

# Regulation of type II collagen, matrix metalloproteinase-13 and cell proliferation by interleukin-1 $\beta$ is mediated by curcumin via inhibition of NF- $\kappa$ B signaling in rat chondrocytes

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**Abstract.** Curcumin possesses strong anti-inflammatory, anti-rheumatoid and anti-oxidative activities, and has the potential to inhibit nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling. Cartilage damage in osteoarthritis (OA) is largely mediated by interleukin-1 $\beta$  (IL-1 $\beta$ ) via activation of various transcription factors, including NF- $\kappa$ B and activator protein-1. The aim of the present study was to determine whether IL-1 $\beta$  induces matrix metalloproteinase-13 (MMP-13) expression and inhibits type II collagen expression, as well as to examine whether cell proliferation may be inhibited by curcumin through the inhibition of NF- $\kappa$ B signaling. The effects of curcumin were investigated in rat articular chondrocyte cell cultures treated with IL-1 $\beta$  in the presence or absence of curcumin or the NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate. Western blotting and reverse transcription-quantitative polymerase chain reaction were conducted to evaluate protein and mRNA expression levels of type II collagen, MMP-13, NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ), phosphorylated-I $\kappa$ B $\alpha$  and NF- $\kappa$ B subunit p65/RelA. Western blotting and immunofluorescence were performed to examine the effects of curcumin on the expression, phosphorylation and nuclear translocation of NF- $\kappa$ B-associated proteins. The effects of curcumin on cell proliferation were evaluated by Cell Counting Kit-8 (CCK-8). Curcumin was demonstrated to inhibit the IL-1 $\beta$ -induced activation of NF- $\kappa$ B by suppressing I $\kappa$ B $\alpha$  phosphorylation and p65/RelA nuclear translocation. These events were associated with the downregulation of MMP-13 expression and the

upregulation of type II collagen expression, both of which are considered to be NF- $\kappa$ B targets. CCK-8 assays revealed that co-treatment with curcumin resulted in increased proliferation in IL-1 $\beta$ -treated chondrocytes. These findings implicated curcumin as a naturally occurring anti-inflammatory agent for the treatment of OA via inhibition of NF- $\kappa$ B signaling.

## Introduction

Osteoarthritis (OA) is a chronic degenerative disease that is characterized by articular cartilage degeneration and subchondral osteophyte formation, and exhibits common symptoms, including joint pain and restricted movement (1). Currently, treatment methods for OA are limited to symptomatic approaches or surgery involving prosthesis implantation, primarily due to the lack of targeted treatments that are able to effectively protect against cartilage destruction (2). Certain available treatments, including glucosamine and chondroitin dietary supplements, have been proven to offer moderate protection, but long-term use is required (3). Consequently, there is an urgent requirement to develop therapeutic and preventive options for OA.

OA is primarily caused by an imbalance between the degradation and the synthesis of cartilage extracellular matrix (ECM). During the pathophysiology of OA, chondrocytes are immersed in an inflammatory environment, which may result in loss of cartilage matrix components. Type II collagen is a predominant and important component of the ECM and interacts with proteoglycans, providing the cartilage with the elasticity and capacity for deformation. The degeneration of cartilage is accompanied by a decrease in type II collagen. Previous studies have demonstrated that the expression of matrix metalloproteinases (MMPs) is significantly increased in the chondrocytes of patients with OA and animal models (4-6). Among the MMPs, only MMP-13 has been demonstrated to degrade the ECM directly, whereas other MMP subtypes require the involvement of MMP-13 (5). An abnormal increase in MMP expression is a major cause of the imbalance between synthesis and degradation of cartilage ECM, leading to gradual erosion of articular cartilage, ulcer formation and cartilage degradation (7,8). Interleukin-1 $\beta$  (IL-1 $\beta$ ) is one of the primary

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inflammatory cytokines and stimulates IL-1 receptors and downstream signaling molecules, which subsequently activate the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) (9-11). The NF- $\kappa$ B signaling pathway is important for the regulation of MMP expression levels (12-14). Previous studies have demonstrated that the inhibition of NF- $\kappa$ B activation by curcumin leads to the inhibition of cyclooxygenase 2 expression and increased MMP-mediated degradation of cartilage, whereas other studies have revealed that curcumin protects cartilage cells by upregulating the expression of type II collagen (15-18). Therefore, preventing the loss of type II collagen and/or inhibiting the synthesis of MMPs may provide promising options to protect against cartilage degradation and may be beneficial for the treatment of OA.

Curcumin is a yellow pigment extracted from Zingiberaceae and Araceae turmeric, which is widely used in foods, cosmetics and drugs. Curcumin exhibits a broad range of properties, including inflammatory response inhibition, antioxidative activity and anti-rheumatoid effects. Previously, curcumin has attracted attention for its potential to inhibit NF- $\kappa$ B signaling (15-17). The aim of the present study was to investigate whether curcumin may reverse the IL-1 $\beta$ -induced downregulation of type II collagen, and whether curcumin was able to inhibit the catabolic effects of MMP-13 by suppressing NF- $\kappa$ B activation and NF- $\kappa$ B-induced gene expression.

## Materials and methods

**Reagents.** Curcumin and collagenase II were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and a 10 mM stock solution was prepared in dimethyl sulfoxide, and diluted with cell culture medium immediately prior to use.

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; ThermoFisher Scientific, Inc.) and 100 IU/ml penicillin, 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

Recombinant human IL-1 $\beta$  was supplied by PeproTech, Inc. (Rocky Hill, NJ, USA). Primary antibodies against type II collagen (AB746) and MMP-13 (AB39012) were purchased from Abcam (Cambridge, UK). Primary antibodies against NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ; 4814S), phosphorylated (p)-I $\kappa$ B $\alpha$  (9246S) and NF- $\kappa$ B p65/RelA (8242S) were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA). The selective NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate (PDTC), primary antibodies against GAPDH (AB8245) and lamin B1 (AB16048), and horseradish peroxidase (HRP)-conjugated secondary antibody (AB7097) were obtained from Abcam. Cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc.).

