Costunolide inhibits matrix metalloproteinases expression and osteoarthritis via the NF-κB and Wnt/β-catenin signaling pathways

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Abstract. Osteoarthritis (OA) is a chronic joint disease involving cartilage erosion and matrix degradation. Costunolide is a sesquiterpene lactone that has been demonstrated to exert anti-inflammatory activities in a wide variety of cells. The aim of the present study was to investigate the effect of costunolide in OA treatment, using rat chondrocytes and an OA rat model, in which animals were subjected to destabilization of the medial meniscus. The results revealed that costunolide (2-6 μM) had no effect on chondrocyte viability or phenotype maintenance. Costunolide decreased the interleukin (IL)-1β-induced upregulation of matrix metalloproteinases (MMPs), inducible nitric oxide synthase, cyclooxygenase-2 and IL-6, and increased the expression of collagen II and transcription factor SOX-9, which were inhibited by IL-1β. Costunolide significantly decreased p65 phosphorylation induced by IL-1β and the translocation of p65 into the nucleus of rat chondrocytes, as observed by western blot analysis and immunofluorescence staining. In addition, activation of the Wnt/β-catenin signaling pathway was inhibited by costunolide, as demonstrated by the level of activation of β-catenin and the transfer of β-catenin into the nucleus induced by IL-1β. *In vivo*, cartilage treated with costunolide exhibited attenuated degeneration and lower Mankin scores compared with the OA group. The present study investigated the anti-osteoarthritic effects of costunolide, which exerted anti-inflammatory activities and inhibited MMPs expression.

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Taken together, these results indicate that costunolide may have a potential value in the treatment of OA.

Introduction

Osteoarthritis (OA) is a common degenerative joint disease associated with progressive loss of articular cartilage, formation of osteophytes and synovial inflammation, particularly in elderly patients (1-3). It is widely recognized that inflammatory cytokines serve pivotal roles in the pathogenesis of OA. Among these, interleukin (IL)-1β is considered the most important inflammatory cytokine in OA. Stimulation of IL-1β leads to decreased expression of collagen II and transcription factor SOX-9 (SOX9), which are phenotypic markers of chondrocytes (4,5). IL-1β may also induce the expression of inducible nitric oxide synthase (INOS) and cyclooxygenase-2 (COX-2) in chondrocytes, which leads to increased levels of nitric oxide (NO) and prostaglandin E2 (PGE2) (6). The action of PGE2 has been confirmed in joint pain and bone resorption (7,8). NO is produced in excess by INOS, and has been demonstrated to increase the production of inflammatory cytokines and matrix metalloproteinases (MMPs) in OA (9,10). Cytokines cause degradation of cartilage matrix by upregulating the expression of MMPs (11).

The MMP family is considered to serve a major role in the pathophysiology of OA, as MMPs lead to the breakdown of the extracellular matrix (ECM) and their expression is increased in the cartilage of patients with OA (12). Among the members of the MMP family, MMP-1, MMP-13 and MMP-3 are indispensable for cartilage degradation. The primary role of MMP-1 and MMP-13 is to degrade aggrecans and collagens, which are the major components of the cartilage matrix (13). Although numerous drugs have been approved for treating this disease, none appear to delay the progression of OA. Corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs) and hyaluronan are drugs currently used for the treatment of OA. However, they cannot prevent subsequent cartilage degradation, but only relieve OA symptoms. Additionally, multiple patients with OA may eventually require surgery. Therefore, there is a necessity for more optimal agents to treat OA (14,15).

Costunolide is a sesquiterpene lactone, which is a group of bioactive compounds that can be isolated from various

plants (16). The pharmacological activities of costunolide include anti-inflammatory (17), antitumor (18), antimicrobial (19) and antioxidant (20) effects. Previous studies focused on the molecular mechanisms of costunolide in effectively decreasing the activation of inflammatory signaling pathways, including the NF-κB and Wnt/β-catenin signaling pathways (21,22). It is well recognized that these pathways are involved in the progression of OA and may be an effective target for OA therapy (22,23). The anti-inflammatory activity of costunolide may exert a potential therapeutic effect on diseases associated with inflammatory mediators. However, to the best of our knowledge, no studies have examined the effects of costunolide in the treatment of OA at present. Therefore, the present study investigated whether costunolide inhibited the progression of OA via the Wnt/β-catenin and NF-κB signaling pathways by analyzing the effects of costunolide in an OA rat model *in vivo* and in rat chondrocytes *in vitro*.

Materials and methods

Reagents. Costunolide (purity >98%; Fig. 1A) was obtained from Nantong Jingwei Fine Chemical Co., Ltd., and was dissolved in dimethyl sulfoxide (DMSO). Rat IL-1β was purchased from R&D Systems, Inc. Chloral hydrate and DMSO were purchased from Sigma-Aldrich (Merck KGaA). FBS, Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin, 0.25% trypsin and collagenase II were obtained from Gibco (Thermo Fisher Scientific, Inc.).

