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Telmisartan Mitigates TNF-α-Induced Type II Collagen Reduction by Upregulating SOX-9

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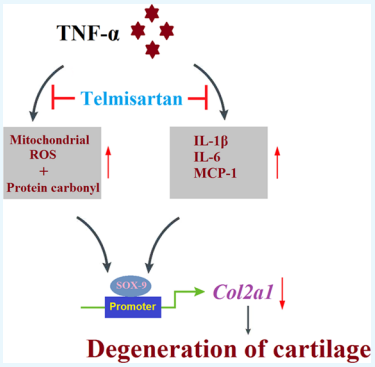
ABSTRACT: The proinflammatory cytokine tumor necrosis factor-α (TNF-α)-induced degradation of extracellular matrix (ECM), such as type II collagen in chondrocytes, plays an important role in the development of osteoarthritis (OA). Telmisartan, an angiotensin II (Ang-II) receptor blocker, is a licensed drug used for the treatment of hypertension. However, the effects of Telmisartan in tumor necrosis factor-α (TNF-α)-induced damage to chondrocytes and the progression of OA are unknown. In this study, we found that treatment with Telmisartan attenuated TNF-α-induced oxidative stress by reducing the levels of mitochondrial reactive oxygen species (ROS) and the production of protein carbonyl in human C28/I2 chondrocytes. Interestingly, Telmisartan inhibited TNF-α-induced expression and secretions of proinflammatory mediators such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemotactic protein 1 (MCP-1). Notably, stimulation with TNF-α reduced the levels of type II collagen at both the mRNA and the protein levels, which was rescued by the treatment with Telmisartan. Mechanistically, we found that Telmisartan restored TNF-α-induced reduction of SOX-9. Silencing of SOX-9 blocked the inhibitory effects of Telmisartan against TNF-α-induced degradation of type II collagen. These findings suggest that Telmisartan might be a potential and promising agent for the treatment of OA.



INTRODUCTION

The degeneration of articular cartilage in osteoarthritis (OA) patients is mainly induced by direct or indirect elements that regulate the synthesis and metabolism of the cartilage matrix.[1] Chondrocytes are the only cells that exist in articular cartilage tissues and play an important role in the processing of cartilage formation, metabolism, and repair.[2] Extracellular matrix (ECM) is mainly secreted by chondrocytes and composed of collagens, glycosaminoglycans (GAGs), and structural proteins, which all maintain the structure and function of the endochondral fibrous grid.[3] Type II collagen is one of the main components of ECM and forms approximately 90% of the total collagen, which makes up the fiber grid structure.[4,5] Cartilage proteoglycans and collagenous fibers (including type II collagens) are the substrates of matrix metalloproteinases (MMPs),[6] the expression level of which can be regulated by endochondral ossification factors (such as hypoxia-inducible factor-α) and inflammatory factors (such as tumor necrosis factor-α (TNF-α)).[7,8] SOX-9 is an important transcriptional factor involved in the development and processing of OA[9] and is found to be significantly downregulated in cartilage tissues of OA patients.[10−12] Loss of proteoglycans, the activation of a disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5), and the degradation of aggrecan can be induced by knocking down the expression of SOX-9,[13] indicating that SOX-9 might be an important transcriptional factor for the protection of cartilage ECM degradation.

Indeed, it has been reported that SOX-9 regulates a specific set of genes in chondrocytes and controls the differentiation of these cells by activating not only cartilage ECM genes but also the genes encoding ECM modification enzymes, membrane receptors, and transporters.[14] However, treatment with TNF-α reduces SOX-9 activity and cartilage matrix gene expression by increasing the activity of nuclear factor-κB (NF-κB) and limiting the availability of p300 in primary cultures of rat chondrocytes.[15] Therefore, targeting SOX-9 using TNF-α inhibitors[16] will be an effective idea for the treatment of OA. Telmisartan (Figure 1A) is a novel antagonist of the angiotensin II (Ang-II) receptor developed by Boehringer Ingelheim and approved by the U.S. Food and Drug Administration in 1998 for the treatment of hypertension.[17] In the clinic, due to its promising antihypertensive effects, inhibitory efficacy on myocardial remodeling, and high

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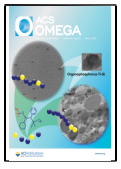