**Chondrocyte isolation and culture.** A total of eight 5-week-old male rats (300-410 g) were purchased from Shanghai SLAC Laboratory Animal, Co., Ltd. and kept in common rabbit cages and fed a standard diet with tap water *ad libitum*. All the rat procedures were approved by the Institutional Care and Use Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. The rats were anaesthetized with pentobarbital sodium (40-45 mg/kg)

and the articular cartilage was isolated from femoral and tibial articular joints of by aseptic dissection following air injection into the ear vein. Following two washes with PBS, the cartilage sections were treated with 2 mg/ml pronase (EMD Millipore, Billerica, MA, USA) in serum-free DMEM at 37°C for 1 h in 5% CO<sub>2</sub>, followed by overnight digestion with 0.25 mg/ml collagenase II dissolved in serum-free DMEM. Isolated chondrocytes were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All experiments were performed on chondrocytes between passage 1 and 3. Fibroblasts were used as control group, according to the manufacturer's protocol for VECTASTAIN® ABC kit (AK-5000) and toluidine blue staining kit (H-5000) were provided by Vector Laboratories (Southfield, MI, USA). The chondrocytic phenotype of the cultured cells was confirmed by toluidine blue staining of glycosaminoglycan and immunocytochemical staining.

**Experimental design.** To exclude stimulation that may be caused by other cytokines or growth factors present in the FBS, chondrocytes were serum-starved and exposed to 10 ng/ml IL-1 $\beta$  for 24 h. Experiments were designed to mimic the cellular events that occur in OA. To acquire the optimum timepoint and concentration of curcumin for the treatment of chondrocytes, chondrocytes were co-treated with either of the following: i) 10 ng/ml IL-1 $\beta$  and 50  $\mu$ M curcumin for various time periods (0, 12, 24, 36 or 48 h); or ii) 10 ng/ml IL-1 $\beta$  and various concentrations of curcumin (0, 25, 50, 75, or 100  $\mu$ M) for 36 h. To investigate p65/RelA translocation and I $\kappa$ B $\alpha$  phosphorylation, chondrocytes were pretreated with 10 ng/ml IL-1 $\beta$  in the presence or absence of 50  $\mu$ M curcumin for 0, 10, 20, 30, 40 or 60 min, after which nuclear and cytoplasmic extracts were prepared using a nuclear and cytoplasmic protein extraction kit (P0028; Beyotime Institute of Biotechnology) following the manufacturer's protocol. The experiments were performed in triplicate.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from cells (1.2x10<sup>6</sup> cells/well) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and 2  $\mu$ g was reverse-transcribed into cDNA using an Advantage® RT-for-PCR kit (639506; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. qPCR was performed using SYBR Green PCR master mix (Takara Bio, Inc., Otsu, Japan), in accordance with the manufacturer's protocols. The primer sequences used are presented in Table I. The PCR conditions were set as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec with a final extension at 72°C for 3 min. mRNA levels were normalized to levels of the endogenous control GAPDH, and the relative expression levels were calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (19). Data were obtained from three independent experiments performed in triplicate.

**Protein extraction and western blot analysis.** Chondrocytes (1.2x10<sup>6</sup> cells/well) were washed twice with ice-cold PBS, lysed with Radioimmunoprecipitation Assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'→3')	Expected size (bp)
GAPDH	F: CCCCAATGTATCCGTTGTG R: CTCAGTGTAGCCCAGGATGC	124
MMP-13	F: CGTTCAAGGAATCCAGTCTCTC R: TCCACATGGTTGGGAAGTTC	231
Type II collagen	F: GCAAGAATCCCGCTCGCA R: TGGGTTGGGGTAGACGCA	158
IκBα	F: GAAATACCCCTCTCCATCTTGC R: ACATCAGCCCCACACTTCAA	298
p65/RelA	F: GACCTGGAGCAAGCCATTAG R: CACTTTGTACACAGCAAGAAGA	285

Bp, base pairs; F, forward; R, reverse; MMP-13, matrix metalloproteinase-13; IL-1β, interleukin-1β; IκBα, nuclear factor-κB inhibitor α.

1 mM phenylmethylsulfonyl fluoride on ice for 30 min, and centrifuged at 16,992  $\times g$  at 4°C for 20 min. Protein concentrations were determined using the Bicinchoninic Acid method (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts (60  $\mu\text{g}$ ) of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, and subsequently incubated with the following primary antibodies: anti-type II collagen (1:200), anti-MMP-13 (1:200), anti-IκBα (1:200), anti-p-IκBα (1:200), anti-NF-κB p65/RelA (1:200), anti-GAPDH (1:1,000) and anti-lamin B1 (1:1,000) at 4°C overnight. Following three washes with Tris-buffered saline containing 0.05% Tween-20, the membranes were incubated with goat anti-rat IgG H&L HRP-conjugated secondary antibody (1:2,000; AB7097) at room temperature for 1 h. The immunoblots were analyzed by Enhanced Chemiluminescence (ECL) with an ECL Plus kit (Beyotime Institute of Biotechnology), and the bands were quantified by ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH expression. Data were obtained from three independent experiments performed in triplicate.

**Immunofluorescence microscopy.** Cells ( $1.2 \times 10^6$  cells/well) were seeded onto glass coverslips in 24-well plates, cultured for 24 h at 37°C, pre-incubated with serum-starved DMEM for 1 h at 37°C, and subsequently stimulated with IL-1β (10 ng/ml) alone, IL-1β (10 ng/ml) and curcumin (50  $\mu\text{M}$ ), or IL-1β (10 ng/ml) and PDTC (0.1 mmol/l) for 30 min at 37°C. The cells were washed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100/PBS for 15 min. Following blocking in PBS containing 10% normal goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 1 h, the cells were incubated with anti-p65/RelA (1:50) overnight at 4°C, followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rat IgG H&L (PE) secondary antibody (1:50; AB7010; Abcam, Cambridge, UK) for 1 h at room temperature. Finally, cells were rinsed in PBS three times and covered with Fluoromount mountant (Gallard-Schlesinger Industries, Garden City, NY,

USA). Fluorescence signals were examined and imaged under a Zeiss Axioskop 40 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) by using Image-J software version 1.45 (National Institutes of Health).