Cell culture. The articular cartilage was harvested from the femoral heads of rats under sterile conditions. The obtained cartilage was cut into small pieces and digested with 0.25% trypsin for 15 min to remove unwanted tissues and cells, followed by digestion with 0.2% collagenase II in an incubator at 37°C for 5 h to obtain dispersed chondrocytes. Subsequently, the chondrocytes were suspended in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Then, the chondrocytes were seeded in tissue culture flasks at 37°C with 95% air and 5% CO_2. These cells were considered to be passage 0 (P0). In order to increase the number of chondrocytes, cells were split at a ratio of 1:3 when cells were at 80% confluence. To avoid phenotype loss, all experiments were conducted using chondrocytes of P2-P3.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 kit (Nanjing KeyGen Biotech Co., Ltd.) was used according to the manufacturer's instructions to assess the cytotoxicity of various concentrations of costunolide. The cells, which were in the logarithmic growth phase, were seeded into 96-well plates ($4x10^3$ cells/ml). The culture medium was replaced with medium containing costunolide (0, 2, 4, 6, 8, 10 or 16 μM) for 24 and 48 h. Then, 10 μl CCK-8 solution was added to each well and incubated at 37°C for 4 h. Following incubation, the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader. Each experiment was repeated 3 times independently.

Safranin O staining. To analyze the effect of costunolide on chondrocyte phenotype changes, safranin O staining was used.

Upon seeding in 12-well plates ($5x10^5$ cells/ml), chondrocytes were treated with different concentrations (0, 2, 4, 6, 8, 10 and 16 μM) of costunolide for 48 h. The cells were then stained with 0.1% safranin O solution for 5 min followed by fixation with 4% paraformaldehyde solution for 10 min (both at room temperature). Images of the cells were captured using a gross camera (ILCE-7M3K; Sony Corporation) upon washing with PBS for 3 times.

Cell treatment. Chondrocytes were plated overnight in 6-well plates at a density of $2x10^5$ cells/well. Next, chondrocytes were preincubated with different concentrations of costunolide (2, 4 and 6 μM) at 37°C for 1 h followed by stimulation with IL-1β (10 ng/ml) for 24 h to analyze the mRNA expression of MMPs, INOS, IL-6 and COX-2. Similarly, other chondrocytes were seeded in 25 cm² flasks ($5x10^5$ cells/ml) to analyze the levels of protein expression.

The cells were also pretreated with costunolide (2, 4 and 6 μM) for 1 h and then stimulated with IL-1β for 30 min to analyze the NF-κB signaling pathway, or for 3 h to analyze the Wnt/β-catenin signaling pathway. Then, total intracellular proteins were extracted used RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) to investigate the activation of the NF-κB and Wnt/β-catenin signaling pathways.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from chondrocytes using a TRIzol® Plus RNA Purification kit (Invitrogen; Thermo Fisher Scientific, Inc.). The absorbance at 260 nm (A260)/A280 ratio was calculated to verify the quality and purity of RNA. Total RNA was used to synthesize cDNA by RT with PrimeScript™ RT Master Mix (Takara Biotechnology Co., Ltd.); the reaction was conducted at 37°C for 15 min, 85°C for 5 sec, and then terminated at 4°C. The mRNA levels of the target gene were analyzed by RT-qPCR using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: 30 sec at 95°C for the initial denaturation, then 40 cycles of 15 sec at 95°C, 32 sec at 60°C and 1 min at 72°C, followed by 5 min at 72°C. The level of target mRNA was normalized to the level of 18S and compared with the control. The primers used are listed in Table I. All gene analyses were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta Cq}$ method (24).

Western blot analysis. Upon washing twice with PBS, 100 μl RIPA lysis buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors was added to stimulated cells. The extracted protein was analyzed by using a BCA quantification kit, and protein (30 μg//lane) was resolved by 10% SDS-PAGE prior to being transferred to a polyvinylidene difluoride membrane. Following blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature, the membranes were incubated overnight at 4°C with antibodies against MMP-3 [rabbit monoclonal antibody (mAb); cat. no. ab52915 Abcam], MMP-9 (rabbit mAb; cat. no. ab76003; Abcam), MMP-13 (rabbit mAb; cat. no. sc-30073; Santa Cruz Biotechnology, Inc.), collagen II (rabbit mAb; cat. no. ab34712; Abcam), GAPDH (rabbit mAb; cat. no. ab70699; Abcam), INOS (rabbit

Table I. Reverse transcription quantitative polymerase chain reaction primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
Rat MMP3	CAGGCATTGGCACAAAGGTG	GATAACCATCCGAGCGACCTTT
Rat MMP9	GCAAACCCTGCGTATTTCCAT	GATAACCATCCGAGCGACCTTT
Rat MMP13	GCAAACCCTGCGTATTTCCAT	GATAACCATCCGAGCGACCTTT
Rat IL-6	AGCGATGATGCACTGTCAGA	GGAACTCCAGAAGACCAGAGC
Rat INOS	CCTTACGAGGCGAAGAAGGACAG	CAGTTTGAGAGAGGAGGCTCCG
Rat COX-2	GAGAGATGTATCCTCCCACAGTCA	GACCAGGCACCAGACCAAAG
Rat 18S	CCTGAGAAACGGCTACCACA	ACCAGACTTGCCCTCCAATG