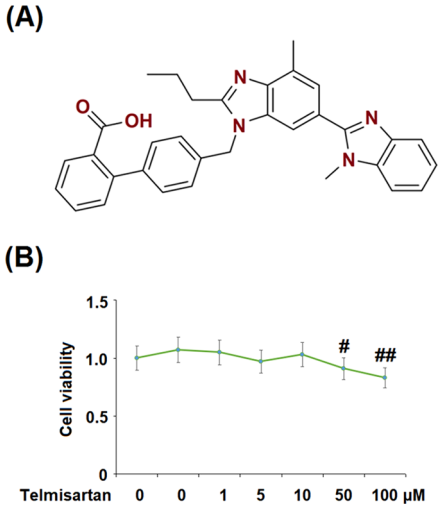

Figure 1. Effects of Telmisartan in cell viability of human C28/I2 chondrocytes. (A) Molecular structure of Telmisartan. (B) Cells stimulated with Telmisartan at concentrations of 0.5, 1, 5, 10, 50, and 100 μM. Cell viability was measured using a cell counting kit-8 (CCK-8) assay (#, ##, $P < 0.05$, 0.01 vs vehicle group).

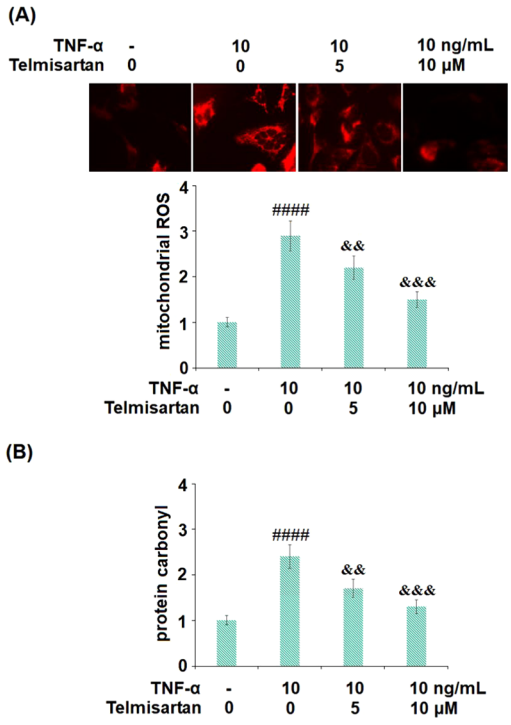

Figure 2. Telmisartan alleviated TNF-α-induced oxidative stress in human C28/I2 chondrocytes. The cells were incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h. (A) Production of mitochondrial ROS. (B) Production of protein carbonyl (####, $P < 0.0001$ vs vehicle group; &&, &&&, $P <$ 0.01, 0.001 vs TNF-α group).

bioavailability, it is widely used for the treatment of hypertension and cardiac remodeling.[18] Telmisartan suppresses the activity of excessively released Ang-II by inhibiting the binding between Ang-II and its receptor, blocking the process of myocardial hypertrophy and protects against heart failure.[19] Recently, significant inhibitory effects of Telmisartan on MMPs and the degradation of ECM have been widely reported.[20,21] In the present study, the protective effect of Telmisartan on TNF-α-induced degradation of ECM in chondrocytes will be investigated to explore the potential therapeutic property of Telmisartan in OA.

RESULTS

Effects of Telmisartan on the Cell Viability of Human C28/I2 Chondrocytes. To screen the optimized concentration of Telmisartan incubated with human C28/I2 chondrocytes, the cells were stimulated with Telmisartan at concentrations of 0.5, 1, 5, 10, 50, and 100 μM. As shown in Figure 1B, as the concentration of Telmisartan increased from 0.5 to 10 μM, no significant difference in the cell viability was observed. However, as the concentration of Telmisartan was elevated to 50 and 100 μM, the cell viability decreased significantly. Therefore, 5 and 10 μM Telmisartan were used in the subsequent experiments.

Telmisartan Alleviated TNF-α-Induced Oxidative Stress. To evaluate the state of oxidative stress in treated C28/I2 chondrocytes, after the cells had been incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h, the production of mitochondrial reactive oxygen species (ROS) and the level of protein carbonyl were detected. As shown in Figure 2A, the level of mitochondrial ROS was significantly elevated by stimulation with TNF-α but greatly suppressed by the introduction of Telmisartan in a dose-dependent manner. In addition, the increased production of protein carbonyl induced by TNF-α was intensely inhibited by the treatment of Telmisartan. These data indicate that the oxidative stress in chondrocytes induced by TNF-α was greatly alleviated by Telmisartan.