**Cell proliferation assay.** Cell proliferation was evaluated using a Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology) in accordance with the manufacturer's protocol. Chondrocytes were treated with IL-1β for 24 h prior to curcumin, and subsequently co-treated with either curcumin or PDTC, a combination of curcumin plus PDTC, or were untreated. Cells ( $6 \times 10^3$  cells/well) were seeded into 96-well plates and incubated for 12, 24, 48 or 72 h at 37°C. Following this, 10  $\mu\text{l}$  of CCK-8 was added to each well (90  $\mu\text{l}$  medium was mixed with 10  $\mu\text{l}$  CCK-8) and the plates were incubated at 37°C for 2 h. Following incubation, the absorbance was measured at a wavelength of 450 nm using an automated plate reader. The cell viability was calculated as a percentage of the viable cells in the curcumin-treated group compared with the untreated control. Each experiment was repeated three times independently.

**Statistical analysis.** All experiments were carried out at least three times, and the data are presented as the mean  $\pm$  standard deviation. Statistical analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Comparisons between experimental and control data were evaluated by Student's t-test or analysis of variance. Multiple groups were compared using analysis of variance, the comparison between groups using LSD test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Regulation of type II collagen and MMP-13 expression in rat chondrocytes by IL-1β.** The chondrocytic phenotype of the isolated rat chondrocytes was confirmed by toluidine blue staining of glycosaminoglycan and immunocytochemical staining of type II collagen (Fig. 1). RT-qPCR and western blot analyses were performed to assess the regulation of MMP-13 and type II collagen mRNA and protein expression



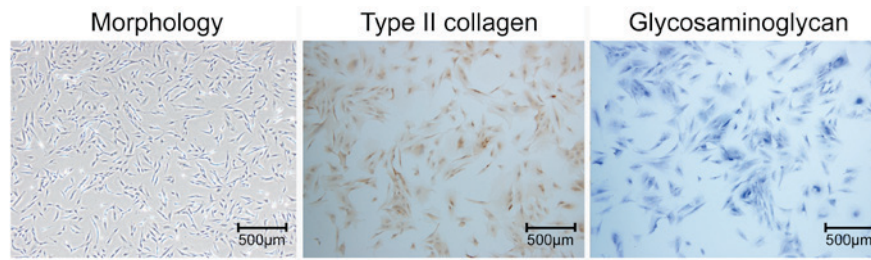


Figure 1. Confirmation of chondrocyte phenotype by toluidine blue staining of glycosaminoglycan and immunocytochemical staining of type II collagen in isolated rat chondrocytes under light microscopy.

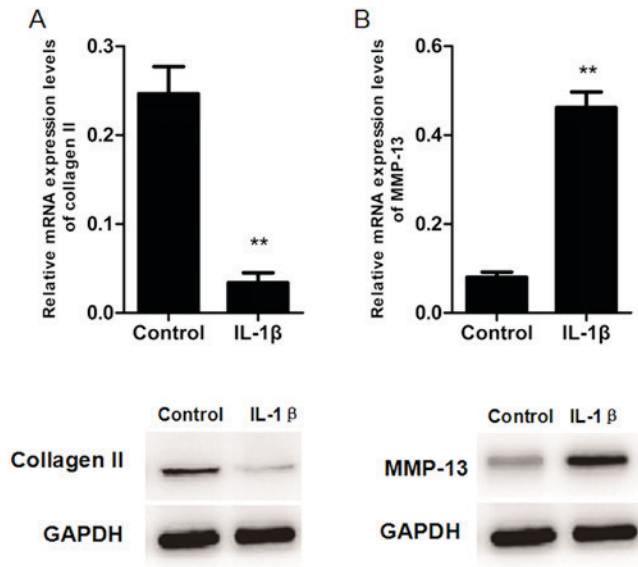


Figure 2. Regulation of type II collagen and MMP-13 by IL-1 $\beta$  in rat chondrocytes. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analyses demonstrating that IL-1 $\beta$  stimulation decreases type II collagen and increases MMP-13 mRNA and protein expression levels. GAPDH served as an internal control. Data are presented as the mean  $\pm$  standard deviation. \*\*P<0.01. IL-1 $\beta$ , interleukin-1 $\beta$ ; MMP-13, matrix metalloproteinase-13.

by IL-1 $\beta$  using chondrocytes that were cultured in the presence or absence of IL-1 $\beta$  for 24 h. IL-1 $\beta$  treatment significantly decreased the expression of type II collagen (Fig. 2A) and markedly increased the expression of MMP-13 (Fig. 2B). These results indicated that IL-1 $\beta$  induces MMP-13 expression, but inhibits type II collagen expression in rat chondrocytes.

*Curcumin inhibits MMP-13 expression and upregulates type II collagen expression levels.* To determine the optimum conditions of curcumin, serum-starved rat chondrocytes were treated with IL-1 $\beta$  (10 ng/ml) for 24 h and subsequently cultured in medium containing 50  $\mu$ M curcumin. The chondrocytes were harvested after 0, 12, 24, 36 or 48 h and the expression levels of MMP-13 and type II collagen proteins were examined by western blot analysis. As shown in Fig. 3, MMP-13 protein expression was significantly decreased, whereas type II collagen was markedly increased in chondrocytes following treatment with curcumin compared with the untreated group (0 h). Moreover, the decrease in MMP-13 expression was lowest at 36 h, and the increase in type II collagen peaked at 48 h.