MMP, matrix metalloproteinase; IL-6, interleukin-6; INOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

mAb; cat. no. ab3523; Abcam), IL-6 (10E5; mouse mAb; cat. no. sc-57315; Santa Cruz Biotechnology, Inc.), COX-2 (D5H5; rabbit mAb; cat. no. 12282; Cell Signaling Technology, Inc.), SOX9 (rabbit mAb; cat. no. ab185966Ab; Abcam), β-actin (mouse mAb; cat. no. ab8226; Abcam), β-catenin (D10A8; rabbit mAb; cat. no. 8480p; Cell Signaling Technology, Inc.), active non-phosphorylated (p)-β-catenin (Ser45; D2U8Y; rabbit mAb; cat. no. 19807S; Cell Signaling Technology, Inc.), transcription factor p65 (p65; C22B4; rabbit mAb; cat. no. 4764S; Cell Signaling Technology, Inc.), p-p65 (Ser536; rabbit Ab; cat. no. 3031; Cell Signaling Technology, Inc.), NF-κB inhibitor α (IκB-α; rabbit mAb; cat. no. 4812; Cell Signaling Technology, Inc.) and p-IκB-α (Ser32; 14D4; rabbit mAb; cat. no. 2859; Cell Signaling Technology, Inc.). All primary antibodies were used at a 1:1,000 dilution. Then, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse secondary antibodies (1:1,000; cat. nos., A0208 and A0216; Beyotime Institute of Biotechnology) was incubated with the membranes at room temperature for 2 h. Protein bands were visualized using an ECL kit (Immobilon Western Chemiluminescent HRP Substrate; cat. no. WBKLS0050; Merck KGaA) and analyzed with Quantity One software v4.6.6 (Bio-Rad Laboratories, Inc.). GAPDH or β-actin were used as controls in all western blot analyses.

Immunofluorescence staining. Upon fixing with 4% paraformaldehyde for 15 min at room temperature, chondrocytes were permeabilized with PBS containing 0.3% Triton X-100 for 15 min and then blocked with 5% BSA for 1 h at room temperature. Chondrocytes were then incubated with rabbit monoclonal anti-p65 antibody (1:500) or rabbit monoclonal anti-β-catenin antibody (1:500) at 4°C overnight, and then incubated with Alexa Fluor 555-labeled Donkey Anti-Rabbit IgG (H+L) (cat. no. A0453; Beyotime Institute of Biotechnology) or Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (H+L) (cat. no. A0423; Beyotime Institute of Biotechnology) secondary antibodies (1:1,000) for 2 h in the dark at room temperature. Cells were counterstained with DAPI (1:1,000) for 5 min and analyzed using a Leica fluorescence microscope (magnification, x100; Leica Microsystems, Inc.).

Animal experiments. Sprague-Dawley rats (6-week-old; Animal Center of Zhejiang University) weighing 1.8-2.4 kg were used in the present study. All rats were housed 3 animals/cage

at room temperature (24±2°C) and at a relative humidity of 55±5% with controlled lighting (12 h light/dark cycle). Food and water were routinely provided in the facility *ad libitum*. There were a total of 30 rats included, and 20 of them underwent surgical destabilization of the medial meniscus (DMM) in the knee joints to construct a rat model of OA. The remaining 10 rats (sham group) received sham surgeries. Prior to surgery, all rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) without observing any signs of peritonitis, and then the effect of the anesthetic was evaluated by measuring the breathing, nerve reflexes and muscle relaxation. A total of 1 week after the surgeries, the rats in the costunolide group were intra-articularly injected with 6 μM costunolide once a week for 8 weeks, while the OA group was injected with the same volume of PBS in both knees under the same conditions. The health and behavior of rats were monitored every day from the first postoperative day until sacrifice. Following the final intra-articular injection of costunolide, rats were euthanized with 100% CO_2. The flow rate of CO_2 was 20% of the chamber volume per minute. Loss of breathing and fading of eye color were monitored during the procedure, which usually takes 2-3 min. Following observation of these events, the flow of CO_2 was maintained for 1 min, and then the animals were removed from the chamber. A combination of criteria was used to confirm death, including lack of pulse, breathing and inability to hear heartbeat by use of stethoscope, in compliance with AVMA guidelines (25). This experiment was conducted according to the National Institutes of Health guidelines (26), and the protocol was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China; approval no. 2015-107).