Telmisartan Alleviated TNF-α-Induced Expression of Inflammatory Factors. We further investigated the concentrations of inflammatory factors in the treated C28/I2 chondrocytes. As shown in Figure 3A, the elevated gene expression levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and monocyte chemotactic protein 1 (MCP-1) induced by stimulation with TNF-α were greatly suppressed by the introduction of Telmisartan. As shown in Figure 3B, compared with the control, the secretion of IL-1β was increased from 130.5 to 532.7 pg/mL by stimulation with TNF-α but suppressed to 401.4 and 322.8 pg/mL by treatment with 5 and 10 μM Telmisartan, respectively. In addition, the concentration of IL-6 in the control, TNF-α, and 5 and 10 μM Telmisartan groups were 76.4, 377.2, 267.8, and 176.3 pg/mL, respectively. Compared to the control, the production of MCP-1 was elevated from 66.6 to 258.9 pg/mL by stimulation with TNF-α but reduced to 187.2 and 137.3 pg/mL by the introduction of 5 and 10 μM Telmisartan, respectively. These data indicate that the severe inflammation in chondrocytes induced by TNF-α was dramatically ameliorated by treatment with Telmisartan.

Telmisartan Restored TNF-α-Induced Reduction of Col2a1 Gene and Type II Collagen. To evaluate the effect of Telmisartan on TNF-α-induced degradation of ECM in chondrocytes, the cells were incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h, followed by measuring the expressions of the Col2a1 gene and type II collagen. As shown in Figure 4A,B, the expressions of the Col2a1 gene and type II collagen were significantly inhibited by stimulation with TNF-α but greatly upregulated by treatment with Telmisartan, indicating a

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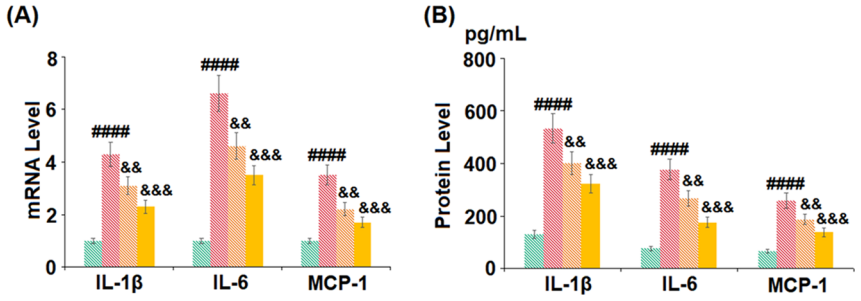

Figure 3. Telmisartan alleviated TNF-α-induced expression of inflammatory factors human C28/I2 chondrocytes. The cells were incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h. (A) mRNA of IL-1β, IL-6, and MCP-1. (B) Secretions of IL-1β, IL-6, and MCP-1 (####, P < 0.0001 vs vehicle group; &&, &&&, P < 0.01, 0.001 vs TNF-α group).

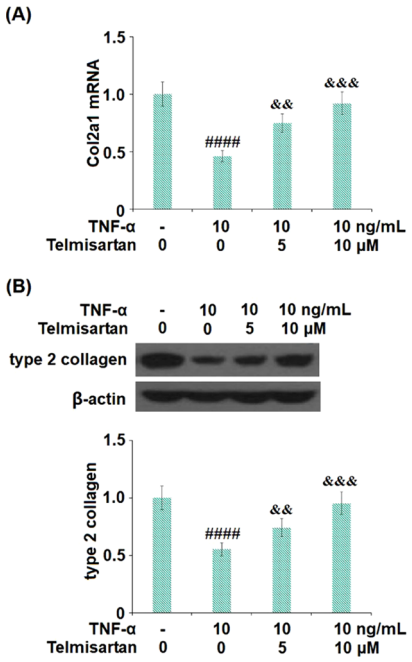

Figure 4. Telmisartan restored TNF-α-induced reduction of Col2a1 gene and type II collagen in human C28/I2 chondrocytes. The cells were incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h. (A) mRNA of Col2a1. (B) Protein of type II collagen (####, P < 0.0001 vs vehicle group; &&, &&&, P < 0.01, 0.001 vs TNF-α group).

