Role of microRNA-449a on IL-1β-induced inflammatory responses in human chondrocytes

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Directed by Professor Jin Woo Lee

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Abstract

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SIRT1 has anti-inflammatory as well as protective effects in chondrocytes. The object of this study was to investigate whether microRNA-449a regulates expression of SIRT1, which inhibits expression of catabolic genes in IL-1β-induced cartilage destruction. MicroRNA-449a expression was determined in OA chondrocytes and IL-1β-induced chondrocytes by real-time PCR. MicroRNA-449a binding sites on the 3'-UTR of SIRT1 mRNA and binding site conservation were examined using microRNA target prediction tools. SIRT1-overexpressing or knockdown chondrocytes were transfected with microRNA-449a mimic or anti-microRNA-449a mimic and stimulated by IL-1β. Expression of catabolic and anabolic genes was examined by real-time PCR and western blotting. Finally, positive effects of anti-microRNA-449a on expression of these genes were confirmed by western analysis of OA chondrocytes.

Expression of microRNA-449a was increased in OA chondrocytes and IL-1β-induced chondrocytes. MMP-13 expression was enhanced, whereas type II collagen and SIRT1 expression were decreased in IL-1β-induced chondrocytes. SIRT1 overexpression resulted in decreased expression of catabolic genes, such as MMPs and ADAMTSs in response to IL-1β, but these effects were moderated by microRNA-449a. Suppression of microRNA-449a by anti-microRNA-449a inhibited expression of catabolic genes despite IL-1β stimulation, but these effects were abolished in SIRT1 knockdown chondrocytes. Furthermore, expression of catabolic genes was decreased and expression of type II collagen as well as SIRT1 was restored by anti-microRNA-449a in OA chondrocytes as well as in IL-1β-induced chondrocytes. Taken together, silencing of microRNA-449a had a protective effect, inhibiting catabolic gene expression and restoring anabolic gene expression, by targeting SIRT1 in IL-1β-induced cartilage destruction.

Key words: microRNA-449a, SIRT1, chondrocyte, IL-1β, osteoarthritis

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I. INTRODUCTION

The destruction of articular cartilage may trigger arthritis associated with a variety of causal factors, such as advanced age, mechanical stress, and genetic factors, as well as inflammation[1,2]. Arthritis can be classified as inflammatory or non-inflammatory. Osteoarthritis (OA), one of the classic age-related diseases, is the most common non-inflammatory arthritis[3]. Nevertheless, inflammation usually accompanies progression of OA and exacerbates cartilage degradation[4]. OA is characterized by cartilage degeneration resulting from an imbalance between anabolic and catabolic activities in cartilage homeostasis[5]. However, completely effective therapeutic measures have not been developed, and treatment remains associated with side effects, such as functional gastrointestinal disorders, providing relief from pain, and not a cure[6]. Therefore, an improved therapeutic strategy is essential to prevent progression of OA[7].

Interleukin-1β (IL-1β), a key pro-inflammatory factor, is one of the mediators of OA[8,9]. IL-1β commonly activates the nuclear factor-kappa B (NF-κB) pathway[10-12] (Figure. 1)[13]. When activated, this pathway is a major cause of inflammation and revolves around the expression of catabolic genes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs), which play a role in cartilage degradation[14]. Ultimately, IL-1β induces degradation of cartilage matrix through activation of extracellular matrix proteases, as well as by repression of synthesis of extracellular matrix proteins. Many studies have focused on therapy using cytokine inhibitors; however, according to recent reports, regulation of expression of specific genes to promote cartilage repair has been considered as a novel strategy for gene therapy applications[15,16].

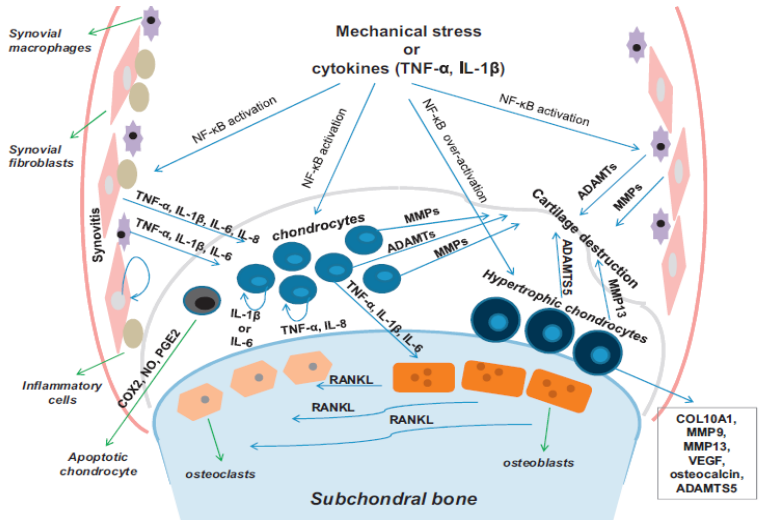

Figure 1. The NF-κB pathway induced by IL-1β in chondrocytes.

Interestingly, the NF-κB pathway, which expresses catabolic genes as well as pro-inflammatory genes, is inhibited by the NAD-dependent deacetylases SIRT1 and SIRT6[17,18]. These enzymes suppress activation of the NF-κB pathway by deacetylation of p65, which is a subunit of the NF-κB transcription complex[19]. Moreover, SIRT1 has various protective effects[20] that regulate apoptosis of chondrocytes and enhance survival of chondrocytes[21,22]. SIRT6 has protective effects against chondrocyte senescence and DNA damage. In particular, although SIRT1 plays a protective role, SIRT1 expression is decreased with stress, aging, and OA[23]. Therefore, I considered that if I was able to regulate expression of SIRT1, it may be a useful target for gene therapy of OA.

MicroRNAs, small noncoding RNAs composed of 20-25 nucleotides, regulate gene expression post-transcriptionally through mRNA degradation or translational suppression by binding to the 3'-UTR of target genes[24]. Recently, it has been reported that microRNAs play important roles in regulating expression of genes associated with human diseases in various biological processes[25,26]. In cartilage biology[27], many researchers have determined that the function of microRNAs is to modulate cartilage degradation[28-30] as well as chondrogenesis[31]. Therefore, attempts have been made to utilize microRNA-based therapeutics in OA[32-34]. Among these, a previous study has shown that microRNA-449a regulates chondrogenesis in human bone marrow-derived mesenchymal stem cells (hBM-MSCs)[35], but the role of microRNA-449a has not been investigated in OA. In addition, some reports have identified that microRNA-449a suppresses the expression of SIRT1 in cancer cells[36], including hepatoma cells,

by directly binding to the 3'-UTR of SIRT1 mRNA[37]. Therefore, I investigated the function of microRNA-449a in OA progression. I selected SIRT1 as microRNA-449a target in chondrocytes and investigated the role of microR-449a in response to IL-1β induction of cartilage degeneration, which promotes OA.

Ⅱ. MATERIALS AND METHODS

1. SW1353 cell culture

The human chondrosarcoma cell line SW1353, also known as a chondrocytic cell line, was maintained in Dulbecco's modified Eagle medium-High Glucose (DMEM-HG; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic antimycotic solution (Gibco). At 80% confluence cells were harvested using 0.05% trypsin-EDTA (Gibco). Cells were washed, centrifuged, resuspended, and seeded in new plates. The medium was replaced every 2-3 days.