Histological examination. Knee joint samples from each group were first fixed in 4% paraformaldehyde and decalcified until becoming soft following ~2 months at room temperature. Subsequently, the samples were dehydrated in a graded alcohol series (95% followed by 100%), embedded in paraffin and cut into 3-μm sections. Paraffin sections were stained with safranin O-fast green (1:100) for ~5 min at room temperature and graded according to the Mankin scoring system (27) to assess the degree of histological change in the different groups. A total of five fields per sample were analyzed for the different groups (magnification, x40; BX51-P; Olympus Corporation).
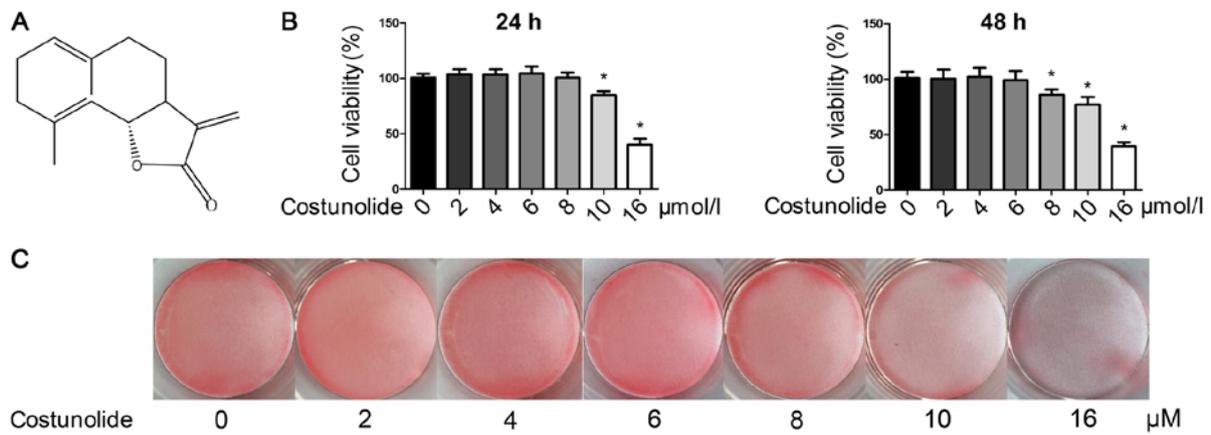
Figure 1. Effect of costunolide on chondrocyte viability and phenotype maintenance. (A) Chemical structure of costunolide. Chemical formula, $C_{15}H_{20}O_2$; molecular weight =232.32 Da. (B) The cytotoxic effect of costunolide on chondrocytes proliferation was determined by a Cell Counting Kit-8 assay at various concentrations for 24 and 48 h. Values are expressed as the mean ± standard deviation of 3 independent experiments (n=3). *P<0.05 vs. 0 μmol/l. (C) Safranin O-stained chondrocytes following treatment with different concentrations of costunolide for 48 h.
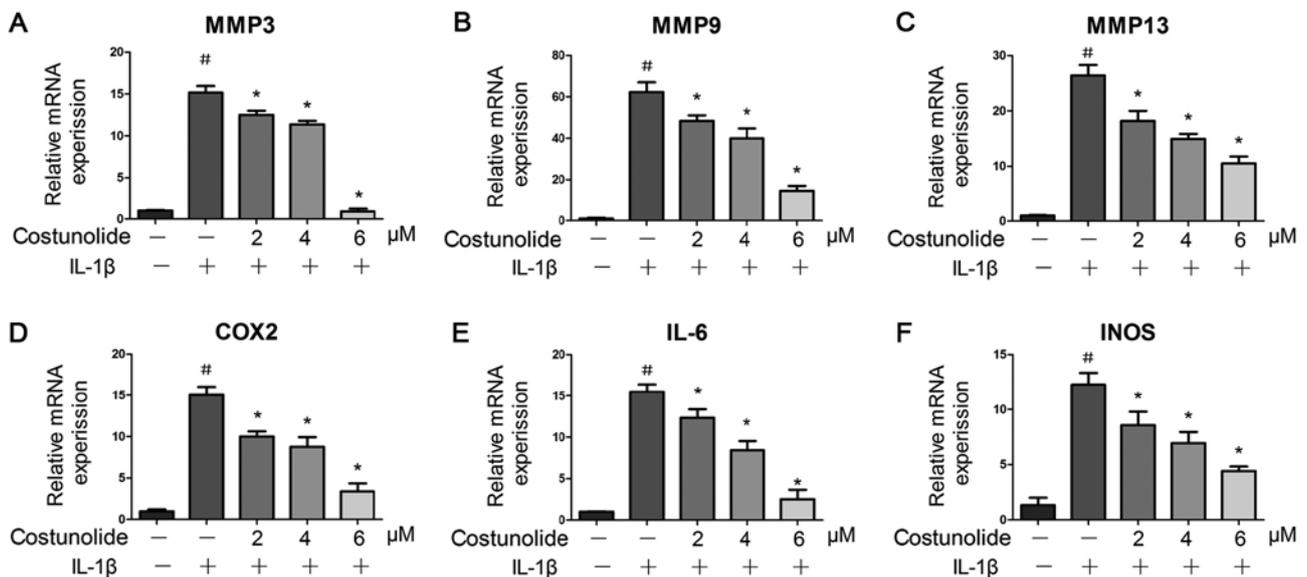

