of AQP9 expression increases IKK phosphorylation in IL-1β-stimulated chondrocytes. To investigate the role of AQP9 in signal transduction, the phosphorylation of ERK, p38 MAPK, JNK and IKK in AQP9-knockdown chondrocytes treated with 10 ng/ml IL-1β was assessed. The results confirmed the time-dependent change in phosphorylation following IL-1β stimulation, and the phosphorylation levels of ERK, p38 MAPK, JNK and IKK were markedly elevated at 30 min (Fig. 3). Therefore, the AQP9-knockdown (si AQP9-1) chondrocytes were treated with IL-1β for 30 min in subsequent experiments. Next, it was observed that the expression levels of ERK, p38 MAPK, JNK and IKK proteins were not altered

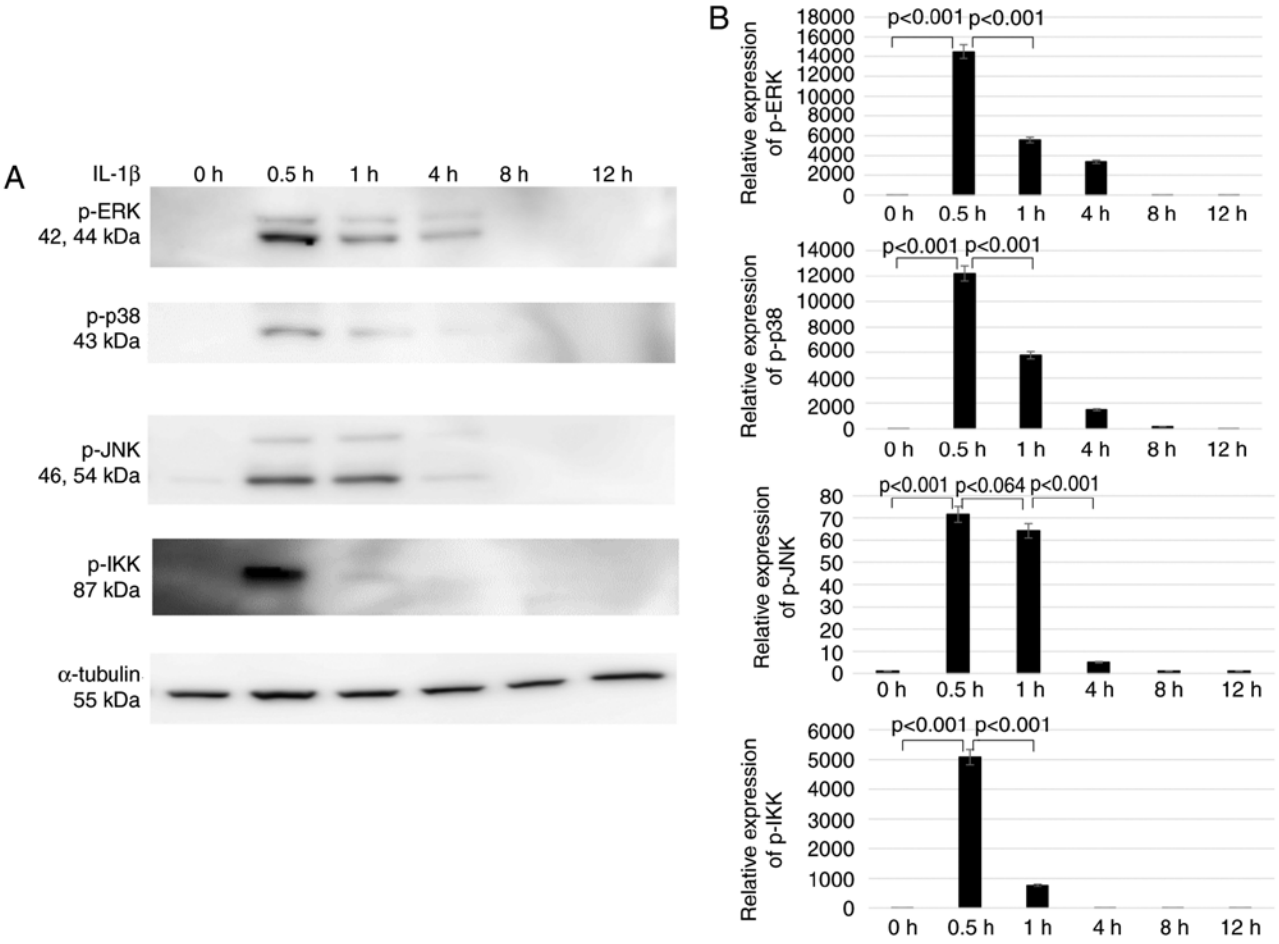

Figure 3. (A) Phosphorylated ERK, p38 MAPK, JNK and IKK protein expression levels were assessed by western blotting following IL-1β treatment for 0-12 h. (B) Protein expression levels were semiquantified (n=5). α-tubulin was used as a control to estimate the protein loading on the gel. IL-1β, interleukin-1β; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; p-, phosphorylated.

in comparison with those in cells treated with the non-specific siRNA control (Fig. 4). However, the AQP9-knockdown cells exhibited significantly lower IKK phosphorylation levels compared with those in the control cells treated with non-specific siRNA. This indicated that AQP9 positively regulates the phosphorylation of IKK, which is a kinase necessary for the activation of nuclear factor (NF)-κB. By contrast, the phosphorylation levels of ERK, p38 MAPK and JNK were similar between the knockdown and control cells (Fig. 4), indicating that AQP9 is not involved in the activation of their corresponding signaling pathways.