Following this, the optimum concentration of curcumin for treatment of IL-1 $\beta$ -treated chondrocytes was determined. Rat chondrocytes were treated 10 ng/ml IL-1 $\beta$  and various concentrations of curcumin (0, 25, 50, 75, or 100  $\mu$ M) for 36 h. As presented in Fig. 4, curcumin markedly upregulated the expression of type II collagen, and significantly inhibited the expression of MMP-13 in IL-1 $\beta$ -pretreated chondrocytes at each concentration compared with the untreated group (0  $\mu$ M). The optimum concentration by which curcumin had the strongest effect on type II collagen and MMP-13 expression levels was determined to be 50  $\mu$ M.

*Curcumin suppresses IL-1 $\beta$ -induced nuclear translocation of p65/RelA.* To investigate the effect of curcumin on IL-1 $\beta$ -induced NF- $\kappa$ B activation, serum-starved chondrocytes were treated with IL-1 $\beta$  in the presence or absence of curcumin for 0-60 min and then subjected to cell component fractionation. The resultant cytoplasmic and nuclear extracts were used for western blot analysis to examine the protein expression of p65/RelA. The results demonstrated that IL-1 $\beta$  treatment led to a decrease in the cytoplasmic expression and an increase in the nuclear translocation of p65/RelA, which accumulated in the nucleus in a time-dependent manner (Fig. 5A). However, upon co-treatment with curcumin, neither the cytoplasmic nor the nuclear expression of p65/RelA exhibited a significant difference at any of the indicated time points compared with IL-1 $\beta$ -treated chondrocytes (Fig. 5B). Protein expression levels of p65/RelA decreased in the cytoplasm (Fig. 5C) and gradually increased in the nucleus (Fig. 5D) over time with IL-1 $\beta$  treatment.

As curcumin may stabilize p65/RelA in the cytoplasm (20), the effect of curcumin on the IL-1 $\beta$ -induced nuclear translocation of p65/RelA was further investigated by immunofluorescence staining. IL-1 $\beta$ -stimulated chondrocytes exhibited clear nuclear translocation of p65/RelA from the cytoplasm (Fig. 6), whereas co-treatment with curcumin and PDTC notably blocked nuclear translocation of p65/RelA. Taken together, these findings suggested that curcumin inhibits IL-1 $\beta$ -induced nuclear translocation of p65/RelA and suppresses IL-1 $\beta$ -induced NF- $\kappa$ B activation in chondrocytes.

*Curcumin inhibits IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  phosphorylation.* The phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  are essential for the nuclear translocation of p65/RelA and the activation of NF- $\kappa$ B signaling. Therefore, the ability of curcumin to inhibit IL-1 $\beta$ -induced phosphorylation and degradation of I $\kappa$ B $\alpha$  was examined. Chondrocytes were

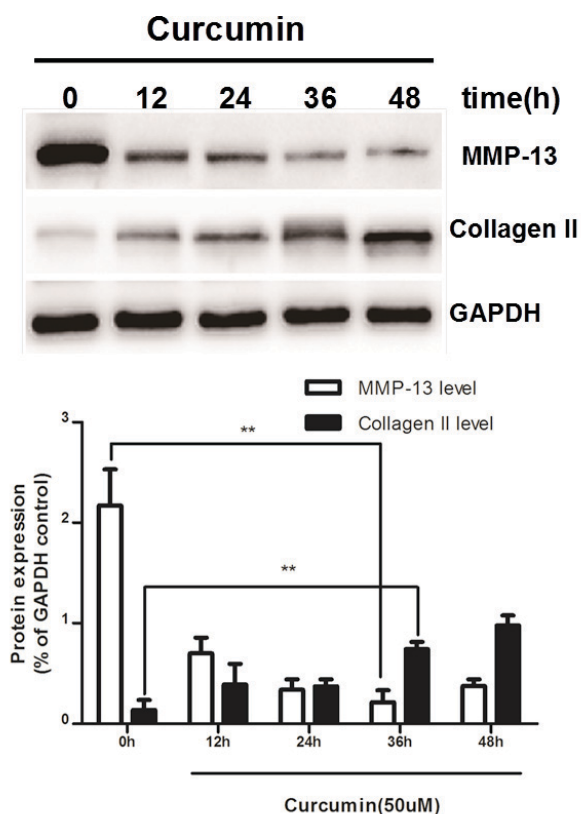


Figure 3. Curcumin inhibits MMP-13 expression and upregulates type II collagen expression in a time-dependent manner. Western blotting and quantification of MMP-13 and type II collagen protein expression levels following curcumin treatment at various time points. GAPDH served as an internal control. Data are presented as the mean  $\pm$  standard deviation; \*\* $P$ <0.01. MMP-13, matrix metalloproteinase-13.

serum-starved and then treated with IL-1 $\beta$  in the presence or absence of curcumin for 0-60 min. The phosphorylation of I $\kappa$ B $\alpha$  in IL-1 $\beta$ -treated chondrocytes increased markedly and peaked at 40 min (Fig. 7A). However, upon co-treatment with IL-1 $\beta$  and curcumin, no significant differences in I $\kappa$ B $\alpha$  phosphorylation were observed compared with IL-1 $\beta$ -treated chondrocytes (Fig. 7B). These findings suggested that curcumin may block IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  phosphorylation in rat chondrocytes.

**Effect of curcumin on chondrocyte proliferation and I $\kappa$ B $\alpha$  phosphorylation.** To elucidate the potential pharmacological effects of curcumin on OA, CCK-8 assays were performed to evaluate cell proliferation. Proliferation of IL-1 $\beta$ -treated chondrocytes was demonstrated to gradually increase following any of the three treatments compared with the control, whereas proliferation gradually decreased in untreated cells. Furthermore, although the proliferation activity of each pharmacologic intervention group increased significantly compared with IL-1 $\beta$ -treated chondrocytes, there were no significant differences in the cell proliferation activity among the groups treated with curcumin, PDTC or curcumin plus PDTC (Fig. 8).