2. Articular cartilage and chondrocyte culture

Human articular cartilage was taken from the knee joints of patients with approval from the institutional review board. Normal articular cartilage was obtained from 10 trauma patients with no previous history of OA. OA articular cartilage was obtained from 7 patients who underwent total knee arthroplasty. The mean age of normal cartilage donor was 60.6 years (age range, 24 - 65 years) and OA cartilage donor was 62.7 years (age range, 45 - 73 years). To isolate chondrocytes, cartilage samples were sequentially digested with 0.1% collagenase (Worthington Biochemical, Lakewood, NJ) and 0.065% hyaluronidase (Worthington Biochemical) and centrifuged at 1,200 rpm for 5 min as previously described[38]. All culture conditions were the same as for SW1353 cells.

3. Transfection of microRNA mimic and IL-1β stimulation

SW1353 cells or chondrocytes were seeded at 1 x 10^5 cells in 6-well plates. After 24 hr of culture, cells were transfected with 100 nM microRNA-449a mimic (Genolution, Seoul, South Korea) or anti-microRNA-449a mimic (ST Pharm, Seoul, South Korea) using Lipofectamine LTX & Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After transfection for 6-8 hr, the medium was replaced with fresh medium with or without IL-1β (R&D Systems, Minneapolis, MN) at 10 ng/ml and then incubated for up to 24 hr. Cells were harvested using 0.05% trypsin-EDTA as described above. MicroRNA mimic sequence are shown in Table 1.

Table 1. A list of microRNA mimic sequence

microRNA	Strand	Sequence (5' → 3')
microRNA-sc	S	UUACCAGACGUGUCUUCACUCCC
	AS	GGGAGUGAAGACACGUCUGGUAA
microRNA-449a	S	UGGCAGUGUAUUGUUAGCUGGU
	AS	ACCAGCUAACAAUACACUGCCA

S: sense strand, AS: anti-sense strand

4. Real-time PCR

Total RNA was extracted from harvested cells using Trizol (Invitrogen) following the protocols provided by the manufacturer. For cDNA synthesis, 2 μg RNA was reverse transcribed using the Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany), and 80 ng cDNA was used to carry out real-time PCR with 2x qPCRBIO SyGreen Mix (PCR Biosystems, London, UK). For quantification of target genes, 18S ribosomal RNA (18s rRNA) was used as control for relative expression. Primer sequences are shown in Table 2. For microRNA cDNA synthesis, 0.25-8 μg RNA was reverse transcribed using the Mir-X™ MicroRNA First-Strand Synthesis Kit (Clontech, Mountain View, CA) following the protocols provided by the manufacturer. U6 small nuclear RNA (snRNA) was used as control for relative expression. The primer sequence of the microRNA-449a was 5'- TGGCAGTGTAT TGTTAGCTGGT -3'.

Table 2. A list of primers used for real-time PCR

Gene symbol		Sequence (5' → 3')
18s rRNA	Forward	ACACGGACAGGATTGACAGATTG
	Reverse	GCCAGAGTCTCGTTCGTTATCG
COX-2	Forward	GGTGCCTGGTCTGATGATGTATG
	Reverse	AGTATTAGCCTGCTTGTCTGGAAC
MMP-8	Forward	GCTGCTTATGAAGATTTTGACAGAG
	Reverse	ACAGCCACATTTGATTTTGCTTCAG
MMP-13	Forward	CCAGACTTCACGATGGCATTG
	Reverse	GGCATCTCCTCCATAATTTGGC
ADAMTS-4	Forward	GAGGAGGAGATCGTGTTTCCA
	Reverse	CCAGCTCTAGTAGCAGCGTC
ADAMTS-5	Forward	GAACATCGACCAACTCTACTCC
	Reverse	CAATGCCCACCGAACCATCT
COL2A1	Forward	CCAGATGACCTTCCTACGCC
	Reverse	TTCAGGGCAGTGTACGTGAAC

5. Western blotting

For protein extraction, cell pellets were suspended in lysis buffer consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS), followed by gentle pipetting and heating at 100°C for 10 min with vortex mixing every 3 min. Lysates were centrifuged at 13,000 rpm for 5-10 min and supernatants were collected. To measure the concentration of extracted protein, I used the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Prior to western blotting, 30 μg protein was mixed with 5× loading dye (Pierce) and heated at 100°C for 7 min. Prepared protein samples were run on 10% SDS polyacrylamide gel electrophoresis (PAGE) gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond, Escondido, CA) for 90 min. Following blocking in 5% skim milk (BD Biosciences, San Jose, CA) for 90 min, the membranes were incubated with primary antibodies at 4°C overnight. Antibodies used were anti-COX2 (BD Biosciences, San Jose, CA, 1:1,000 in 1% skim milk); anti-SIRT1 and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1,000 in 1% skim milk); and anti-COL2A1 (Santa Cruz Biotechnology, 1:500 in 1% BSA). Finally, membranes were developed using enhanced chemiluminescence (ECL) solution (Amersham, Buckinghamshire, UK).

6. Viral infection

Lentiviruses encoding SIRT1 or short hairpin RNA targeting SIRT1 (shSIRT1) and control shRNA (shcontrol) were transduced into chondrocytes. To produce viral supernatants, 293 FT cells were transfected with lentivirus using Lipofectamine LTX & Plus Reagent (Invitrogen) according to the manufacturer's instructions. Medium was changed 6 hr after transfection. Supernatants were harvested 48 hr after the medium change, filtered with 0.45 μm syringe filter (Millipore, Billerica, MA), centrifuged, and stored at 4°C overnight. Chondrocytes were infected with the purified lentiviruses for 24 hr and, after puromycin (5 μg/ml) selection, the lentivirus-infected chondrocytes were used in experiments.

7. Histological analysis

Articular cartilage from human knee joints was fixed in 3.7% formaldehyde, embedded in paraffin, and sectioned. Sections were deparaffinized in xylene, rehydrated in an ethanol series, and stained sequentially. For hematoxylin and eosin (H&E) staining, the sections were stained with hematoxylin for 3 min and eosin for 30 sec. For safranin O staining, the sections were stained with hematoxylin for 1 min, 0.02% fast green for 5 min, and 0.1% safranin O for 30 min.

8. Statistical analysis

All experiments were performed in triplicate using samples from at least three donors. The independent sample *t*-test was used for the detection of differences between the two groups. The statistical significance of the differences among three or more groups was calculated using one-way analysis of variance with Tukey's post hoc analysis. $p < 0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Stimulation of catabolic genes under conditions in the IL-1β-induced cartilage destruction

To confirm conditions of IL-1β-induced cartilage destruction, I treated SW1353 cells with IL-1β at various concentration (0.1, 1 and 10 ng/ml) for 24 hr before treatment of chondrocytes and then expression of catabolic genes were examined by real-time PCR and western blotting. At a concentration of 10 ng/ml, the expression of COX-2 mRNA and protein, known as inflammatory marker, effectively increased (Figure 2A, B). The expression of representative cartilage-degrading genes, such as MMPs and ADAMTSs, also increased (Figure 3A). Interestingly, a consistent time-dependent increase in miR-449a expression was observed in SW1353 cells (Figure 3B).

