MicroRNA-558 regulates the expression of cyclooxygenase-2 and IL-1β-induced catabolic effects in human articular chondrocytes

S.J. Park, E.J. Cheon, H.A. Kim

Division of Rheumatology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Kyunggi, Republic of Korea

**SUMMARY**

Objective: Cyclooxygenase-2 (COX-2) is a major prostaglandin E2 (PGE2) synthetic enzyme and is involved in the pathogenesis of chronic inflammation and pain in osteoarthritis (OA). The objective of this study was to directly address whether microRNA (miR)-558 can control the interleukin (IL)-1β-mediated induction of COX-2 and catabolic effects in human articular chondrocytes.

Materials and methods: Total RNA was extracted from the cartilage tissues of normal and OA donors or cultured human articular chondrocytes. The expression of miR-558 was quantified by TaqMan assay. To investigate the repressive effect of miR-558 on COX-2 expression, human chondrocytes and chondrogenic SW1353 cells were transfected with mature miR-558 or an antisense inhibitor (anti-miR-558). The expression of COX-2 protein was determined by Western blot analysis and the involvement of miR-558 in IL-1β-induced catabolic effects was examined by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). Direct interaction between miR-558 and the putative site in the 3′-untranslated region (UTR) of COX-2 messenger RNA (mRNA) was validated by luciferase reporter assay.

Results: Normal human articular cartilage expressed miR-558, and its expression was significantly lower in OA cartilage. Stimulation with IL-1β led to a significant reduction in miR-558 expression in normal and OA chondrocytes. IL-1β-induced activation of MAP kinase (MAPK) and nuclear factor-kB (NF-kB) decreased miR-558 expression and induced COX-2 expression in chondrocytes. The overexpression of miR-558 directly suppressed the luciferase activity of a reporter construct containing the 3′-UTR of COX-2 mRNA and significantly inhibited IL-1β-induced upregulation of COX-2, while treatment with anti-miR-558 enhanced IL-1β-induced COX-2 expression and reporter activity in chondrocytes. Interestingly, IL-1β-induced activation of NF-kB and expression of matrix metalloproteinase (MMP)-1 and MMP-13 was significantly inhibited by miR-558 overexpression.

Conclusion: These findings demonstrated that cartilage homeostasis is influenced by miR-558, which directly targets COX-2 and regulates IL-1β-stimulated catabolic effects in human chondrocytes.

© 2013 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

**Introduction**

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by progressive destruction of articular cartilage, and pain resulting in disability. It is widely accepted that the development of OA is associated with excessive production of inflammatory cytokines such as interleukin (IL)-1β. Published evidence indicates that IL-1β has a pivotal role in cartilage matrix destruction via upregulation of the production of proteases, such as matrix metalloproteinase (MMPs), and downregulation of the synthesis of proteoglycan and collagen. It can also induce excessive cyclooxygenase-2 (COX-2) expression, leading to elevated production of prostaglandin E2 (PGE2). Although very low levels of PGE2 may have anabolic functions in cartilage, nanomolar to micromolar concentrations produced by OA tissues are predominantly catabolic, leading to an inhibition of proteoglycan synthesis, increased MMP-13 and increased type II collagen degradation. Thus, PGE2, which is found at high levels in synovial fluid in OA patients and OA animal models, is regarded as a possible therapeutic target in OA. Non-steroidal anti-inflammatory drugs (NSAIDs), the main function of which is to inhibit COX and to impair production of PGE2, have long been used to treat pain and inflammation associated with OA.
microRNAs (miRNAs) are endogenous non-coding RNAs containing 20–25 nucleotides that can regulate gene expression by binding the 3′-untranslated region (UTR) of their target messenger RNA (mRNA), leading to translational repression or mRNA cleavage. To date, many reports have demonstrated that miRNAs function in diverse biological processes, including cell differentiation, proliferation, and development. Alterations in miRNA expression are closely related to human diseases, such as heart disease and arthritis. Additionally, an important role of miRNA in cartilage development was demonstrated by a report examining cartilage-specific Dicer-null mice, which lack an essential component for the biogenesis of miRNA. Dicer-null plates showed greatly reduced chondrocyte proliferation and accelerated hypertrophic differentiation, leading to severe skeletal growth defects and premature death. Genetic modification of miR-140 influenced the development of OA in model animals, with miR-140 (-/-) mice showing proteoglycan loss and fibrosis of articular cartilage.