The effects of curcumin and PDTC on the phosphorylation of I $\kappa$ B $\alpha$  were further examined. Consistent with the previous data (Fig. 2B), curcumin and PDTC inhibited the

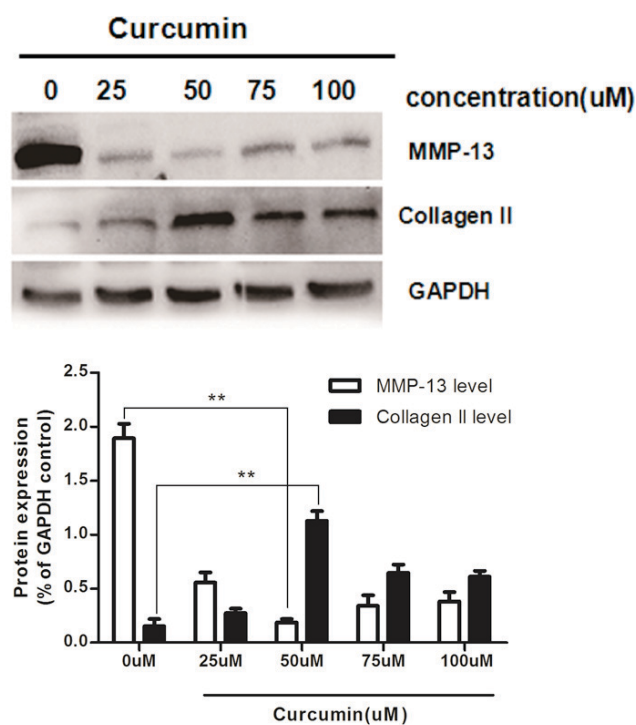


Figure 4. Curcumin inhibits MMP-13 expression and upregulates type II collagen expression in a concentration-dependent manner. Western blotting and quantification of MMP-13 and type II collagen protein expression levels were examined following treatment with various concentrations of curcumin. GAPDH served as an internal control. Data are presented as the mean  $\pm$  standard deviation; \*\* $P$ <0.01. MMP-13, matrix metalloproteinase-13.

expression of p-I $\kappa$ B $\alpha$  and MMP-13 proteins, but upregulated type II collagen protein expression levels compared with the control (Fig. 9A); no significant changes to I $\kappa$ B $\alpha$  expression were observed. Curcumin and PDTC treatments also led to the downregulated expression of MMP-13 and p65/RelA mRNA, and upregulated type II collagen mRNA expression levels compared with the control; no significant changes to I $\kappa$ B $\alpha$  expression were observed compared with the control. (Fig. 9B). Co-treatment of IL-1 $\beta$ -stimulated chondrocytes with curcumin and PDTC did not cause further inhibition of MMP-13 expression or upregulation of type II collagen expression beyond that caused by treatment with curcumin or PDTC alone. Notably, no significant differences were observed in cell proliferation or the expression levels of MMP-13 and type II collagen in the pharmacological intervention groups, and chondrocytes co-treated with curcumin plus PDTC did not exhibit enhanced effects in proliferation or MMP-13 and type II collagen expression levels compared with cells treated with curcumin or PDTC alone, suggesting that the regulation of chondrocytes by curcumin and PDTC may follow the same underlying molecular mechanism.

## Discussion

OA involves the breakdown of joint tissues in response to a number of factors, including aging, stress and trauma. The OA process can be simulated experimentally by stimulating chondrocytes with IL-1 $\beta$  or TNF- $\alpha$ , which serve prominent