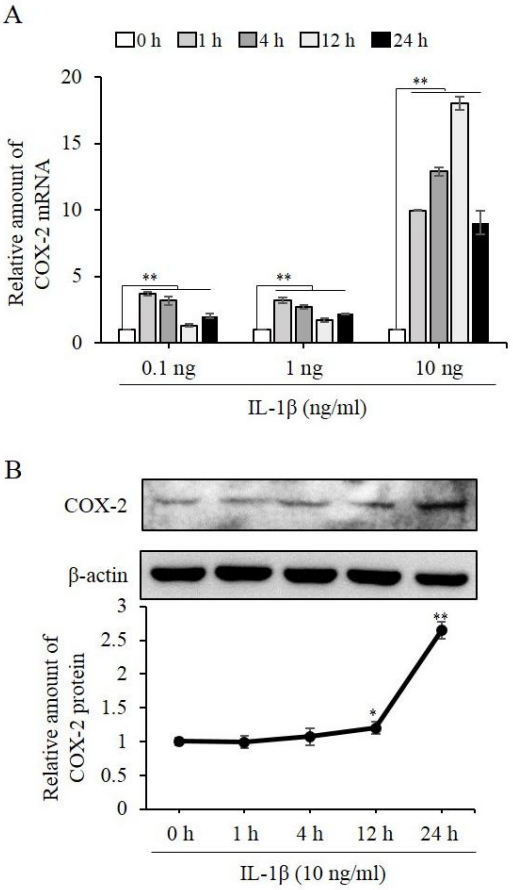

Figure 2. Expression of COX-2 under conditions in the IL-1β-induced cartilage destruction. (A) Real-time PCR analysis of expression of COX-2 in chondrocytic cell line SW1353 treated with the indicated concentration of IL-1β (0.1, 1 and 10 ng/ml) for 24 hr. (B) Western analysis (top) and quantitation (bottom) of expression of COX-2 after treatment with 10 ng/ml IL-1β for 24 hr. β-actin was used as an endogenous mRNA control. Data represent mean ± SD of triplicate samples. *P*-values were calculated compared to IL-1β treatment at 0 h. *$p < 0.05$, **$p < 0.01$

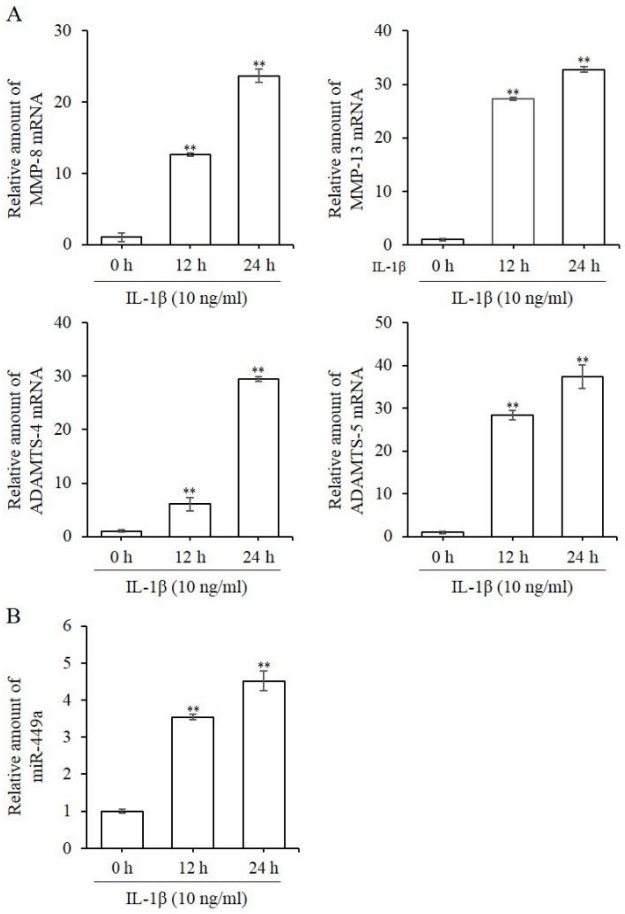

Figure 3. Expression of catabolic genes and miR-449a by IL-1β stimulation in the SW1353. (A) Real-time PCR analysis of expression of catabolic genes encoding the main enzymes associated with cartilage destruction. β-actin was used as an endogenous mRNA control. (B) Relative expression level of miR-449a in SW1353 cells exposed to IL-1β for the indicated times. U6 was used as internal control. Data represent mean ± SD of triplicate samples. *P*-values were calculated compared to IL-1β treatment at 0 h. $^{**}p < 0.01$

2. Expression of miR-449a in OA chondrocytes and IL-1β-induced chondrocytes

To investigate the expression of miR-449a in normal cartilage and OA cartilage-derived chondrocytes, I performed microRNA-specific real-time PCR. MiR-449a expression was increased approximately 7-fold in OA chondrocytes compared with normal chondrocytes (Figure 4A). To confirm whether the expression of miR-449a was regulated by IL-1β in chondrocytes, I treated chondrocytes with IL-1β (10 ng/ml) for 24 hr and then determined expression level of miR-449a. Stimulation with IL-1β resulted in an approximately 6-fold increase in the expression of miR-449a compared with normal chondrocytes (Figure 4B).

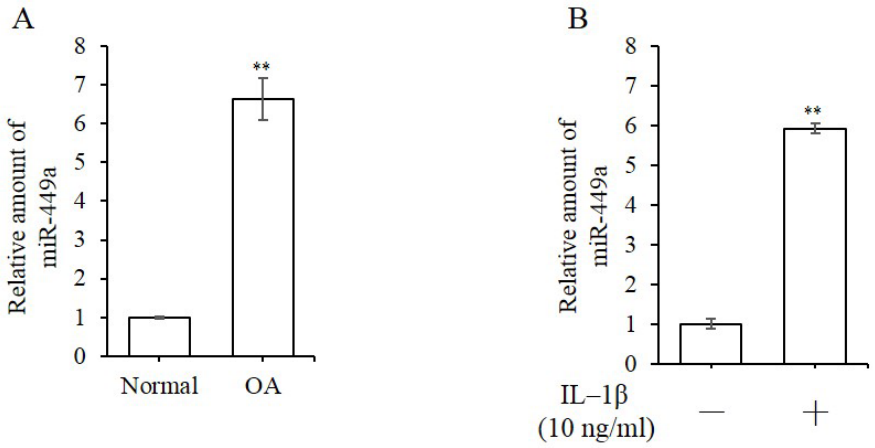

Figure 4. Expression of miR-449a in OA chondrocytes and IL-1β-induced chondrocytes. (A) Relative expression level of miR-449a expression in OA cartilage-derived chondrocytes compare to normal cartilage-derived chondrocytes and (B) in chondrocytes incubated in the presence or absence of IL-1β at 10 ng/ml for 24 hr. U6 was used as internal control. Data represent mean ± SD of triplicate samples. *P*-values were calculated compared to normal or IL-1β non-stimulated chondrocytes.**$p < 0.01$

3. Inverse correlation between expression of catabolic genes and SIRT1

First, to confirm that cartilage samples acquired from patients show characteristics of normal and OA cartilage, I performed histological analysis of human articular cartilage samples using H&E and Safranin O staining. I identified surface-crack, surface-fibrillation and chondrocyte clusters indicated arrows in OA cartilage using H&E staining. Also surface-fibrillation as well as reduction of proteoglycan indicated little triangles was increased in OA compare to normal cartilage using Safranin O staining (Figure 5).