Inhibition of IKK phosphorylation decreases catabolic gene expression in chondrocytes. To examine the involvement of the NF-κB signaling pathway in the induction of MMP-3, MMP-13 and ADAMTS-5 expression by IL-1β treatment, the chondrocytes were subsequently treated with an inhibitor of IKK phosphorylation, BMS-345541. RT-qPCR demonstrated that, in cells treated with 10 ng/ml IL-1β for 24 h, the IKK inhibitor suppressed *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA levels in a concentration-dependent manner. More specifically, 10 μM BMS-345541 resulted in significantly lower *MMP-3* (0.19%; P<0.001), *MMP-13* (0.98%; P<0.001) and *ADAMTS-5* (24%; P=0.032) mRNA levels compared with those in cells treated only with IL-1β (Fig. 5).

AQP9 and inflammatory markers are highly expressed in OA cartilage. Subsequent to safranin O and fast green staining, human OA cartilage exhibited mid-zone excavation and lower proteoglycan content compared with the normal cartilage (Fig. 6A). Histological evaluation was performed using the histopathology grading system by Mankin *et al* (30), indicating that the average score of the OA cartilage was significantly higher compared with that of normal cartilage (Fig. 6B).

Immunohistological analysis demonstrated that IL-1β, AQP9 and p-IKK were highly expressed in the surface layer of OA cartilage (Fig. 6C-E). As shown in Fig. 6F, the numbers of IL-1β-, AQP9- and p-IKK-positive cells were significantly higher (P<0.001) in OA cartilage as compared with those in normal tissue. In OA and normal cells, the IL-1β-positive cell rates were 43.3 and 17.9%, respectively. Similarly, the AQP9-positive cell rates were 36 and 17.1%, respectively, while the p-IKK-positive cell rates were 38.8 and 7.14%, respectively.