Figure 2. Effects of costunolide on IL-1β-induced gene expression. Levels of (A) MMP-3, (B) MMP-9, (C) MMP-13, (D) COX-2, (E) IL-6 and (F) INOS were measured by reverse transcription-quantitative polymerase chain reaction. The chondrocytes were pretreated for 1 h with various concentrations of costunolide (0, 2, 4 and 6 μM) and then stimulated with or without IL-1β (10 ng/ml) for 24 h. The data are expressed as the mean ± standard deviation of three experiments (n=3). *P<0.05 vs. samples stimulated with IL-1β in the absence of costunolide. #P<0.05 vs. the control group. MMP, matrix metalloproteinase; COX2, cyclooxygenase-2; IL, interleukin; INOS, inducible nitric oxide synthase.

Immunohistochemistry. Immunohistochemical analyses were performed to evaluate MMP-13 and COX-2 expression on cartilage. The tissue sections were permeabilized with xylene for 10 min twice and then rehydrated in a graded alcohol series. Then, the sections were treated with pepsin for 20 min for antigen retrieval after the peroxidase activity in the samples had been quenched by incubation with 3% H_2O_2 for 10 min. The sections were then incubated with primary antibodies (1:500) against MMP-13 (rabbit mAb; cat. no. sc-30073; Santa Cruz Biotechnology, Inc.) and COX-2 (rabbit mAb; cat. no. 12282; Cell Signaling Technology, Inc.) overnight at 4°C following blocking with 5% BSA for 1 h at room temperature. HRP-conjugated secondary antibodies (1:1,000) were then added to the sections for 1 h at room temperature, and 3,3'-diaminobenzidine (1:1,000; Sigma-Aldrich; Merck