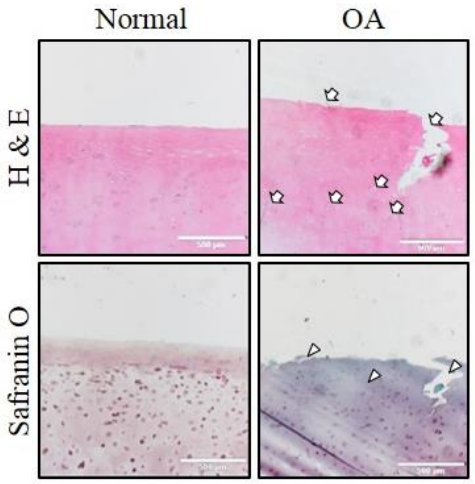

Figure 5. Comparison of normal and OA cartilage. Comparison of phenotypes of normal and OA cartilage after H&E (top) and Safranin O staining (bottom). Arrows indicated surface-fibrillation, surface-crack and chondrocyte clusters in OA cartilage compared to normal cartilage using H&E staining. Little triangles indicated surface-fibrillation, surface-crack as well as reduction of proteoglycan (proteoglycan stained red color) in OA cartilage compared to normal cartilage using Safranin O staining. Scale bars, 500 μm.

Second, I identified the expression of major cartilage-related genes, including those responsible for catabolic and anabolic functions. The expression of MMP-13, a typical cartilage-degrading collagenase, was markedly increased, whereas expression of type II collagen, a main cartilaginous extracellular matrix protein contributing to cartilage formation, was reduced in IL-1β-induced chondrocytes as well as in OA chondrocytes (Figure 6). Moreover, the expression of SIRT1, a predicted target of miR-449a, was also reduced in OA chondrocytes and IL-1β-induced chondrocytes. Expression of SIRT1 was decreased in a time-dependent manner in SW1353 cells treated with IL-1β (Figure 6B). These results indicate an inverse correlation of miR-449a and SIRT1 expression in OA chondrocytes and IL-1β-induced chondrocytes. Third, to clarify the potential for regulation of SIRT1 expression by miR-449a, I used microRNA target prediction tools such as TargetScan, miRanda and miRBase. I identified two miR-449a binding sites in the 3'-UTR of SIRT1 mRNA and conservation of the miR-449a seed sequence in various species (Figure 7A, B). These results suggest that miR-449a regulates the expression of SIRT1.

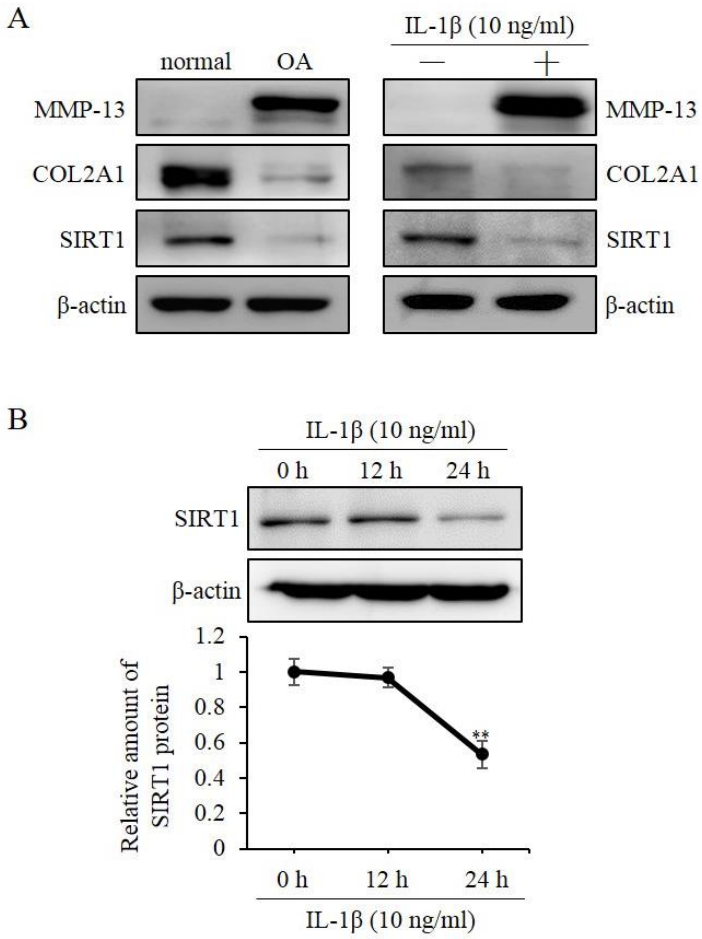

Figure 6. Expression of representative catabolic and anabolic genes as well as SIRT1. (A) Western analysis of expression of MMP-13, type II collagen (COL2A1) and SIRT1 in OA chondrocytes and IL-1β-induced chondrocytes compared with normal chondrocytes. (B) Western analysis (top) and quantitation (bottom) of expression of SIRT1 in SW1353 cells after stimulation with IL-1β for the indicated times. *P*-values were calculated compared to IL-1β treatment at 0 h. $^{**}p < 0.01$

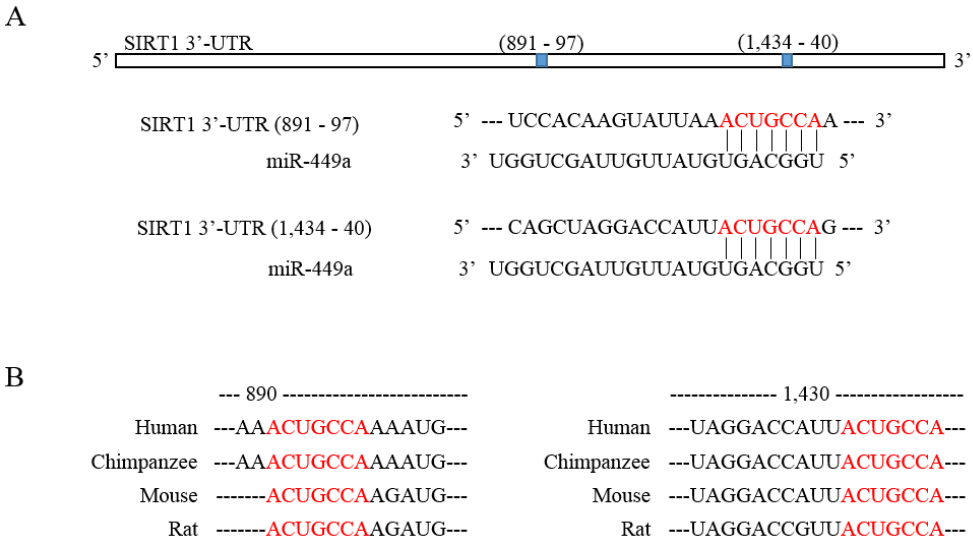

Figure 7. Bioinformatic prediction of the seed sequence of miR-449a in the 3'-UTR of SIRT1 mRNA. (A) Results for bioinformatic prediction of two miR-449a binding sites on the 3'-UTR of SIRT1 mRNA and the full length of human SIRT1 3'-UTR is 1,797 base pairs (bp). The binding site of miR-449a (indicated red letters on the sequence) is located at 891-897 bp and 1,434-40 bp on the 3'-UTR of SIRT1 mRNA. (B) Results for conservation of miR-449a seed sequences (indicated red letters on the sequence) in the indicated species using microRNA target prediction tools such as TargetScan, miRanda and miRBase.