Catabolic markers are highly expressed in OA cartilage. To confirm the expression of catabolic markers in human OA cartilage tissue, immunohistochemical detection of MMP-3, MMP-13 and ADAMTS-5 was then performed. The immunohistological analysis revealed that all three proteins were highly expressed in the surface layer of OA cartilage (Fig. 7A-C). As shown in Fig. 7D, the numbers of MMP-3-, MMP-13- and

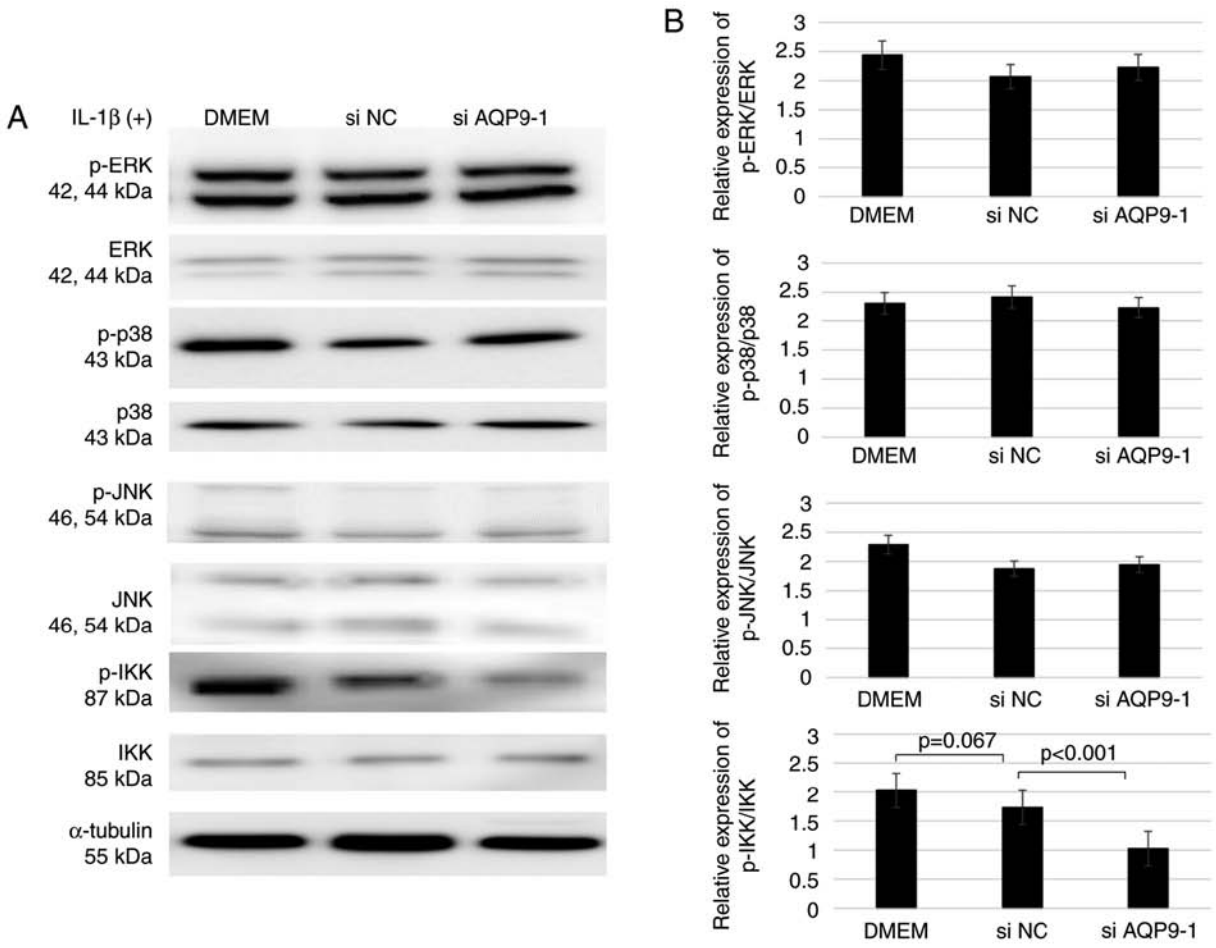

Figure 4. Levels of p-ERK, ERK, p-p38 MAPK, p38 MAPK, p-JNK, JNK, p-IKK and IKK following IL-1β stimulation of chondrocytes transfected with siAQP9 or non-specific siNC, as determined by western blotting. (A) Western blots and (B) semiquantified results of protein expression levels are shown (n=5). Each full length protein of ERK, p38MAPK, JNK and IKK was used as the control to estimate the protein loading on the gel. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; p-, phosphorylated; IL-1β, interleukin-1β; AQP9, aquaporin 9; si, small interfering RNA; NC, negative control; DMEM, Dulbecco's modified Eagle's medium.