In a previous report, miR-558 was reported to be significantly downregulated by IL-1β in human OA chondrocytes. MiR-558 was first identified in human colorectal cells and subsequently found to be upregulated in irradiated fibroblasts and noted for targeting genes involved in cell cycle checkpoints and apoptosis. Using the miRNA target prediction algorithms, we found that miR-558 has the potential to regulate COX-2 expression. In this study, we found that miR-558 regulates COX-2 expression, suggesting its potential role in cartilage homeostasis. These findings indicate that reduced miR-558 expression in cartilage may contribute to the increased catabolic pathway characteristic of OA development and identify miR-558 as a promising new target for OA cartilage protection.

Materials and methods

Recombinant human IL-1β and antibodies against COX-2 and MMP-1 were purchased from R&D systems (Minneapolis, MN, USA). Antibodies against microsomal PGE synthase-1 (mPGES1) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Antibodies against phospho-JNK (p-JNK), p-p38, phospho-inhibitor of κB α (p-IκBα) and phospho-extracellular signal-regulated kinases (p-ERK) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MMP-13 and β-actin were obtained from Abcam (Cambridge, UK) and Sigma (St Louis, MO, USA), respectively. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059 [a Mitogen-activated protein kinase kinase (MEK)-1/2 inhibitor], SB203580 [a p38 MAP kinase (MAPK) inhibitor], SP600125 [a JNK inhibitor] and SN50 [a nuclear factor-κB (NF-κB) inhibitor] were purchased from Calbiochem (San Diego, CA, USA).

Patients

OA cartilage was obtained from knee joints at the time of total knee replacement surgery from patients (n = 20, mean ± standard deviation (SD) age 71.23 ± 7.10 years) diagnosed according to the criteria of the American College of Rheumatology. Normal cartilage was taken from patients with no previous history of OA or rheumatoid arthritis (RA) who underwent total hip replacement surgery due to fracture of the femoral neck (n = 20, mean ± SD age 72.12 ± 9.78 years). Normal cartilage was selected in areas with no surface irregularity while OA cartilage was selected in areas showing gross erosion. The mean modified Mankin grades from OA and normal cartilages were 7.5 ± 1.3 (median 8) and 1.9 ± 0.5 (median 1). The collection and use of human samples were reviewed and approved by the institutional review board of Hallym University Sacred Heart Hospital (Anyang, Korea).

Cell culture

To isolate primary human chondrocytes, cartilage was dissected and subjected to sequential digestion with pronase and collagenase in serum-free modified Eagle’s medium (DMEM, Life Technologies, Inc., MD) until the fragments were digested. First passage-human chondrocytes at 80% confluence were used for all experiments.

Human chondrogenic SW1353 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin. During the culture period, cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air and the medium was changed every 2–3 days.

Transfection of miRNAs

Human chondrocytes were transfected with the mature type of hsa-miR-558 (Dharmacon, Chicago, IL, USA; 5′-UGACUGCU-5′) or the antisense inhibitor (anti-miR-558, Dharmacon), at a concentration of 50 nM, using the calcium phosphate precipitation method. Forty-eight hours after transfection, cells were stimulated with IL-1β for the indicated time and used for further analysis. SW1353 cells were seeded in 12- or 24-well plates. The following day, cells were transfected with miR-558 or anti-miR-558 at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer’s instructions. For the following experiments, cells were used 48 h after transfection. Nonspecific control miR (miR-Control, Dharmacon) was used as a control for off-target effects.