4. The role of SIRT1 regulated by miR-449a in chondrocytes in response to IL-1β

To identify the role of SIRT1 under conditions of IL-1β-induced cartilage destruction, I overexpressed SIRT1 in chondrocytes using a lentiviral vector. Chondrocytes were infected with lentiviral vector encoding SIRT1. After puromycin selection, the cells were transfected with miR-sc (scrambled control) or miR-449a and treated with IL-1β. I confirmed significant overexpression of SIRT1 and efficient transfection of miR-449a using western blotting and microRNA-specific real-time PCR, respectively (Figure 8A, B). The expression of catabolic genes, such as MMPs and ADAMTSs, was increased by IL-1β stimulation of chondrocytes (Figure 9). However, these increases were prevented by SIRT1 overexpression, and the inhibitory effect of SIRT1 overexpression was eliminated by miR-449a targeting SIRT1. These results suggest that miR-449a regulates SIRT1 expression and function.

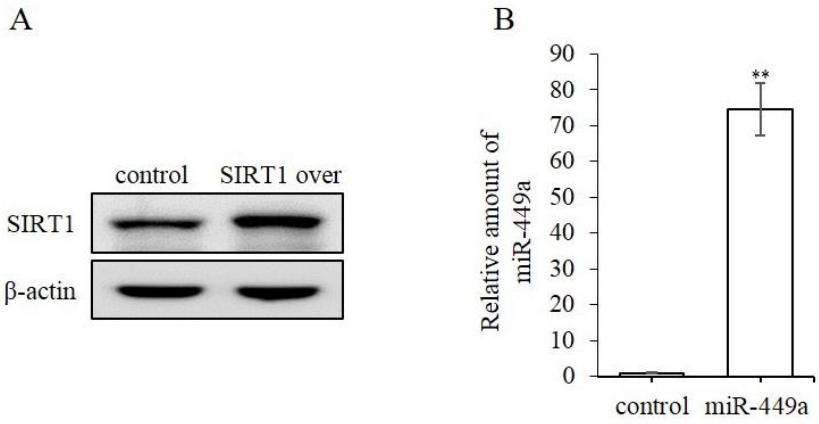

Figure 8. Confirmation of efficient overexpression of SIRT1 and transfection of miR-449a in chondrocytes. (A) Western blotting of SIRT1 expression in SIRT1-overexpressing chondrocytes transduced with lentiviral SIRT1 expression vector. (B) Real-time PCR analysis of relative expression level of miR-449a in SIRT1-overexpressing chondrocytes after transfection with miR-449a. U6 was used as internal control. Data represent mean ± SD of triplicate samples. *P*-values were calculated compared to control. $^{**}p < 0.01$

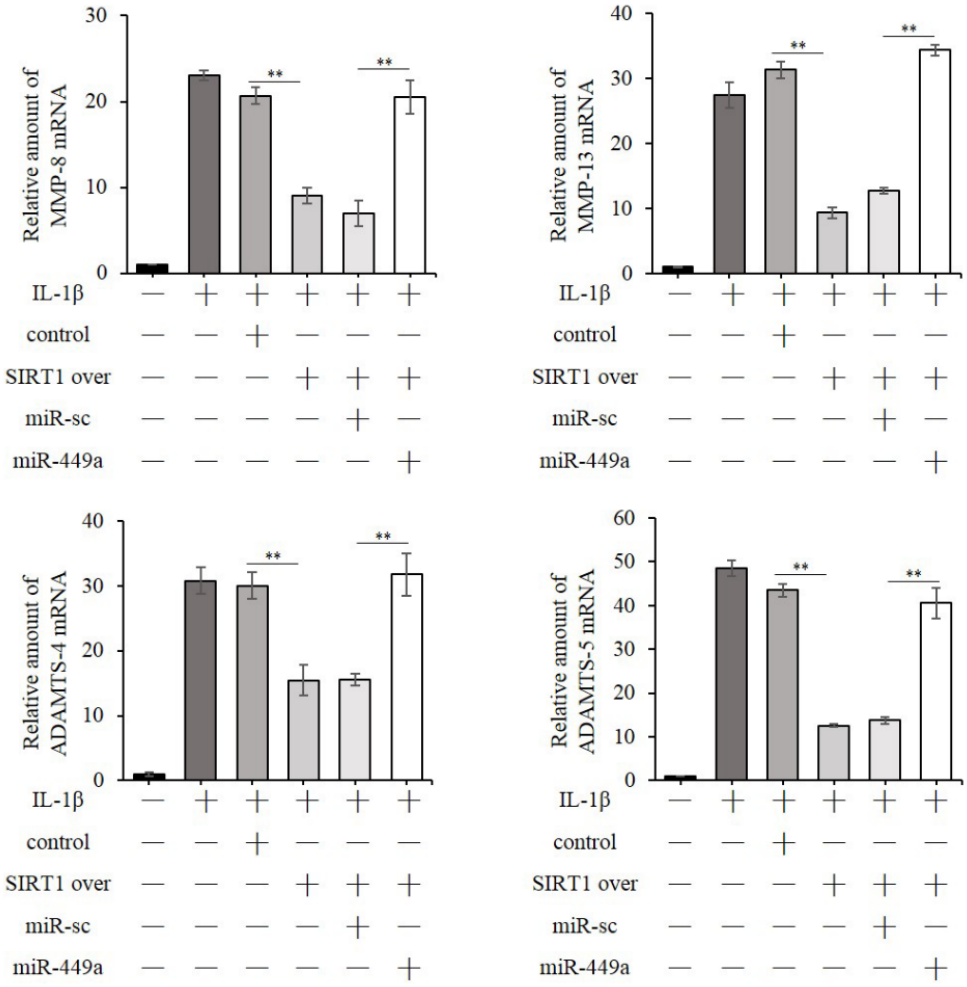

Figure 9. Effect of miR-449a on SIRT1-overexpressing chondrocytes. Real-time PCR analysis of expression of catabolic genes in vector control or SIRT1-overexpressing chondrocytes (SIRT1 over) transfected with miR-sc or miR-449a and then stimulated by IL-1β. β-actin was used as an endogenous mRNA control. Data represent mean ± SD of triplicate samples. $^{**}p < 0.01$

5. Suppression of IL-1β-induced cartilage destruction in chondrocytes by inhibition of miR-449a targeting SIRT1

To investigate whether increased expression of miR-449a in response to IL-1β regulates the expression of cartilage-degrading genes through SIRT1, I performed shRNA-mediated SIRT1 knockdown in chondrocytes. As shown in Figure 10A, effective knockdown of SIRT1 was confirmed by western blotting. I transfected control and SIRT1 knockdown chondrocytes with anti-miR-sc or anti-miR-449a and then treated with IL-1β. As a result, the expression of primary anabolic gene type II collagen was reduced by IL-1β stimulation, whereas type II collagen expression was restored by anti-miR-449a even though chondrocytes were induced by IL-1β (Figure 10B). Interestingly, SIRT1 knockdown chondrocytes showed no enhancement of type II collagen expression. On the contrary, the increased expression of catabolic genes induced by IL-1β was decreased by anti-miR-449a, but this repression of catabolic gene expression consistently had no effect on SIRT1 knockdown chondrocytes (Figure 11). Therefore, these results suggest that the effect of miR-449a is modulated by SIRT1, which then regulates the expression of anabolic as well as catabolic genes.