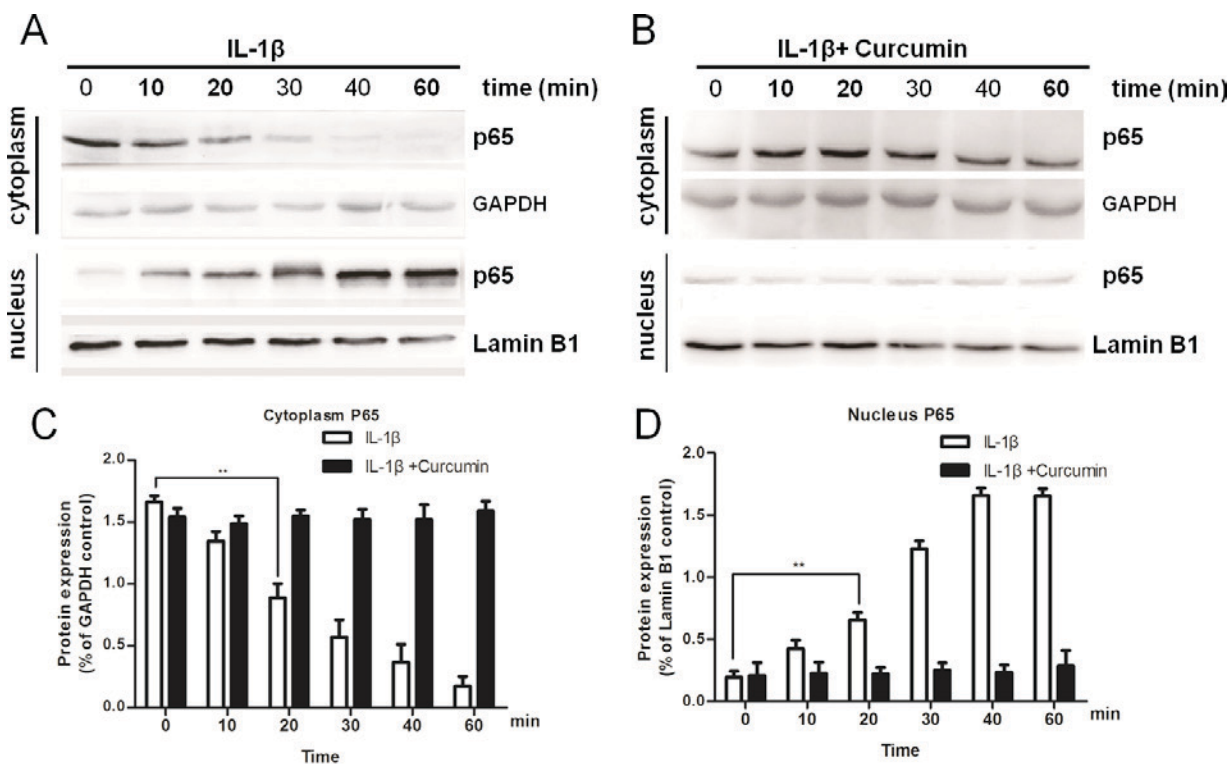


Figure 5. Curcumin inhibits IL-1 $\beta$ -induced nuclear translocation of nuclear factor- $\kappa$ B subunit p65/RelA. Western blot analysis of p65/RelA protein expression levels in chondrocytes treated with either (A) IL-1 $\beta$  alone or (B) IL-1 $\beta$  and curcumin for various durations. Quantification of (C) cytoplasmic and (D) nuclear p65/RelA protein expression levels. GAPDH and lamin B1 served as internal controls. Data are presented as the mean  $\pm$  standard deviation; \*\*P<0.01. IL-1 $\beta$ , interleukin-1 $\beta$ .

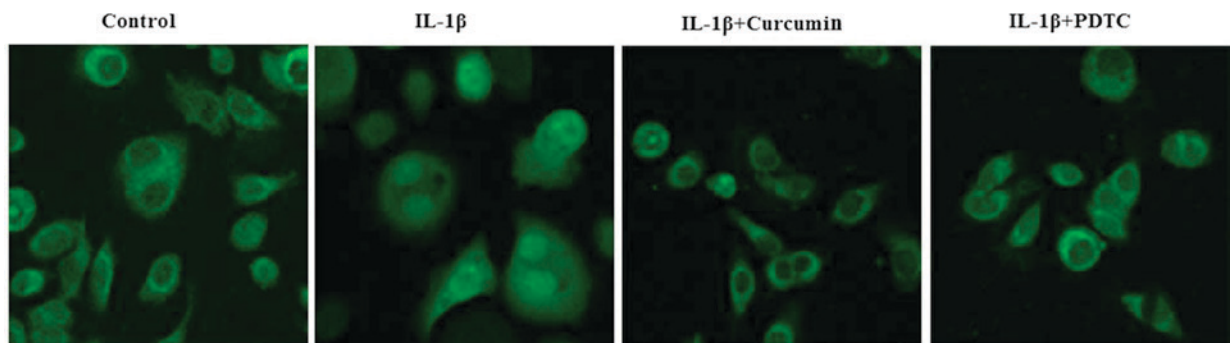


Figure 6. Immunofluorescence staining of nuclear factor- $\kappa$ B subunit p65/RelA. Curcumin and PDTC block IL-1 $\beta$ -induced nuclear translocation of p65/RelA. PDTC, pyrrolidine dithiocarbamate; IL-1 $\beta$ , interleukin-1 $\beta$ .

roles in the articular cartilage catabolism (21-23). Type II collagen is a predominant and important component of the cartilage matrix, and a decrease in type II collagen expression is one of the hallmarks of cartilage degeneration. Type II collagen occupies the vast majority of space in healthy cartilage tissue. During the process of cartilage tissue degeneration, type II collagen proteins and polysaccharides are primarily destroyed by decomposition, and protein contents become clearly decreased. In the present study, the effects of curcumin on the expression of type II collagen in IL-1 $\beta$ -stimulated rat chondrocytes were investigated. The results revealed that the IL-1 $\beta$ -inhibited expression of type II collagen was markedly reversed by curcumin, suggesting that curcumin may have a protective effect on IL-1 $\beta$ -induced cartilage degeneration.

IL-1 $\beta$  induces inflammatory conditions and increases the production of protein-degrading enzymes including MMPs, particularly collagenase-2 (24). Remodeling and breakdown processes of the cartilage matrix are primarily regulated by MMPs, which lead to cleavage of the ECM components. MMP-13 has a higher affinity for cleaving the ECM than other MMPs, and is regarded as a crucial enzyme for cartilage degradation during the progression of OA (25,26). Inhibition of MMPs may prevent the loss of cartilage ECM and cartilage degradation. Therefore, to investigate the preventive effect of curcumin on cartilage degradation, IL-1 $\beta$  was used to stimulate chondrocytes. As expected, IL-1 $\beta$  treatment significantly induced MMP-13 expression, leading to degradation of the ECM produced by the chondrocytes corresponding to the protein contents of type II collagen. Curcumin suppressed