Quantitative real-time polymerase chain reaction (PCR) analysis of COX-2 and miR-558 expression

Total cellular RNA was extracted from cultured human chondrocytes using TRIzol reagent (Invitrogen, CA, USA). miRNA was purified with the mirVana miRNA isolation kit, according to the manufacturer’s instructions (Ambion, Austin, TX, USA). For some studies, cartilage from normal and OA donors were milled to a powder in liquid nitrogen and total RNA and miRNA were prepared as described above. Expression levels of COX-2 mRNA were quantified using the SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA) using a LightCycler 2.0 Instrument (Roche Diagnostics, Indianapolis, IN, USA). GAPDH was used as a reference gene. Primer sequences were as follows: COX-2, forward 5′-TGG GAA GCC TTC TCT AAC CTC-3′ and reverse 5′-TGA TGA CAT CAA -3′; miR-558, forward 5′-TGC TGT TTT TTA CCA TAC CTA-3′ and reverse 5′-GGT CCA CGC ATG TGG GCC AT-3′. Expression of mature miRNA was quantified using TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA, USA). Purified miRNA was reverse transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) and miRNA-specific stem-loop RT primers (Applied Biosystems). Real-time PCR was performed using a StepOnePlus Real-time PCR System (Applied Biosystems) in a 10 μl PCR mixture containing 2 μl RT product, 5 μl TaqMan Universal PCR Master Mix, 0.2 μl TaqMan probe and 10 μl forward and reverse primers. RNU6B was used as an internal control for miRNA detection.

Immunohistochemical analysis

Formalin-fixed and paraffin-embedded cartilage specimens were sectioned and stained with Safranin O for histological
evaluation. For immunohistochemical staining of COX-2, the paraffin sections were dewaxed, rehydrated and treated with 0.05% trypsin/EDTA solution for 10 min at 37°C. Intrinsic peroxidase activity was blocked with 3% hydrogen peroxide and sections were incubated with 1.5% normal goat serum for 30 min and then with a 1:150 dilution of polyclonal antibody against COX-2 for 16 h at 4°C. The sections were rinsed and incubated sequentially with biotinylated secondary antibody for 30 min and Vectastain ABC reagent (Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature. All sections were developed with the ImmPACT 3′,3′-Diaminobenzidine (DAB) peroxidase substrate kit (Vector) and counterstained with Mayer’s hematoxylin. Rabbit immunoglobulin G (IgG) was used as the negative control.

**ELISA**

The cumulative production of PGE2 and MMP-13 in the culture supernatants was quantified using the PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) and proMMP-13 immunoassay kit (R&D Systems), respectively, according to the manufacturer’s instructions.

**Western blot analysis**

For protein extraction, culture supernatants were collected and cells were washed with cold PBS and lysed at 4°C in lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitors). Proteins were resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in TBST (Tris-buffered saline plus 0.1% Tween 20), membranes were incubated with primary antibodies against COX-2, mPGE2, MMP-1, p-p38, p-JNK, p-IκBα, p-ERK or β-actin. After washing, primary antibodies were detected with HRP-conjugated secondary anti-rabbit antibodies. The blots were developed using an ECL chemiluminescence kit (Santa Cruz).