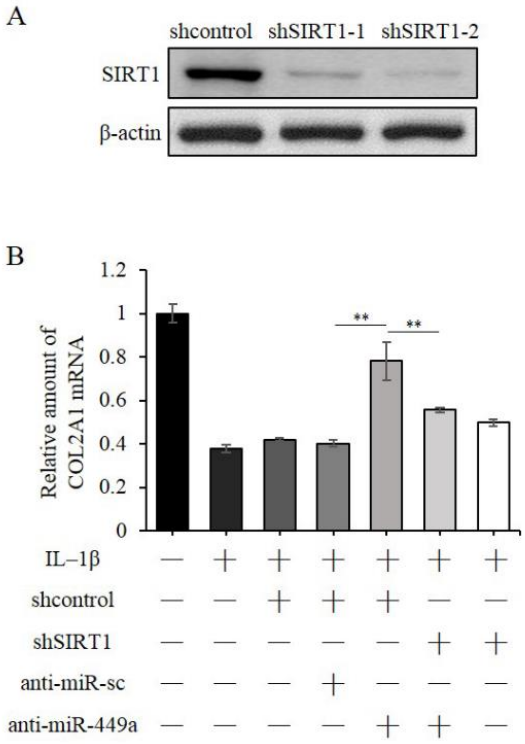

Figure 10. Confirmation of efficient shRNA-mediated knockdown of SIRT1 in chondrocytes. (A) Western blotting of SIRT1 in the shRNA-mediated SIRT1-knockdown chondrocytes. (B) Real-time PCR analysis of expression of type II collagen in shRNA-control (shcontrol) or SIRT1-knockdown chondrocytes (shSIRT1) transfected with anti-miR-sc or anti-miR-449a after IL-1β stimulation. β-actin was used as an endogenous mRNA control. Data represent mean ± SD of triplicate samples. $^{**}p < 0.01$

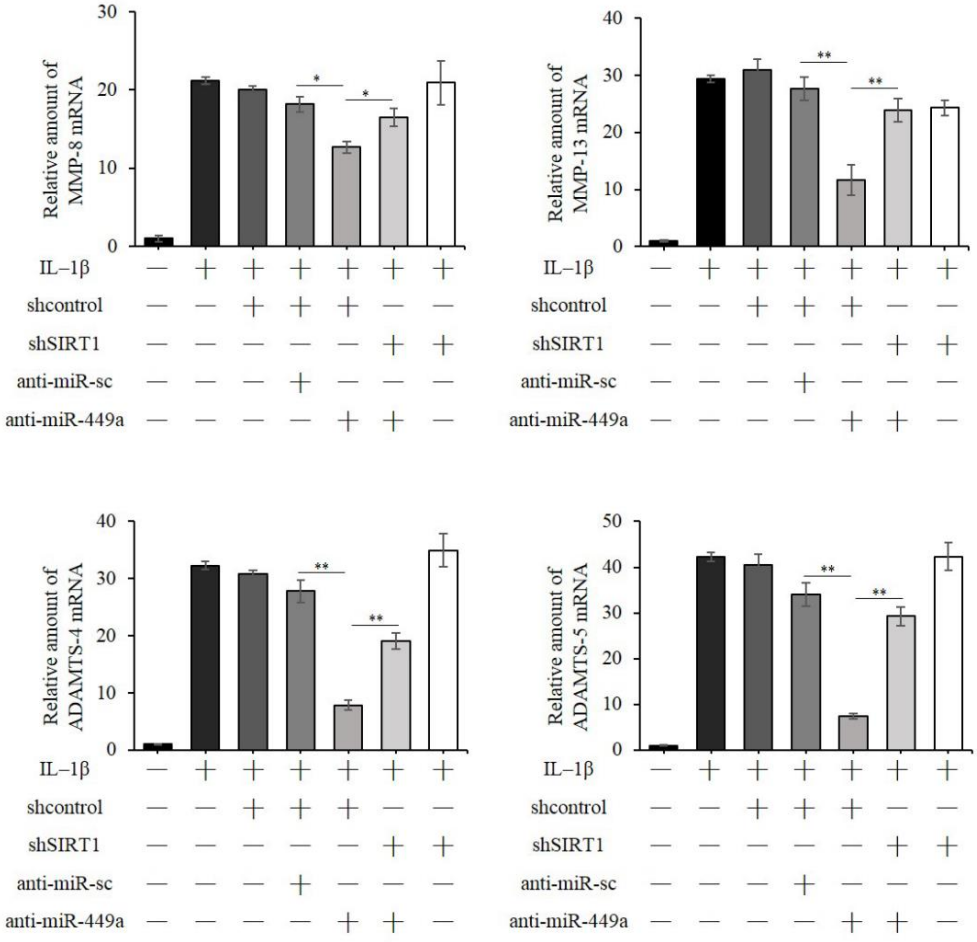

Figure 11. Effect of anti-miR-449a on IL-1β stimulation in chondrocytes with SIRT1 knockdown. Real-time PCR analysis of expression of catabolic genes in shRNA-control (shcontrol) or SIRT1-knockdown chondrocytes (shSIRT1) transfected with anti-miR-sc or anti-miR-449a after IL-1β stimulation. β-actin was used as an endogenous mRNA control. Data represent mean ± SD of triplicate samples. *$p <$ 0.05, **$p < 0.01$

6. Regulation of catabolic genes in IL-1β-induced chondrocytes and the restoration of anabolic genes and SIRT1 in OA chondrocytes by anti-miR-449a

I observed regulation of catabolic gene expression with inhibition of miR-449a by anti-miR-449a in IL-1β-induced chondrocytes. As a result, the expression of catabolic genes such as MMPs and ADAMTSs was decreased by anti-miR-449a, despite IL-1β induction of chondrocytes (Figure 12A). Moreover, the expression of the representative catabolic gene MMP-13 was increased by IL-1β stimulation, but it was decreased by anti-miR-449a in IL-1β-induced chondrocytes (Figure 12B). Conversely, the expression of representative anabolic gene type II collagen and SIRT1 was decreased by IL-1β stimulation but, in the same pattern, was restored by anti-miR-449a.