KGaA) was used as a chromogenic agent at room temperature. A total of five fields per sample were analyzed for the different groups (magnification, x400; BX51-P; Olympus Corporation).

Statistical analysis. All data are presented as the mean ± standard deviation of 3 experiments used GraphPad Prism 5 (GraphPad Software, Inc.). One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of costunolide on chondrocyte viability and phenotype maintenance. To evaluate the cytotoxicity of costunolide, chondrocytes were treated with various concentrations of
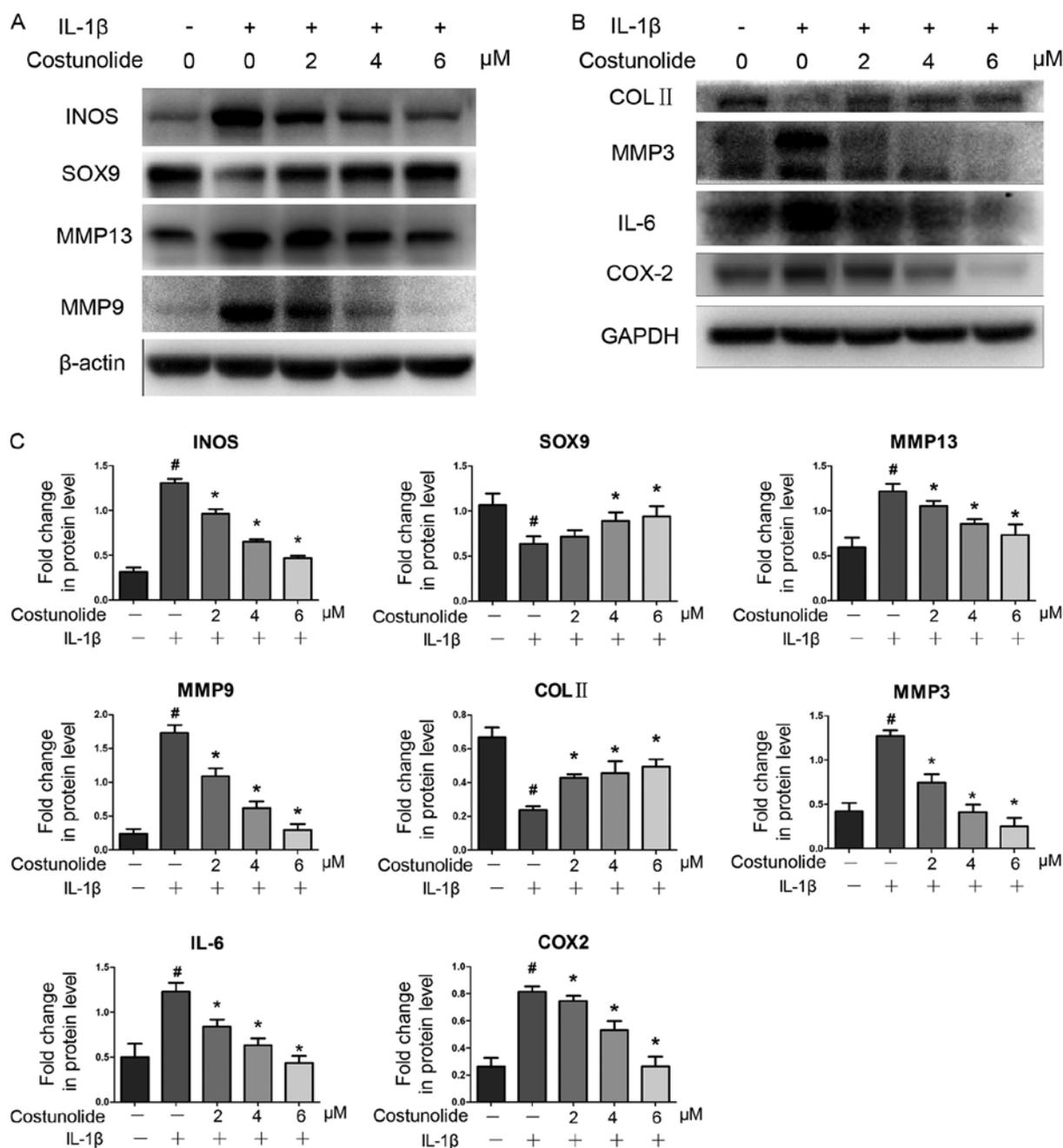
Figure 3. Effects of costunolide on inflammation-associated protein expression. IL-1β-pretreated chondrocytes were incubated with various concentrations of costunolide for 24 h in the presence (+) or absence (-) of IL-1β. (A) Representative gel of INOS, SOX9, MMP-13 and MMP-9 expression. (B) Representative gel of COL II, MMP-3, IL-6 and COX-2 expression. (C) Densitometric quantification of the protein concentrations. The data are presented as the mean ± standard deviation of three experiments (n=3). *P<0.05 vs. samples stimulated with IL-1β in the absence of costunolide. #P<0.05 vs. the control group. INOS, inducible nitric oxide synthase; SOX9, transcription factor SOX-9; MMP, matrix metalloproteinase; IL, interleukin; COL II, collagen II; COX2, cyclooxygenase-2.