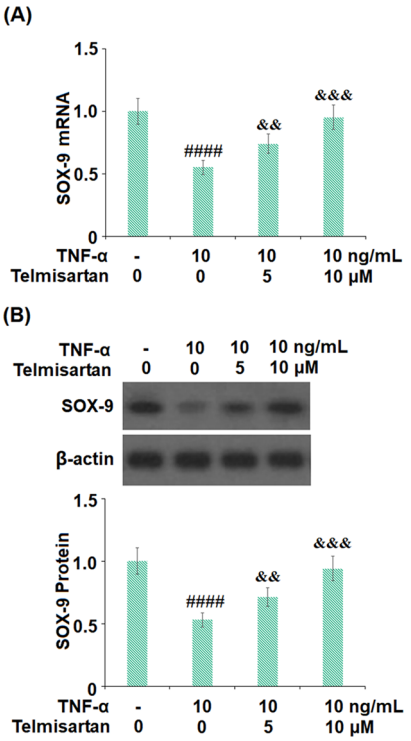

Figure 5. Telmisartan restored TNF-α-induced reduction of SOX-9 in human C28/I2 chondrocytes. The cells were incubated with TNF-α (10 ng/mL) in the presence or absence of Telmisartan (5 and 10 μM) for 24 h. (A) mRNA of SOX-9. (B) Protein of SOX-9 (####, P < 0.0001 vs vehicle group; &&, &&&, P < 0.01, 0.001 vs TNF-α group).

potential protective effect of Telmisartan against ECM degradation in chondrocytes induced by TNF-α.

Telmisartan Restored TNF-α-Induced Reduction of SOX-9. We further investigated the impact of Telmisartan on the expression level of OA-related transcriptional factor SOX-9. As shown in Figure 5, SOX-9 was intensely downregulated in chondrocytes by stimulation with TNF-α but greatly elevated by the introduction of Telmisartan in a dose-dependent manner, indicating that the protective effect of Telmisartan against TNF-α-induced injury to human C28/I2 chondrocytes might be related to the upregulation of SOX-9.

Silencing of SOX-9 Abolished the Protective Effects of Telmisartan against TNF-α-Induced Reduction of the Col2a1 Gene and Type II Collagen. To verify the potential mechanism, we established the SOX-9 knockdown chondrocytes by transfecting cells with siRNA targeting SOX-9, followed by stimulation with TNF-α (10 ng/mL) or Telmisartan (10 μM) for 24 h. As shown in Figure 6A, our findings indicate that exposure to TNF-α significantly reduced the expression of the Col2a1 gene, which was rescued by treatment with Telmisartan. However, silencing of SOX-9 remarkably abolished the protective effects of Telmisartan. Consistently, Western blot results in Figure 6B indicate that the knockdown of SOX-9 impaired the beneficial effects of Telmisartan against TNF-α-induced reduction of type II collagen at the protein levels. These data verified that the protective effect of Telmisartan against TNF-α-induced injury to human C28/I2 chondrocytes was mediated by SOX-9.

DISCUSSION

It is reported that oxidative stress is involved in the pathogenesis of OA by mediating the cellular signaling pathway, regulating the senescence and apoptosis of chondrocytes, accommodating the synthesis and metabolism of ECM, and inducing inflammation in the synovial

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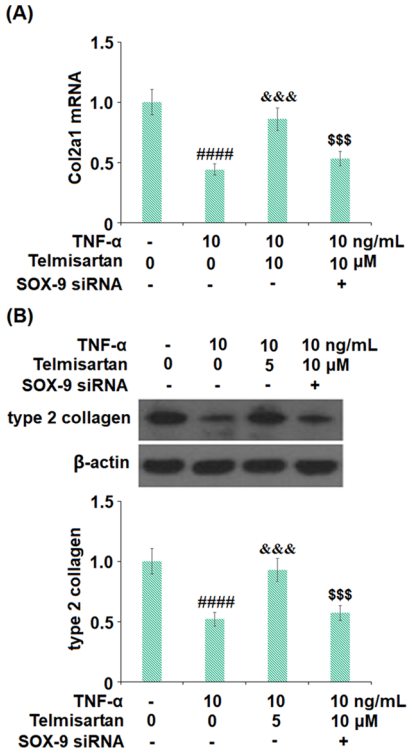