ADAMTS-5-positive cells were significantly higher (P<0.001) in OA cartilage compared with those in normal tissue. The MMP-3-positive cell rates were 40.4 and 16% in OA and normal cells, respectively, the MMP-13-positive cell rates were 41.9 and 12.5%, and the ADAMTS-5-positive cell rates were 39.7 and 13.3%.

Discussion

The current study demonstrated that AQP9 and inflammatory markers are highly expressed in OA cartilage tissue, and that downregulation of AQP9 expression in normal human chondrocytes reduced the IL-1β-induced expression of catabolic genes. Matsushima *et al* (34) reported elevated AQP9 expression levels in systemic inflammatory response syndrome patients and suggested that AQP9 may be associated with F-actin polymerization, which would implicate it in the morphologic and functional changes observed in this syndrome. In addition, Mesko *et al* (35) reported that changes in AQP9 expression are universal markers of chronic inflammation and may be one of the causes of this condition in autoimmune disease patients. Nagahara *et al* (28) also reported the presence of *AQP9* mRNA in synovial tissues from patients with OA and rheumatoid arthritis. The present study suggested that the elevated expression levels of AQP9 in OA joints may increase the expression of catabolic factors, such as MMPs and ADAMTSs. It is likely that AQP9 mediates, at least partly, the IL-1β-induced expression of MMP-3, MMP-13 and ADAMTS-5. Furthermore, the current findings indicate that AQP9 positively regulates the phosphorylation of IKK, which is known to activate NF-κB. Based on these results, it can be hypothesized that IL-1β first upregulates AQP9, which then induces the expression levels of MMP-3, MMP-13 and ADAMTS-5 by activating the NF-κB cascade.

NF-κB is one of the transcription factors with a central role in immunoreaction and is known to be activated by various stimuli, including stress, cytokines and ultraviolet rays (36). It is involved in numerous physiological phenomena, such as acute and chronic inflammation, cell proliferation and apoptosis. Defective control of NF-κB activity leads to inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, asthma and chronic obstructive pulmonary disease (37,38), as well as cancer and septic shock (39). Recently, Wang *et al* (40) reported that knockdown of AQP9 expression attenuated brain edema through the inhibition of the NF-κB signaling pathway. To determine the mechanism through which