**Luciferase constructs and reporter assay**

To create the wild-type COX-2 3′ UTR–Luc reporter plasmid (COX-2 3′ UTR), a fragment of the 3′UTR of the COX-2 gene, including the predicted miR-558-binding site, was PCR-amplified using the following primer sets: forward 5′-CTC GAG TCA AGC CTG CCT ACC TG C ATG CTG TTC C-3′ and reverse 5′-GCG GCC GGC CTA GCC TCT TTG CAT C-3′, and then cloned into the psiCHECK™-2 vector downstream of the firefly luciferase gene with Xhol and NotI. To produce a reporter plasmid with a mutated miR-558-binding site (COX-2 mut 3′ UTR), the 3′ UTR of COX-2 was amplified with the following primer set: forward 5′-GCA AGT TTG CAG GTA AAC CTG GAA GCA GCA CTG C-3′ and reverse 5′-GCA GTC CTG CCT GGA GCA GGA GCA GCA GCA CTG C-3′. The PCR mixture consisted of 0.7 μl of expanded long range enzyme mix (Roche, Mannheim, Germany), 10 μl of 5× expanded long range buffer, 100 ng of plasmid template, 100 nM of primers, 3 μl of DMSO and 2.5 μl of dNTPs (10 mM). PCR cycling conditions were as follows: 92°C for 30 s, 55°C for 1 min, 68°C for 10 min and a final extension at 68°C for 10 min. After PCR, 20 μl of the reaction was digested with DpnI at 37°C for 1 h and 10 μl was transformed into DH5 alpha Escherichia coli to prepare the mutant construct plasmids. All constructs were confirmed by sequencing (Cosmogenetech, Seoul, Korea). Cells were co-transfected with wild-type- or mutant type-COX-2 3′ UTR–Luc reporter plasmid and miR-Control or miR-558 using Lipofectamine PLUS™ reagent. Cell lysates were harvested 48 h after transfection and luciferase activity was assayed with the Dual-Glo Luciferase Assay system (Promega, Madison, WI, USA), according to the manufacturer’s instructions.

To assess modulation of IL-1β–driven NF-κB activation by miR-558, SW1353 cells were co-transfected with NF-κB luciferase reporter plasmid (pNF-κB-Luc, plasmid containing a NF-κB binding site; Stratagen, Grand Island, NY, USA) and internal control plasmid pTK-Renilla–luciferase reporter plasmid (Promega), miR-Control or miR-558 using Lipofectamine PLUS™ reagent. Forty-eight hours after transfection, cells were stimulated with IL-1β for 24 h and luciferase activity was analyzed by the above methods.

**Statistical analysis**

The data are expressed as means ± 95% confidence intervals (CIs). Statistical analysis was carried out using Kruskal–Wallis test for multiple comparison and P < 0.05 was considered to indicate statistical significance. Mann–Whitney U test or Wilcoxon signed-rank test were used for two group comparisons and Bonferroni P-values were used to account for multiple testing. All experiments were performed using samples from at least three different donors with duplicate or triplicate replication as indicated in the figure legends.

**Results**

**The expression of miR-558 and COX-2 in normal and OA cartilage**

To identify the miRNAs targeting the 3′UTR of COX-2 mRNA, we evaluated numerous miRNAs reported to be regulated by IL-1β in chondrocytes using miRNA target prediction algorithms, such as miRanda (http://www.microna.org), TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/). We found that among IL-1β–responsive miRNAs, miR-558 has the potential to regulate COX-2 expression. To assess the potential involvement of miR-558 in the OA process, we initially attempted to compare its expression level between normal and OA cartilage. As shown in Fig. 1A, miR-558 expression was significantly reduced in OA cartilage compared with normal cartilage. Conversely, the high expression of COX-2 mRNA and protein in OA cartilage compared with normal cartilage was confirmed by quantitative real-time PCR and immunohistochemical analysis, respectively [Fig. 1(B) and (C)].

**The reverse correlation between IL-1β–regulated COX-2 and miR-558 expression in normal and OA chondrocytes**

Next, we verified the regulation of miR-558 expression by IL-1β, a pivotal proinflammatory cytokine in OA cartilage. Monolayer cultured human chondrocytes were treated with IL-1β and the expressions of miR-558 and COX-2 were determined by quantitative real-time PCR and Western blot analysis, respectively. In normal chondrocytes, IL-1β stimulation resulted in a significant downregulation of miR-558 at 12 h. MiR-558 expression was repressed with IL-1β treatment starting at 1 ng/ml [Fig. 2(A)]. In OA chondrocytes, miR-558 expression was significantly repressed after 6 h with IL-1β stimulation and the suppression was significant at a 1 ng/ml concentration [Fig. 2(B)]. In contrast, COX-2 mRNA expression was increased by IL-1β stimulation in a dose-dependent manner in both cells [Fig. 2(C) and (D)]. Similarly, COX-2 protein expression was increased with IL-1β stimulation in time- and dose-dependent manners [Fig. 2(E) and (F)]. Therefore, these results suggest that abnormally reduced miR-558 expression in OA cartilage and in response to IL-1β appear to correlate with increased COX-2 expression. Faster upregulation of COX-2 mRNA than miR-558 downregulation in response to IL-1β also indicates that additional mediators are involved in the regulation of COX-2.
The roles of IL-1β signal pathways on the regulation of miR-558 and COX-2 expression in human chondrocytes