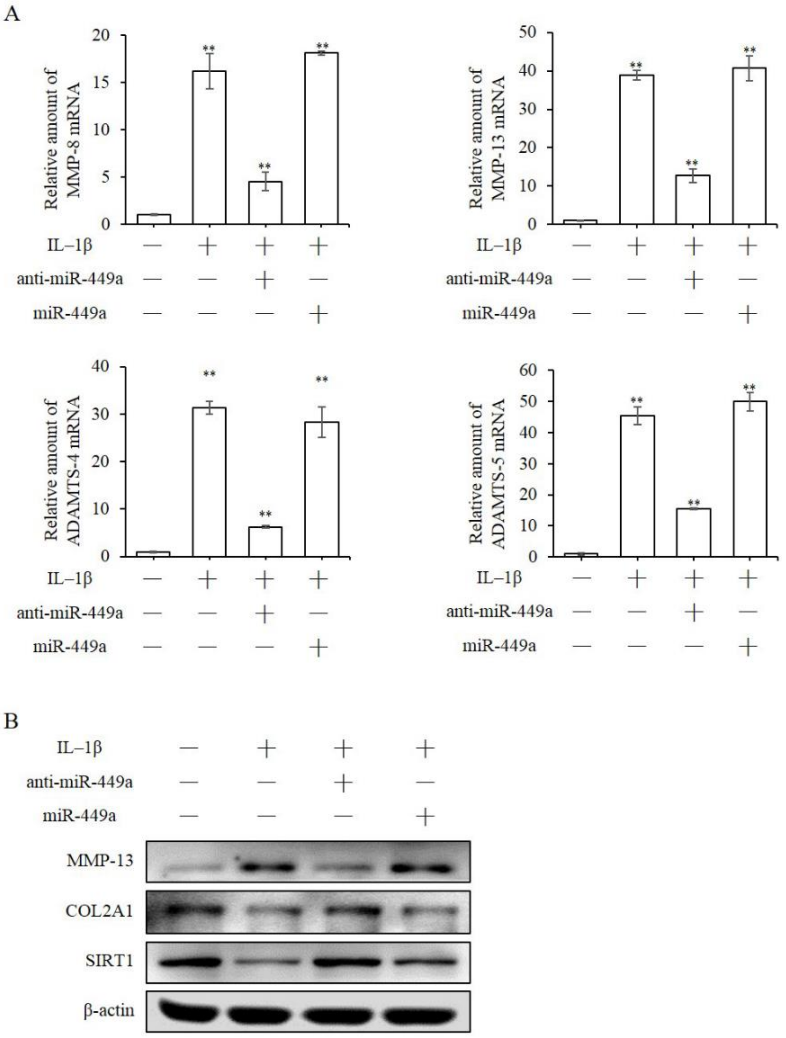

Figure 12. Effect of anti-miR-449a in IL-1β-induced chondrocytes. (A) Real-time PCR analysis of expression of catabolic genes in IL-1β-induced chondrocytes. β-actin was used as an endogenous mRNA control. (B) Western blotting of SIRT1, catabolic and anabolic genes expression in IL-1β-induced chondrocytes. Data represent mean ± SD of triplicate samples. *P*-values were calculated compared to control. $^{**}p < 0.01$

Finally, to comfirm the effect of miR-449a inhibition by anti-miR-449a, I transfected with anti-miR-449a in OA chondrocytes. As shown in Figure 13, the expression of MMP-13 was dramatically decreased by anti-miR-449a, whereas the expression of SIRT1 and type II collagen was restored in OA chondrocytes. This study suggests that inhibition of miR-449a by anti-miR-449a restores expression of anabolic gene as well as suppresses expression of catabolic genes in response to IL-1β. Increased expression of miR-449a in response to IL-1β stimulation decreases expression of SIRT1, which inhibits NF-κB pathway activation, causing cartilage destruction (Figure 14). Therefore, anti-miR-449a is a potential therapeutic target for IL-1β-induced cartilage destruction.

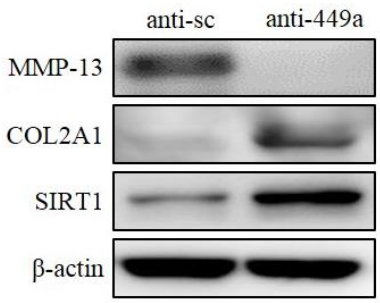

Figure 13. Effect of anti-miR-449a in OA chondrocytes. Western blotting of SIRT1, catabolic and anabolic genes expression in OA chondrocytes transfected with anti-miR-sc (anti-sc) or anti-miR-449a (anti-449a).

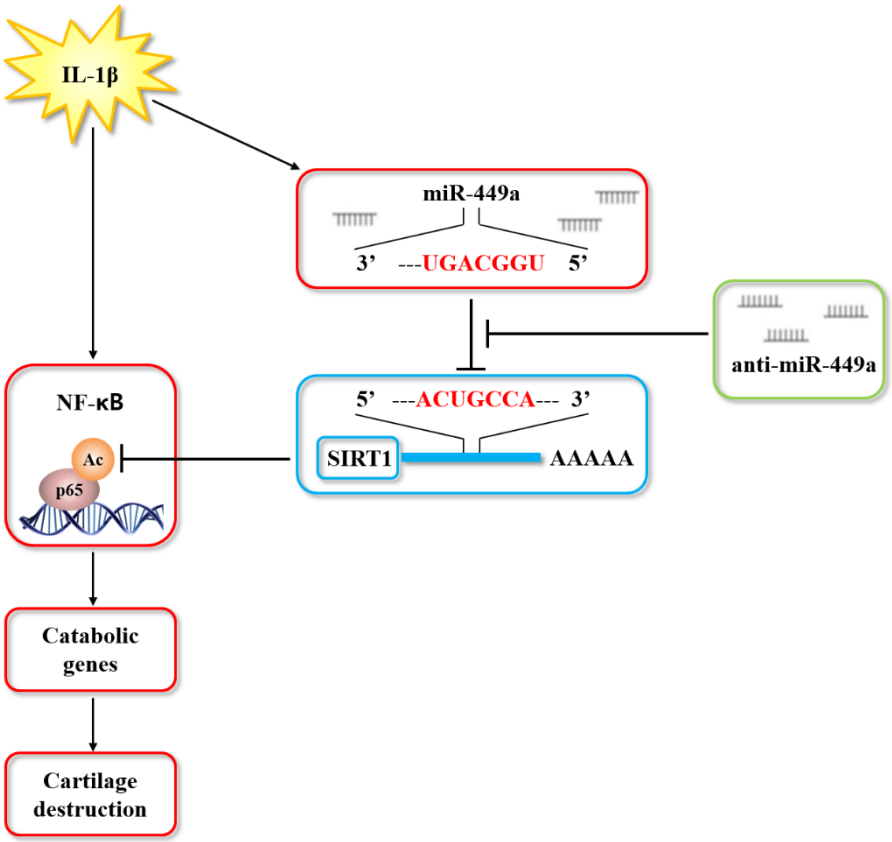

Figure 14. Proposed model of miR-449a activity in chondrocytes. A diagram showing the molecular mechanism of miR-449a targeting SIRT1 in IL-1β-induced cartilage destruction. IL-1β activates NF-kB pathway and induces expression of catabolic genes resulting in cartilage destruction. Also, IL-1β increases expression of miR-449a targeting SIRT1, which inhibits activation of NF-κB pathway and expression of catabolic genes. Suppression of miR-449a by anti-miR-449a can restore expression of SIRT1 and regulate expression of catabolic genes in IL-1β-induced cartilage destruction.

IV. DISCUSSION

In this study, I found that the expression of microRNA-449a was increased in response to IL-1β, causing cartilage destruction. When the function of microRNA-449a, which targets SIRT1, was inhibited by anti-microRNA-449a, the expression of cartilage catabolic genes, such as MMPs and ADAMTSs, was suppressed despite IL-1β induction of chondrocytes. Further, the expression of type II collagen, a major cartilage anabolic gene, and SIRT1, a microRNA-449a target gene, was increased. These effects are the results of anti-microRNA-449a-mediated restoration of SIRT1 expression under conditions of IL-1β-induced cartilage destruction as well as in OA chondrocytes.

Usually, pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor-α (TNF-α) induce the cartilage matrix degradation and inflammation associated with OA[10]. Also, these cytokines are implicated in the NF-κB pathway, which affects initiation of cartilage destruction and development of OA[39]. Although IL-1β and/or TNF-α have interaction effect, the activity level of these cytokines are different depending on the specimens of OA cartilage[40]. Hence, to determine which cytokine has a greater inflammatory effect on chondrocytes, I treated chondrocytes and chondrocytic SW1353 cells with IL-1β and TNF-α. I found that IL-1β was more effective in inducing the inflammatory conditions than TNF-α (data not shown). Therefore, I used IL-1β to induce *in vitro* conditions of cartilage destruction.