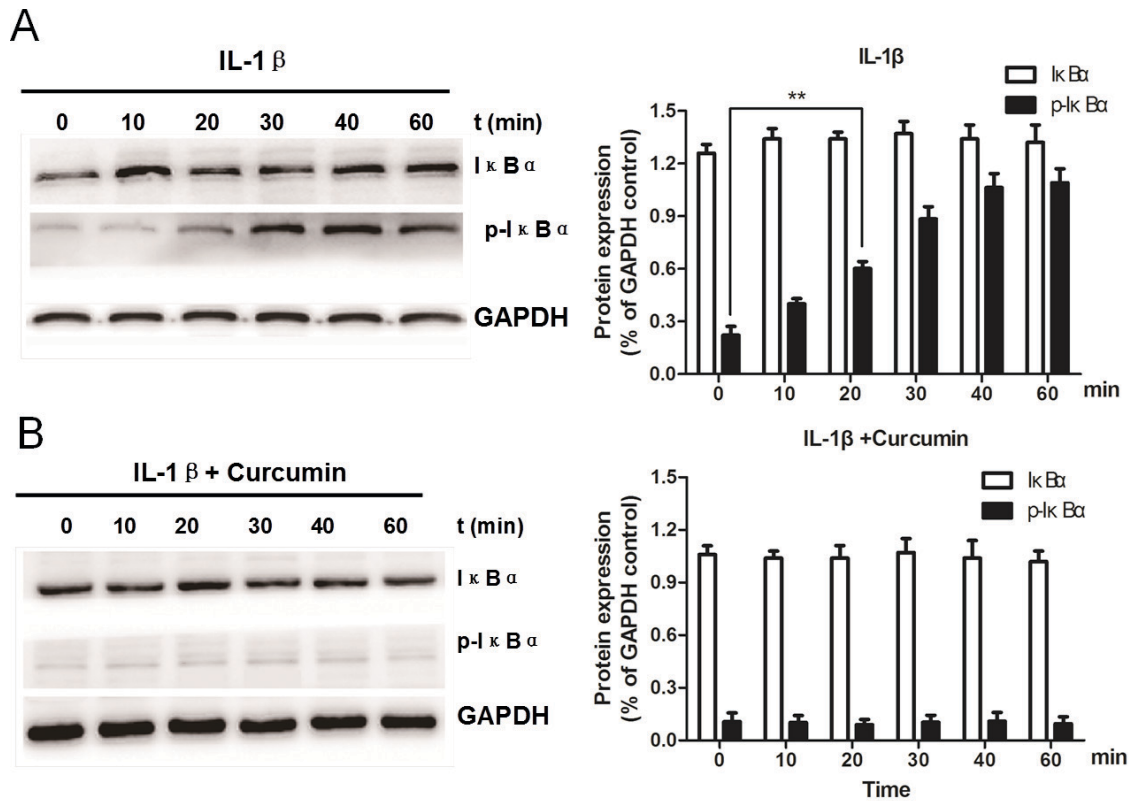


Figure 7. Curcumin inhibits IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  phosphorylation in rat chondrocytes. Western blot analysis and quantification of I $\kappa$ B $\alpha$  and p-I $\kappa$ B $\alpha$  protein expression levels following treatment with either (A) IL-1 $\beta$  alone or (B) IL-1 $\beta$  and curcumin for various durations. GAPDH served as an internal control. Data are presented as the mean  $\pm$  standard deviation; \*\*P<0.01. IL-1 $\beta$ , interleukin-1 $\beta$ ; I $\kappa$ B $\alpha$ , nuclear factor- $\kappa$ B inhibitor  $\alpha$ ; p, phosphorylated.

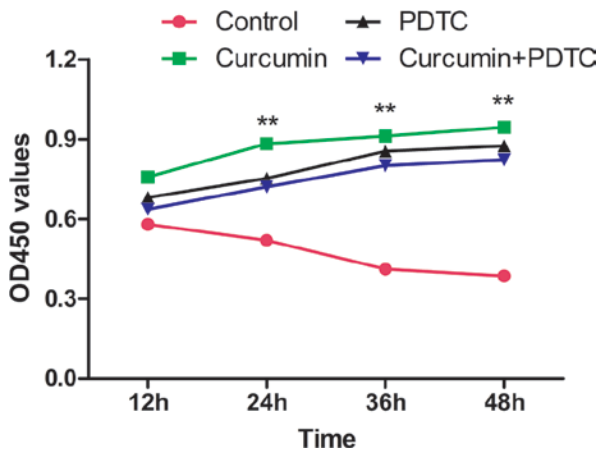


Figure 8. Effect of curcumin on chondrocyte proliferation activity. Isolated rat articular chondrocytes were treated with 10 ng/ml interleukin-1 $\beta$  for 24 h, and subsequently co-treated with 50  $\mu$ M curcumin, 0.1 mmol/l PDTC, or curcumin plus PDTC. Proliferation of chondrocytes at 12, 24, 36 and 48 h was assessed by Cell Counting kit-8 assays. Data are presented as the mean  $\pm$  standard deviation. \*\*P<0.01. PDTC, pyrrolidine dithiocarbamate; OD450, optical density 450.

the expression of MMP-13 in IL-1 $\beta$ -stimulated chondrocytes. Thus, curcumin may inhibit cartilage degradation during inflammatory factor-mediated joint degeneration.

During the treatment of OA, it is important to maintain healthy cartilage matrix metabolism and reduce cartilage degradation. In the future, the most effective treatment for OA will be one that both blocks inflammatory factor-mediated

cartilage destruction and improves the stability of the cartilage matrix. The results of the present study indicated that curcumin had a protective effect on cartilage by reversing the IL-1 $\beta$ -induced decrease in type II collagen and inhibiting IL-1 $\beta$ -induced increase in MMP-13 expression. Taken together, as a small-molecule chemopreventive agent, curcumin may have a role in the treatment of OA.

Curcumin suppresses NF- $\kappa$ B activation via direct inhibition of I $\kappa$ B $\alpha$  phosphorylation, which induces the retention of NF- $\kappa$ B in the cytoplasm and thus interferes with NF- $\kappa$ B binding to DNA to regulate the transcription of target genes (17,18,27). In the inactive state, the p65/RelA subunit of NF- $\kappa$ B is retained in the cytoplasm, whereas activated p65/RelA is immediately translocated into the nucleus where it binds to DNA and regulates the transcription of its target genes (28). In the present study, IL-1 $\beta$ -induced nuclear translocation of p65/RelA in rat chondrocytes was clearly observed by cell component fractionation analyses and immunofluorescence staining. Furthermore, curcumin blocked IL-1 $\beta$ -induced nuclear translocation of p65/RelA, presumably by inhibiting IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  phosphorylation. The present study further demonstrated that NF- $\kappa$ B signaling was involved in the regulation of type II collagen and MMP-13 expression levels in IL-1 $\beta$ -stimulated chondrocytes by using an inhibitor of NF- $\kappa$ B activation. Clutterbuck *et al* (29,30) reported that treatment with curcumin at concentrations >100  $\mu$ M for 48 h or 5 days led to the death of chondrocytes, and that the release of glycosaminoglycan by tissues cultured *in vitro* was suppressed