To investigate which IL-1β signaling pathways regulate the expression of miR-558 and COX-2, human chondrocytes were pretreated for 2 h with NF-κB inhibitor SN50 (5 μM) or various MAPK inhibitors, such as MEK-1/2 inhibitor PD98059 (10 μM), p38 MAPK inhibitor SB203580 (1 μM) and JNK inhibitor SP600125 (10 μM), and then stimulated with IL-1β for 6 or 24 h. In response to IL-1β stimulation, the expression of miR-558 was markedly decreased and pretreatment with MAPK inhibitors and NF-κB inhibitor attenuated the inhibitory effects of IL-1β on miR-558 expression [Fig. 3(A)]. In contrast, IL-1β stimulation resulted in a significant increase of COX-2 mRNA and protein expression and treatment with NF-κB and MAPK inhibitors suppressed IL-1β-induced COX-2 expression in human chondrocytes [Fig. 3(B) and (C)]. Consistent with the effect of COX-2 expression, IL-1β-induced PGE2 production was significantly inhibited by NF-κB and MAPK inhibitors [Fig. 3(D)]. These results suggest that IL-1β-stimulated activation of NF-κB and MAPK signaling pathways may be necessary for the suppressive effect of IL-1β on miR-558 expression in human chondrocytes.

Negative regulation of COX-2 and PGE2 production by miR-558 in human chondrocytes and SW1353 cells

To determine whether miR-558 regulates COX-2 protein levels, human OA chondrocytes and chondrogenic SW1353 cells were transfected with mature- or anti-miRNA specific for miR-
miR-558 targets the 3′UTR of COX-2 mRNA

To understand the molecular mechanisms that underlie miR-558-mediated regulation of COX-2 expression, we analyzed the sequences in the 3′-UTR of human COX-2 mRNA in detail. Bioinformatic predictions showed that the 3′-UTR of human COX-2 mRNA contains a potential miR-558-binding site with 7-mer seeds and this putative miR-558-binding site and its adjacent sequences are highly conserved among the vertebrates [Fig. 5(A)]. To determine if miR-558 inhibits COX-2 gene expression by binding to the predicted target site in the 3′-UTR of COX-2 mRNA, we generated a wild-type COX-2 3′UTR construct containing a putative miR-558-binding site using psiCHECK™-2 vector. Co-transfection of COX-2 3′UTR luciferase reporter plasmids with miR-558 resulted in a significant reduction of luciferase activity [Fig. 5(B)]. In contrast, transfection with anti-miR-558 enhanced the reporter activity of COX-2 3′UTR in SW1353 cells [Fig. 5(B)]. Next, to further confirm the direct interaction of miR-558 and COX-2 3′UTR, we generated a mutant reporter plasmid (COX-2 mut 3′UTR), in which the binding sequence in the 3′UTR of COX-2 was mutated. As anticipated, a mutation in the miR-558 binding sequence led to a complete abrogation of miR-558-mediated repression of COX-2 3′UTR reporter activity [Fig. 5(C)]. These data demonstrate that miR-558 reduced the luciferase activity by binding to COX-2 3′UTR and COX-2 is a bonafide target of miR-558.
Suppression of IL-1β-stimulated catabolic effects by miR-558 overexpression in human chondrocytes and SW1353 cells