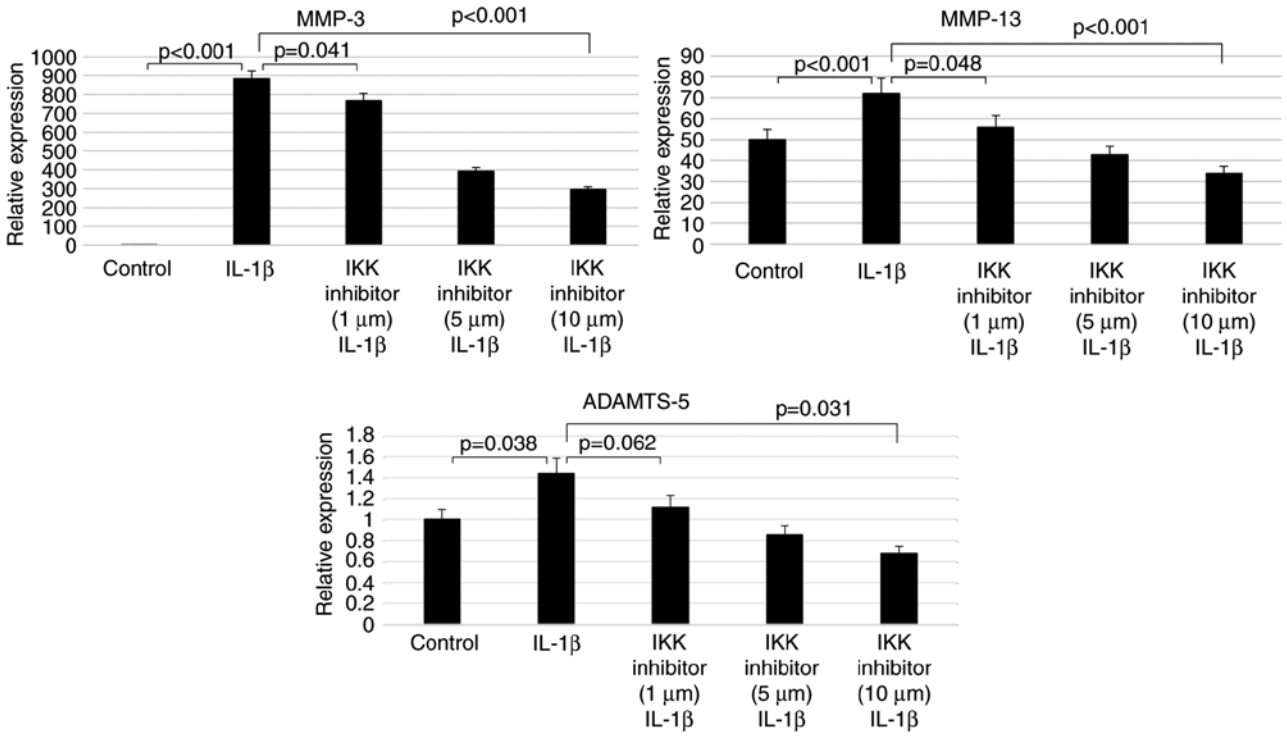

Figure 5. Levels of *MMP-3*, *MMP-13* and *ADAMTS-5* mRNA following IL-1β stimulation in the presence or absence of 1, 5 or 10 μM BMS-345541 (a IKK inhibitor) that was added 30 min before IL-1β, as quantified by quantitative polymerase chain reaction. Chondrocytes not treated with IL-1β treatment were used as controls (n=5). MMP, matrix metalloproteinase; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; IL-1β, interleukin-1β; IKK, IκB kinase.