costunolide (0, 2, 4, 6, 8 and 16 μM) and a CCK-8 assay was performed 24 or 48 h later. As demonstrated in Fig. 1B, the results of CCK-8 indicate that concentrations ≤6 μM had no noticeable toxic effects on the viability of chondrocytes after 24 or 48 h. Chondrocyte phenotype was detected by safranin O staining, and the images revealed that costunolide did not affect the loss of safranin O staining at concentrations ranging from 0-6 μM (Fig. 1C). Therefore, the subsequent experiments were performed with 2, 4 and 6 μM of costunolide to avoid cytotoxicity, and 6 μM costunolide was used for the animal experiments.

Effects of costunolide on gene expression and protein levels in chondrocytes. Release of inflammatory mediators and matrix degradation are representative features of OA. Therefore, the present study evaluated the effect of costunolide on the expression of inflammatory genes including INOS, IL-6 and COX-2, and matrix-degrading genes including MMP-3, MMP-9 and MMP-13, at the mRNA (Fig. 2A-F) and protein levels (Fig. 3A-C). IL-1β significantly upregulated the mRNA and protein expression levels of INOS, IL-6, COX-2, MMP-3, MMP-9 and MMP-13, whereas pretreatment with costunolide resulted in significant inhibition of IL-1β induction at the
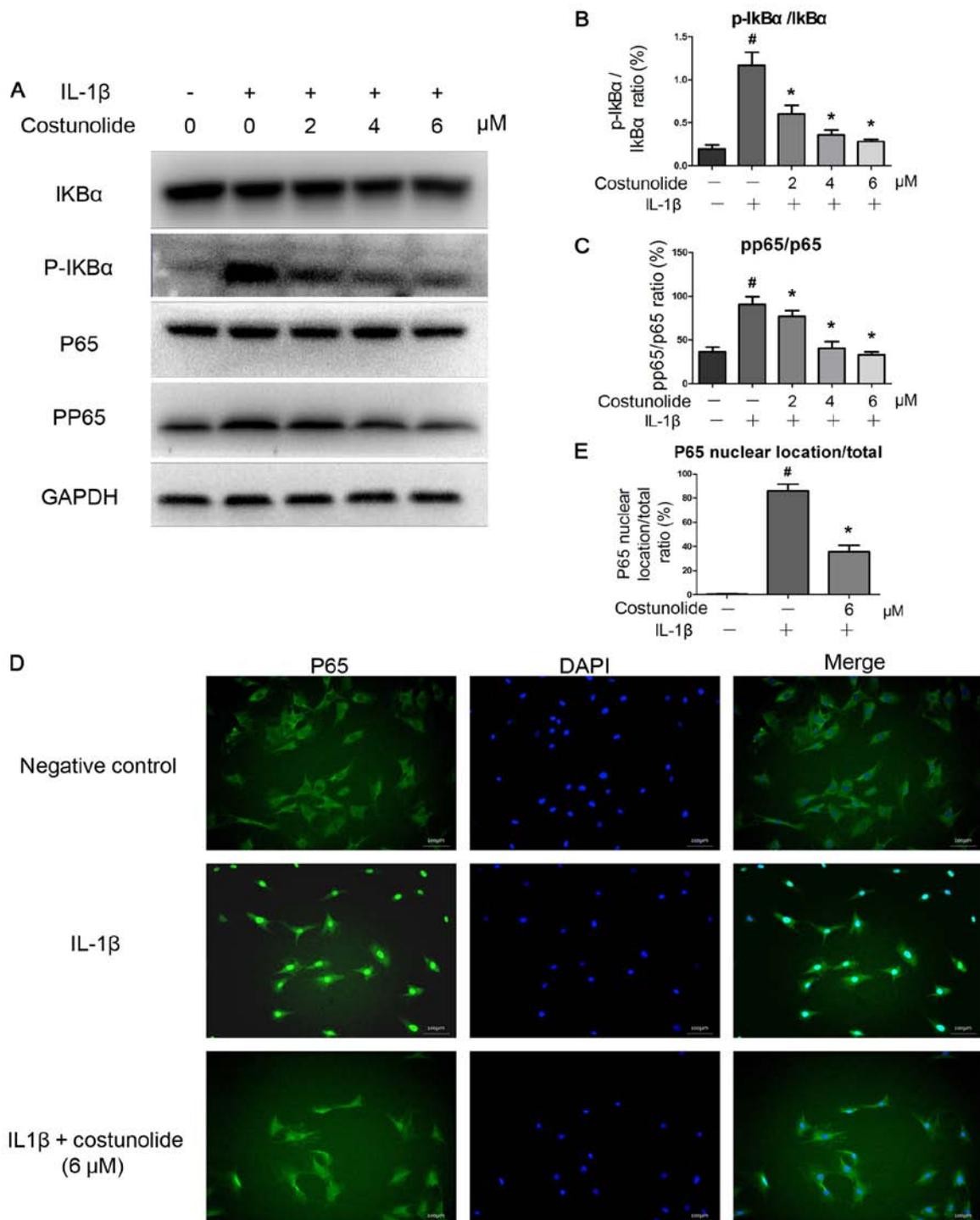
Figure 4. Effect of costunolide on IL-1β-induced NF-κB signaling activation in chondrocytes. Chondrocytes were pretreated with costunolide (0, 2, 4 and 6 μM) for 1 h followed by IL-1β (10 ng/ml) for 30 min. (A) Western blot analysis of the effect of costunolide on the NF-κB signaling pathway. (B and C) The expression of p-p65 was compared to p65 as well as p-IKBα and IKBα. (D) The nuclear translocation of p65 was detected by the immunofluorescence combined with DAPI staining. Blue, DAPI; green, p65. Scale bar=100 μM. (E) Quantitative data for p65 nuclear distribution/total ratio as photographed in. (D) The data are typical results from three independent experiments with similar results (n=3). *P<0.05 compared with samples stimulated with IL-1β in the absence of costunolide. #P<0.05 vs. cell culture without costunolide or IL-1β. IL, interleukin; p, phosphorylated; IκB-α, NF-κB inhibitor α.

mRNA and protein levels. Western blot analysis revealed that costunolide reversed the downregulation of SOX9 and collagen II at the protein level, which was induced by IL-1β (Fig. 3A-C). Therefore, the effect of costunolide involved the inhibition of the IL-1β-induced expression of matrix-degrading genes while maintaining chondrocyte gene expression *in vitro* to protect rat chondrocytes.