We found miR-558 to be capable of repressing COX-2, which is involved in a variety of catabolic responses in OA, so we next evaluated the involvement of miR-558 in other IL-1β-induced effects in chondrocytes. For these studies, human chondrocytes and SW1353 cells were transfected with mature-miR-558 or control-miR and then stimulated with IL-1β for the indicated time periods. The regulatory effects of miR-558 on IL-1β-induced catabolic pathways were determined by Western blot analysis and luciferase reporter assay. Overexpression of miR-558 significantly inhibited the phosphorylation of IκBα in human chondrocytes and SW1353 cells [Fig. 6(A)]. Likewise, in the luciferase reporter assay, we confirmed that IL-1β-stimulated NF-κB activity was markedly suppressed by overexpression of miR-558 in SW1353 cells [Fig. 6(B)]. However, miR-558 overexpression did not affect IL-1β-induced activation of MAPK including JNK, p38 and ERK in both cells [Fig. 6(A)].

Next, the effects of miR-558 on IL-1β-induced production of MMPs, which are major proteases responsible for the degradation of extracellular matrix, mainly collagen, were investigated. Interestingly, miR-558 overexpression significantly reduced the expression of MMP-1 and MMP-13, while transfection with anti-miR-558 showed increases in the expression of MMP-1 and MMP-13 in IL-1β-stimulated human chondrocytes and SW1353 cells [Fig. 6(C) and (D)]. Altogether, our results suggest that miR-558 is an additional crucial regulator of cartilage homeostasis, which is perturbed by IL-1β in human chondrocytes.

Discussion

In this study, we investigated miRNA-mediated post-transcriptional control of COX-2 expression in human chondrocytes. We demonstrated that miR-558, a recently identified miRNA regulated by the potent proinflammatory cytokine IL-1β, selectively and directly regulates COX-2 expression by targeting the 3′UTR of COX-2 mRNA. Overexpression of miR-558 markedly suppressed IL-1β-induced COX-2 and PGE2 production, while inhibition of miR-558 function by anti-miR-558 enhanced IL-1β-induced COX-2 expression.

miRs, as small-molecule regulators of gene expression, are involved in diverse physiological and pathological processes, including OA and inflammation. Identification of miRNA with a functional role in cartilage homeostasis has been attempted using expression profiling and a gene network approach integrating miRNA, proteomic and clinical data.

Distinct miRNAs regulating catabolic pathways in chondrocytes include miR-9, miR-22, miR-27a and b regulating MMP-13, while miR-140 is closely related to enhanced production of COX-2 in OA cartilage. They also showed that reduction of miR-199a expression in OA cartilage and in response to IL-1β is closely related to enhanced production of COX-2 in OA cartilage.

Currently, there are a number of computational algorithms for miRNA target prediction which helps to comprehend the specific
COX-2 is a target of miR-558. (A) Seed-matched sequences for miR-558 in the 3'UTR of COX-2 mRNA and a high level of sequence conservation of their cross species were identified by computational algorithms. (B and C) The luciferase reporter assay for the 3'UTR of COX-2 mRNA in the presence of miR-558. SW1353 cells were co-transfected with wild-type COX-2 3'UTR reporter plasmid or mutated 3'UTR reporter plasmid and miR-Control, miR-558 or anti-miR-558. Forty-eight hours after transfection, luciferase activities were measured. The ratio of reporter (firefly) to control (renilla) was plotted. Each dot represents a mean value from triplicate experiment of one donor. Horizontal lines indicate the mean ± 95% CI of triplicate experiments from each sample obtained from three different donors. *P < 0.01 vs control.

Fig. 5.