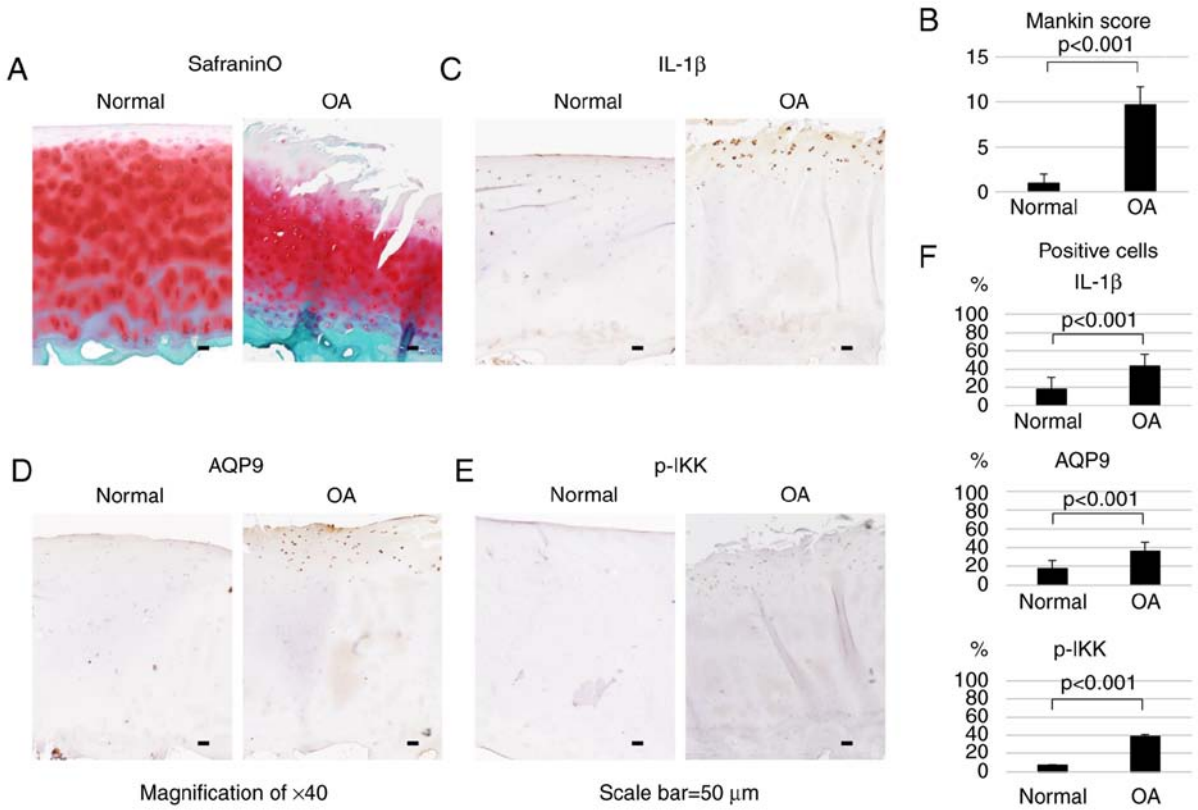

Figure 6. Immunohistological assessment of normal and OA human cartilage. (A) Normal and OA samples were stained with safranin O and then counterstained with fast green. In addition, sections were stained with (B) IL-1β, (C) AQP9 and (D) p-IKK antibodies, and counterstained with hematoxylin. (E) Histological evaluation based on Mankin grading. (F) Percentages of IL-1β-positive, AQP9-positive and p-IKK-positive cells. Normal cartilage samples were obtained from patients with femoral neck fractures who were undergoing femoral head replacement surgery. OA cartilage samples were obtained from the femoral condyle of patients undergoing total knee arthroplasty for primary OA (n=3). Magnification, x40. OA, osteoarthritis; IL-1β, interleukin-1β; AQP9, aquaporin 9; IKK, IκB kinase.