A few microRNAs that closely involved in OA or cartilage degeneration have been reported. Akhtar et al. and Meng et al. respectively reported that microRNA-27b and microRNA-320 regulated expression of MMP-13 in IL-1β-induced chondrocytes[31,41]. In OA chondrocytes, microRNA-558 and microRNA-199a were studied the function that regulating expression of COX-2[42,43] and microRNA-125b regulated expression of ADAMTS-4[29]. These microRNAs directly bind to catabolic genes and focused on the inhibition of catabolic gene expression. Although these microRNAs have not been mentioned expression level of anabolic gene such as type II collagen, we demonstrated that microRNA-449a has been able to regulate expression of anabolic gene as well as catabolic gene by targeting SIRT1 associated with regulation of catabolic and anabolic genes.

Generally, previous studies investigating the function of microRNA reported in IL-1β-induced cartilage destruction conditions. Among several microRNAs, microRNA-449a has been reported to be a gene regulator related to cell cycle[36] and as a tumor suppressor in various cancers, including ovarian cancer[44], gastric cancer[45], lung cancer[46], and bladder cancer[47]. Although several studies investigated functions of microRNA-449a, only one investigated a role in cartilage and showed that microRNA-449a regulates expression of lymphoid enhancer-binding factor-1 (LEF-1), which is a critical gene known and a direct target of β-catenin in wnt signaling during chondrogenesis in hBM-MSCs [35]. To identify the effect of microRNA-449a in chondrocytes, I transfected chondrocytes with microRNA-449a or anti-microRNA-

449a and then stimulated the cells with IL-1β. Interestingly, the expression of LEF-1 did not change with IL-1β treatment (data not shown).

Lize et al. reported that microRNA-449a diminished the expression of SIRT1[36], and Zhang et al. reported that the microRNA-449 family regulates the expression of SIRT1 by direct binding of microRNA-449 to the 3'-UTR of SIRT1 mRNA in hepatoma cells[37]. SIRT1 plays an inhibitory role in IL-1β-induced cartilage destruction associated with OA, a common form of arthritis aggravated by IL-1β-activating NF-κB pathway[8-12]. These effects were inhibited by SIRT1, which has a protective function in chondrocytes, but also aggravated by disruption of SIRT1[17,48]. However, many studies have reported that expression of SIRT1 gradually decreases for various reasons, such as aging, mechanical stress, stimulation of inflammatory cytokines, and severe OA, although genetic regulatory factors remain unknown[1,2]. To my knowledge, this is the first study to investigate the regulatory role of microRNA-449a in the expression of SIRT1 in OA chondrocytes associated with IL-1β-induced cartilage destruction.

Ⅴ. CONCLUSION

I found that expression of microRNA-449a and cartilage catabolic genes, such as MMPs and ADAMTSs, was increased, whereas expression of SIRT1 and anabolic gene type II collagen was decreased in IL-1β-induced chondrocytes. These expression patterns were restored when microRNA-449a was inhibited by anti-microRNA-449a, despite IL-1β stimulation. Taken together, these data suggest that inhibition of microRNA-449a is able to restore the expression of SIRT1 and anabolic gene as well as suppress the expression of catabolic genes in IL-1β-induced chondrocytes. Therefore, anti-microRNA-449a may be a useful regulator and therapeutic agent for prevention and treatment of cartilage destruction such as occurs in OA.

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ABSTRACT (IN KOREAN)

사람 연골세포의 IL-1β에 의한 염증 반응에서 microRNA-449a의 역할

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SIRT1 은 연골세포를 보호하는 효과뿐만 아니라 항염증 효과를 나타낸다. 본 연구에서는 IL-1β 자극에 의해 유도된 염증 환경에서, MMPs 또는 ADAMTSs 와 같은 이화작용 유전자들의 발현 억제가 가능한 SIRT1 의 발현을 microRNA-449a 가 조절할 수 있는지 조사하고자 하였다.

MicroRNA 특이적인 real-time PCR 을 통해, 골관절염 환자의 관절연골에서 얻은 연골세포에서뿐만 아니라 IL-1β 의 자극을 받은 연골세포에서 microRNA-449a 의 발현이 증가함을 확인하였다. Western

blotting 을 통해, 대표적인 연골 기질 파괴 효소인 MMP-13 의 발현은 증가한 반면에 제 2 형 콜라겐과 SIRT1 의 발현은 감소됨을 확인하였으며, 이는 골관절염 연골세포와 IL-1β 의 자극을 받은 연골세포에서 일관된 패턴을 나타내었다. 게다가, microRNA 표적 예상 프로그램을 이용하여 SIRT1 mRNA 의 3'-UTR 부분에 microRNA-449a 결합부위가 존재함을 확인하였으며, 또한 이 결합부위가 다양한 종에서 보존되고 있음을 확인하였다. 이러한 결과는 microRNA-449a 가 SIRT1 의 발현을 조절할 수 있음을 시사한다.

이후, 분자적 메커니즘을 알아보고자 연골세포에 SIRT1 을 과발현 시키거나 SIRT1 의 발현을 억제시켰다. SIRT1-과발현 또는 SIRT1-결손 연골세포에 microRNA-449a mimic 또는 anti-microRNA-449a mimic 을 형질 주입한 후, IL-1β 를 처리하였다. Real-time PCR 과 western blotting 을 통해 이화작용 (MMPs, ADAMTSs 등) 또는 동화작용 (제 2 형 콜라겐) 유전자의 발현을 확인하였다. 그 결과, SIRT1-과발현 연골세포에서는 IL-1β 자극에도 이화작용 유전자들의 발현이 억제되었다. 그러나 SIRT1 과발현에 의한 이화작용 유전자들의 발현 억제 효과는, microRNA-449a 에 의해 제거되었다. 이와는 반대로, SIRT1-결손 연골세포에서는 IL-1β 자극에 이화작용 유전자들의 발현이 증가되었다. 그러나 anti-microRNA-449a 를 이용하여 microRNA-449a 의 기능을

억제하였을 때, 이화작용 유전자들의 발현은 현저히 감소되었다. 게다가 제 2 형 콜라겐과 SIRT1 발현이 회복됨을 관찰하였다. 더구나, 골관절염 연골세포에서 anti-microRNA-449a 에 의해 이화작용 유전자들의 발현이 감소되었으며, 이와 반대로 제 2 형 콜라겐과 SIRT1 의 발현은 효과적으로 증가됨을 확인하였다.

요약하면, anti-microRNA-449a 를 이용한 microRNA-449a 의 기능 억제는 SIRT1 의 발현을 증가시킴으로써, IL-1β 자극을 받은 연골세포와 골관절염 연골세포에서 이화작용 유전자들의 발현을 억제하였을 뿐만 아니라 제 2 형 콜라겐과 SIRT1 의 발현을 회복시켰다. 종합해보면, anti-microRNA-449a 는 SIRT1 의 발현을 조절함으로써, 점진적인 연골파괴에 의해 야기되는 골관절염의 치료 표적이 될 것으로 사료된다.

핵심되는 말: microRNA-449a, SIRT1, 연골세포, IL-1β, 골관절염