Our results suggest that in human OA cartilage, the level of miR-558 is upregulated in mesenchymal cells derived from aged rhesus macaque bone marrow and in fibroblasts irradiated with high doses of radiation. TargetScan contains more than 270 predicted targets of miR-558, some of which are relevant to the maintenance of cartilage homeostasis and involved in the reciprocal regulatory loop. Similar negative regulation of miRNAs in chondrocytes and SW1353 cells. These results suggest that miRNA may amplify its own effect by targeting a set of genes in a common pathway or protein complex. On the other hand, activation of NF-κB by IL-1β significantly inhibited miR-558 expression, suggesting a reciprocal regulatory loop. Similar negative regulation of miRNAs in chondrocytes by activated MAPKs and NF-κB was also reported previously. These results suggest that miRNA may constitute a regulatory motif that acts as both a transcription factor and a microRNA regulator of COX-2 in human chondrocytes. We identified the putative binding sequence of miR-558 in the 3'UTR of COX-2 mRNA located ~1,400 bp downstream of the stop codon of the COX-2 gene, demonstrating that miR-558 is a bona fide regulator of COX-2 in chondrocytes. Although selective COX-2 inhibitors may hamper catabolic cytokine-induced cartilage degeneration, considering the side effect profiles of these agents, understanding of its specific and detailed regulatory mechanism is required.

In IL-1β-stimulated human chondrocytes, treatment with anti-miR-558 enhanced COX-2 expression, but did not affect PGE2 production. The expression of mPGES-1 was not regulated by transfection of miR-558 and anti-miR-558. It has been known that mPGES-1 is a major down-stream enzyme of COX-2 in PGE2 biosynthesis and regulates IL-1β-induced PGE2 upregulation. Interestingly, we observed that overexpression of miR-558 inhibited IL-1β-mediated activation of the catabolic pathway rather broadly, including NF-κB and production of MMP-1 and MMP-13 in human chondrocytes and SW1353 cells. These results suggest that miRNA may amplify its own effect by targeting a set of genes in a common pathway or protein complex. On the other hand, activation of NF-κB by IL-1β significantly inhibited miR-558 expression, suggesting a reciprocal regulatory loop. Similar negative regulation of miRNAs in chondrocytes by activated MAPKs and NF-κB was also reported previously. These results suggest that miRNA may constitute a regulatory motif that acts as both a transcription factor and a mutual negative feedback network, highlighting the importance of elucidating the pathways regulated by miRNA.

The present study has some limitations: First, owing to the difficulty in obtaining normal knee and OA hip cartilages from the elderly in Korea, differences in expression of miR-558 and COX-2 according to joint sites could not be ascertained. Although there may be joint specific distinctions, recent study showed that osteoarthritic gene expression patterns in cells derived from hip or knee joint ex vivo and in primary culture were not significantly different. Second, we used
chondrocytes of passage 1 culture. By minimizing the duration of monolayer culture using high density plating, we could minimize dedifferentiation, although a slight degree of de-differentiation could not be avoided which might have influenced our data. Finally, the regulation conferred by miRNA is usually redundant, meaning that IL-1β regulates numerous miRNAs in addition to miR-558, while COX-2 is regulated by miRNAs other than miR-558. Thus confinement of the regulatory loop of IL-1 induced COX-2 to miR-558 would lead to a reductionist point of view.

In conclusion, we found that miR-558 functions as a negative regulator of IL-1β-mediated catabolic responses by repressing COX-2 expression and catabolic signaling pathways in human chondrocytes. To explore potential application of miR-558 as a therapeutic agent, further study is needed to evaluate the effectiveness and safety of miR-558 in vivo, and technological advance to enhance its efficacy of delivery using viral vector or nanoparticle-based system are needed.

**Author contributions**

Su Jin Park and Hyun Ah Kim conceived the study, its design, data analysis, and manuscript drafting and editing; Su Jin Park and Eun Jeong Cheon performed research. All authors approved the final version of the manuscript.

**Role of the funding source**

This study was supported by a grant from the Korea Healthcare Technology R & D Project, Ministry of Health & Welfare, Republic of Korea (grant no. A084026, A100736). Study sponsors had no involvement in study design, collection, analysis and interpretation of data, the writing of the manuscript or in the decision to submit the manuscript for publication.

**Conflict of interest**

The authors have no conflicts of interest to declare in regard to the manuscript entitled: *MicroRNA-558 regulates the expression of cyclooxygenase-2 and IL-1β-induced catabolic effects in human articular chondrocytes.*

**Acknowledgments**

We thank In Young Park for assistance with histological processing.

**References**