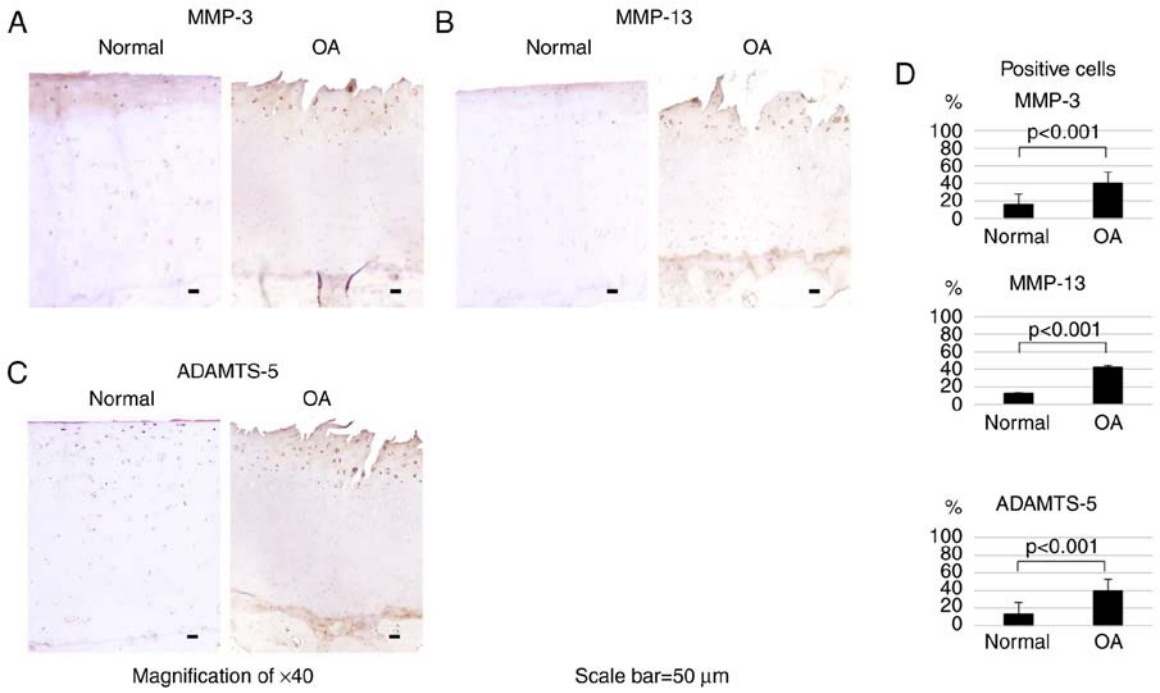

Figure 7. Expression levels of catabolic markers in normal and osteoarthritis human cartilage. Sections were stained for (A) MMP-3, (B) MMP-13 and (C) ADAMTS-5 antibodies, and counterstained with hematoxylin. (D) Percentages of MMP-3-, MMP-13-, and ADAMTS-5-positive cells (n=3). Magnification, x40. MMP, matrix metalloproteinase; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; IL-1β, interleukin-1β.

AQP9 affects catabolic gene expression in IL-1β-stimulated cells, the present study investigated various proteins associated with signaling, and the results revealed that the depletion of AQP9 significantly reduced the abundance of p-IKK. As the IKK complex serves a central role in regulating NF-κB activity, the current results suggest that AQP9 functions as a signal transduction regulator of NF-κB activity by positively regulating the phosphorylation of IKK, which allows NF-κB to mediate the ability of IL-1β to increase the levels of p-IKK and, in turn, induce the expression of MMP-3, MMP-13 and ADAMTS-5. This is supported by the observation that inhibition of IKK phosphorylation reduced MMP-3, MMP-13 and ADAMTS-5 expression in response to IL-1β stimulation. Notably, previous studies have reported that certain AQPs function as inflammatory signal potentiators, enhancing the release of inflammatory cytokines, such as IL-1β, in response to increased activation of NF-κB (27,41). Together, these data suggest that AQPs are involved in both triggering the inflammatory response and mediating its effects on catabolic gene expression.

However, several limitations of the current study require further elaboration. First, the correlation between the upregulation of AQP9 and the NF-κB signaling pathway needs clarification. Notably, it has been reported that inflammatory factors increase *AQP4* mRNA levels through the Toll-like receptor 4 (TLR4) signaling pathway in the cortex and astrocytes, which culminates in the activation of NF-κB, as is the case with all TLR pathways (42). Thus, there may be other factors linking AQP9 and NF-κB. Furthermore, AQP9 overexpression models to confirm the results of inflammation increase and *in vivo* experiments were not tested. In the future, these experiments will be performed to confirm the comprehensive functions of AQP9 in chondrocytes.

In conclusion, the data of the present study demonstrated that AQP9 and inflammatory markers are highly expressed in OA cartilage and that AQP9 downregulation results in decreased catabolic gene expression in response to IL-1β stimulation through NF-κB signaling. Thus, AQP9 may be an attractive target for the treatment of inflammatory joint diseases, such as OA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KT conceived the study, participated in its design, performed statistical analysis and data interpretation, and drafted the manuscript. SHay conceived the study, participated in its design and the acquisition of data, performed statistical analysis and data interpretation, and drafted the manuscript. TM and SHas participated in human sample collection and the drafting of the manuscript. MH, NC and SKih conceived the study and partici-

pated in its design and the acquisition of data. KT, SKir, YK and MT contributed to data acquisition. KN and RK participated in designing the study and helped revise the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Kobe University Graduate School of Medicine (Kobe, Japan) on March 19th, 2015 (no. 1721), and all patients provided informed consent prior to participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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