Figure 6. Silencing of SOX-9 abolished the protective effects of Telmisartan against TNF-α-induced reduction of Col2a1 gene and type II collagen in human C28/I2 chondrocytes. The cells were transfected with SOX-9 siRNA, followed by stimulation with TNF-α (10 ng/mL) or Telmisartan (10 μM) for 24 h. (A) mRNA of Col2a1. (B) Protein of type II collagen (####, $P < 0.0001$ vs vehicle group; &&&, $P < 0.001$ vs TNF-α treatment group; $$$, $P < 0.001$ vs TNF-α+Telmisartan group).

membrane.[22] Regularly, the ROS secreted by the catalyzation of NADPH oxidase in chondrocytes are maintained at a certain level and function as important intracellular signal transduction molecules regulating the apoptosis of chondrocytes, the synthesis and degradation of ECM, and the production of inflammatory factors. When chondrocytes are stimulated by inflammatory cytokines or extracellular high oxygen density, excessive release of ROS is induced, contributing to the synthesis of nitric oxide (NO). As a consequence, the synthesis of extracellular proteoglycans is directly inhibited and the expressions of inflammatory factors, such as MMPs, interleukins, and tumor necrosis factors, are upregulated indirectly through activation of the NF-κB signaling pathway.[23] In addition, the irreversible injury to DNA is induced by ROS by acting on the genetic materials in chondrocytes,[24] aggravating the instability of telomerase and triggering the senescence and apoptosis of chondrocytes.[25] In the present study, we established the in vitro injury model on chondrocytes by incubating cells with TNF-α, which was verified by the activated state of oxidative stress and elevated production of inflammatory factors. After treatment with Telmisartan, the activated state of oxidative stress and severe inflammation in chondrocytes induced by TNF-α were significantly reversed, indicating a potential protective effect of Telmisartan on injured chondrocytes. In our future work, the potential therapeutic effect of Telmisartan on OA will be further verified by establishing the OA animal model and treating it with Telmisartan.

Almost all of the components of ECM can be damaged by excessively released ROS, finally contributing to the disruption of cartilage structure. It has been reported that under high oxygen concentration, the collagen molecule is attacked by ROS, resulting in the disruption of the primary, secondary, and tertiary structures of the collagen molecule to stop the collagens from assembling into collagenous fibers, destroying the network structure of the original collagenous fibers, and enhancing the hydrophobicity. Finally, the loss of proteoglycans is induced.[26] Glycosaminoglycans, such as proteoglycan and hyaluronic acid, can also be directly attacked by ROS, giving rise to the disruption of the molecular structure and glucosidic bonds, decreased viscosity, and declined water content. Finally, the injury to the subchondral bone and cartilage inflammation are aggravated.[27] In the present study, in addition to severe inflammation, the degradation of ECM was also induced by stimulation with TNF-α and greatly reversed by treatment with Telmisartan, indicating a potential protective effect of Telmisartan on TNF-α-induced degradation of ECM. Hypothetically, the protective property of Telmisartan on TNF-α-induced degradation of ECM might be related to the excessively released ROS. In our future work, this hypothesis will be further verified by introducing the activator of oxidative stress into the incubation system to better understand the protective effect of Telmisartan on ECM degradation.

SOX-9 is an important transcriptional factor involved in the maintenance of the homeostasis of the ECM in cartilage tissues. Wang[28] reported that the gene expression of chondrocytes ECM could be promoted by Alendronate by regulating the SP-1/SOX-9 axis. Xu[29] reported that IL-1β-induced reduction of extracellular matrix was significantly prevented by agonism of GPR120 by the upregulation of SOX-9. In the present study, we found that the decreased expression level of SOX-9 in chondrocytes induced by TNF-α was greatly reversed by Telmisartan. The protective effects of Telmisartan against TNF-α-induced reduction of the Col2a1 gene and type II collagen in human C28/I2 chondrocytes were dramatically abolished by the silencing of SOX-9, indicating that the upregulation of SOX-9 was involved in the therapeutic mechanism of Telmisartan. In our future work, we will further verify the involvement of SOX-9 in the pharmacodynamic animal experiments by co-administrating a specific inhibitor of SOX-9.