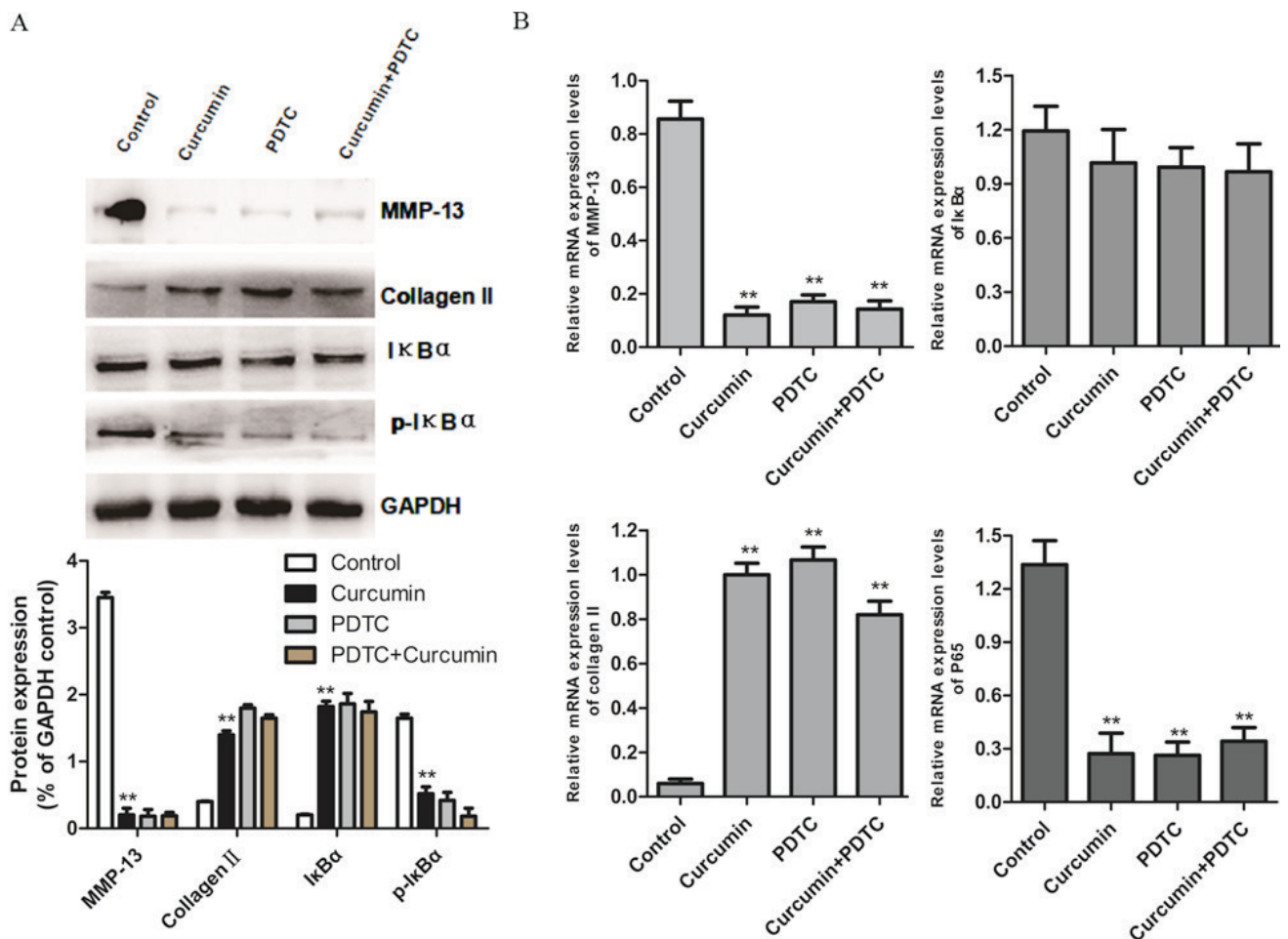


Figure 9. Effect of curcumin and PDTC on protein and mRNA expression levels in interleukin-1 $\beta$ -treated chondrocytes. (A) Western blot analysis of MMP-13, type II collagen, I $\kappa$ B $\alpha$  and p-I $\kappa$ B $\alpha$  protein expression levels. GAPDH served as an internal control. (B) Quantification of MMP-13, type II collagen, I $\kappa$ B $\alpha$  and NF- $\kappa$ B subunit p65/RelA mRNA expression levels, as assessed by reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean  $\pm$  standard deviation; \*\*P<0.01. PDTC, pyrrolidine dithiocarbamate; NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B $\alpha$ , NF- $\kappa$ B inhibitor  $\alpha$ ; p, phosphorylated; MMP-13, matrix metalloproteinase-13.

by curcumin at high concentrations, which subsequently inhibited the proliferation of chondrocytes. However, no significant effects of 50  $\mu$ M curcumin treatment on survival and migration of chondrocytes were identified in the present study (data not shown), thereby indicating the safety of the curcumin concentration employed.

A previous study demonstrated that multiple signaling pathways are involved in the regulation of MMPs, including the p38, extracellular signal-regulated kinase, c-Jun N-terminal kinase, activator protein-1 and NF- $\kappa$ B signaling pathways (31). However, it remains unclear whether a single or numerous signaling pathways are involved in this process. PDTC is an antioxidant that inhibits the degradation of I $\kappa$ B $\alpha$  by blocking the *de novo* phosphorylation of I $\kappa$ B $\alpha$ , thereby preventing NF- $\kappa$ B activation. The present study primarily focused on the effects of curcumin on the NF- $\kappa$ B signaling pathway. The results revealed that curcumin, in addition to PDTC, inhibited IL-1 $\beta$ -induced I $\kappa$ B $\alpha$  phosphorylation and subsequent nuclear translocation of p65/RelA, supporting the hypothesis that curcumin may be an effective treatment for OA through the inhibition of NF- $\kappa$ B signaling. The present results further revealed that curcumin had similar effects as PDTC in decreasing the

expression of MMP-13 and increasing the expression of type II collagen in IL-1 $\beta$ -stimulated chondrocytes. Furthermore, co-treatment with curcumin and PDTC did not cause further inhibition of MMP-13 or upregulation of type II collagen compared with either treatment alone, suggesting that the NF- $\kappa$ B signaling pathway may be the primary molecular pathway for the effects of curcumin on IL-1 $\beta$ -stimulated chondrocytes.

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