Taken together, for the first time, we illustrated that treatment with Telmisartan mitigated TNF-α-induced damages to human C28/I2 chondrocytes. Telmisartan alleviated both oxidative stress and inflammatory responses caused by TNF-α. Importantly, it mitigated TNF-α-induced reduction of the *Col2a1* gene and type II collagen protein by upregulating SOX-9. This suggests that Telmisartan is a potential therapeutic agent against OA.

MATERIALS AND METHODS

Cell Culture and Treatment, siRNA Transfection. Human C28/I2 chondrocytes were purchased from the Merck Millipore (California) and cultured in Dulbecco's modified Eagle's medium (DEME) (Thermo, Massachusetts) added with 10% fetal bovine serum (FBS) (Thermo, Massachusetts), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo, Massachusetts) at 37 °C and 5% CO_2. To knock down the expression level of SOX-9 in the C28/I2 chondrocytes, the cells were transfected with specific

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siRNA designed against SOX-9 and a transfection reagent lipofectamine 3000 (Thermo, Massachusetts). The cells were then incubated with TNF-α (#ab259410, Abcam) in the presence or absence of Telmisartan (5 and 10 μM) (#ab120831, Abcam) for 24 h.

CCK-8 Assay. To determine the cell viability of the treated C28/I2 chondrocytes, a CCK-8 assay was performed. Briefly, the cells were incubated with 100 μL of CCK-8 reagent, followed by incubation at 37 °C for 2 h. Finally, the absorbance at 450 nm in each well was evaluated using a microplate reader (Thermo, Massachusetts) for the calculation of cell viability.

MitoSOX Red Staining. To detect the mitochondrial ROS level in treated C28/I2 chondrocytes, mitoSOX red staining was performed. First, a 5 mM stock solution was prepared by dissolving 50 μg of MitoSOX dye (Thermo, Massachusetts) in 13 μL of dimethyl sulfoxide (DMSO), followed by addition into a serum-free medium, which was further diluted into a 5 μM working solution. The treated C28/I2 chondrocytes were incubated in a 5 μM working solution at 37 °C for 15 min. The images of the staining were taken using a confocal microscope (Leica, Weztlar, Germany), followed by washing with phosphate-buffered saline (PBS) buffer.

Real-Time PCR Analysis. After collecting the treated C28/I2 chondrocytes, the total RNA was isolated using the TRIzol reagent (Thermo, Massachusetts), followed by reverse transcription into cDNA utilizing the cDNA synthesis kit (Thermo, Massachusetts). Subsequently, RT-PCR was conducted using the SYBR Premix Ex Taq II system (Sigma-Aldrich, California), followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Lastly, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of target genes, with GAPDH taken as the internal control gene.

Western Blot Analysis. After collecting the total protein from the treated C28/I2 chondrocytes using the radioimmunoprecipitation assay (RIPA) buffer, the proteins were quantified with a BCA kit (Beyotime, Shanghai, China). Subsequently, the proteins were loaded and separated with the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to the PVDF membrane (Millipore, Massachusetts). After being blocked with 5% bovine serum albumin (BSA) to remove the nonspecific binding proteins, the membrane was incubated with primary antibody against type II collagen (1:1000, Abcam, Cambridge, U.K.), SOX-9 (1:1000, Abcam, Cambridge, U.K.), and GAPDH (1:1000, Abcam, Cambridge, U.K.) at 4 °C overnight, followed by incubation with secondary antibody (1:2000, Abcam, Cambridge, U.K.) at room temperature for 1.5 h. Finally, the immunoreactive signal was visualized using the LAS-3000 Image Analyzer (Tokyo, Japan) after three washes with the PBST buffer.

ELISA Assay. The treated C28/I2 chondrocytes were centrifuged to collect the supernatant, which was further centrifuged to remove the impurities. Subsequently, the supernatant was applied for the detection of protein carbonyl, IL-1β, IL-6, and MCP-1 using the commercial enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, California) according to the instructions of the manufacturer.[30]

Statistical Analysis. The data are reported as mean ± standard deviation (SD). All experiments were performed at least three times. A one-way analysis of variance and posthoc Tukey's test were performed using GraphPad Prism 7 (GraphPad, California) software. For all analyses, a *P*-value <0.05 was considered to indicate statistical significance.

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Notes

The authors declare no competing financial interest.

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