IL-1β activated the NF-κB signaling pathway. However, costunolide was able to decrease the IL-1β-induced activation of the NF-κB signaling pathway. Western blot analysis demonstrates decreased levels of p-p65 compared with the IL-1β group (Fig. 4A). The levels of p-IκBα were downregulated by costunolide in a dose-dependent manner, which was induced by IL-1β (Fig. 4A). Costunolide significantly inhibited the increase
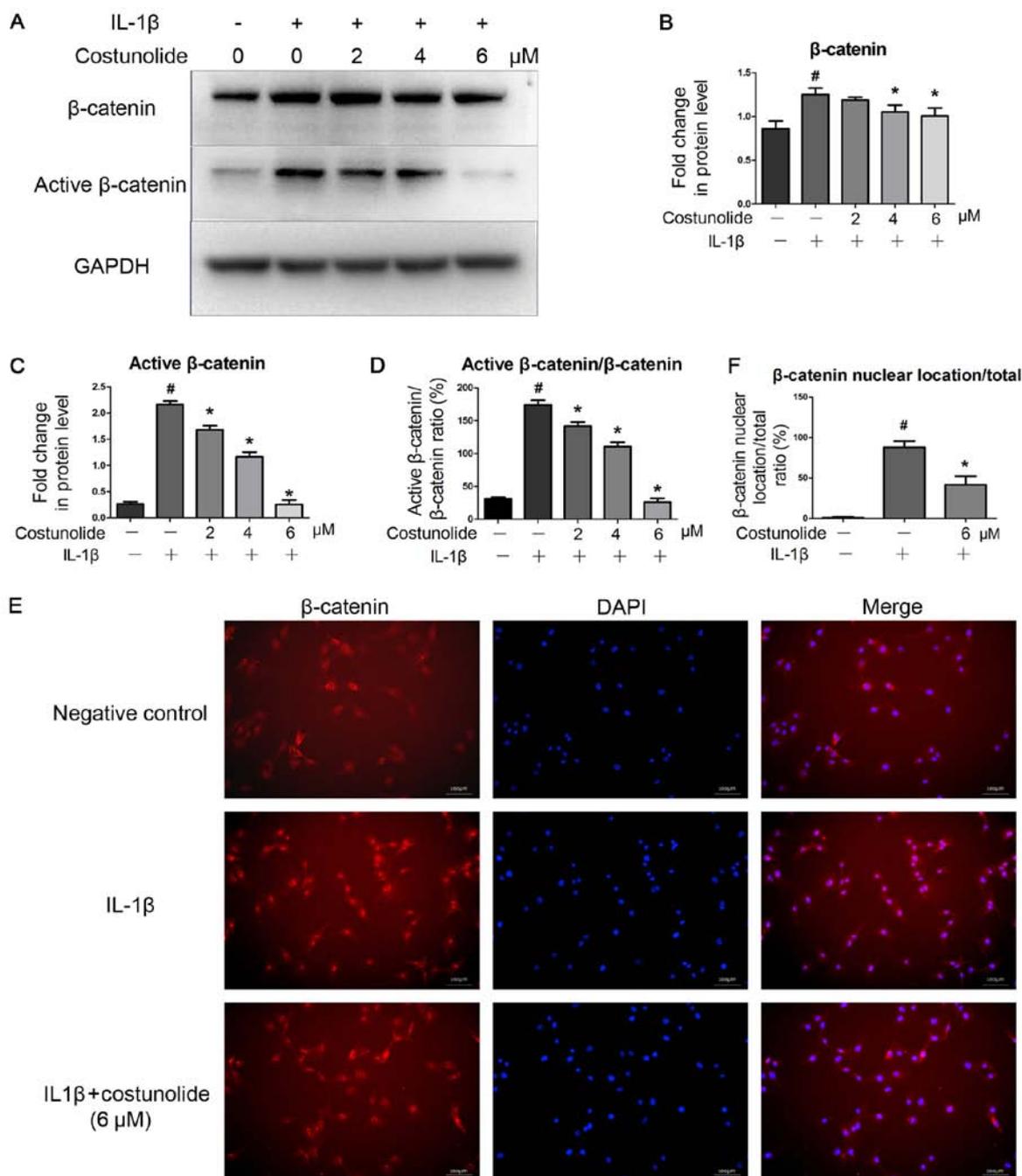
Figure 5. Effect of costunolide on IL-1β-induced Wnt/β-catenin signaling activation in chondrocytes. Chondrocytes were pretreated with costunolide (0, 2, 4 and 6 µM) for 1 h followed by IL-1β (10 ng/ml) for 3 h. (A) Western blot analysis of the effect of costunolide on the Wnt/β-catenin signaling pathway. (B and C) The expression of β-catenin and non-p-β-catenin was compared with that of GAPDH. (D) The ratio of active β-catenin was compared with β-catenin. (E) Immunofluorescence analysis of β-catenin nuclear translocation. Scale bar=100 µM. (F) Quantitative data of β-catenin nuclear distribution/total ratio as photographed in. (E) The data in the figures represent the mean ± standard deviation of three experiments (n=3). #P<0.05 vs. the control group; *P<0.05 vs. IL-1β alone treatment group. IL, interleukin.

of the p-p65/p65 and p-IκBα/IκBα ratios induced by IL-1β stimulation (Fig. 4B and C). Furthermore, immunofluorescence staining revealed that IL-1β-induced p65 translocation into the nucleus was significantly inhibited by pretreatment with 6 µM costunolide (Fig. 4D and E). These results demonstrated that costunolide effectively inhibited IL-1β-induced NF-κB signaling activation in rat chondrocytes *in vitro*.

Effect of costunolide on IL-1β-induced Wnt/β-catenin activation in chondrocytes. To confirm the inhibitory effects

of costunolide on the Wnt/β-catenin signaling pathway, β-catenin protein levels and its distribution in chondrocytes were determined by western blot analysis and immunofluorescence staining. IL-1β stimulation significantly activated the Wnt/β-catenin signaling pathway by inhibiting the degradation of β-catenin, which was suppressed by costunolide in a concentration-dependent manner (Fig. 5A and B). Active β-catenin (non p-β-catenin) levels were also decreased by costunolide treatment (Fig. 5A and C). In addition, a decrease in the active β-catenin: β-catenin ratio was also observed
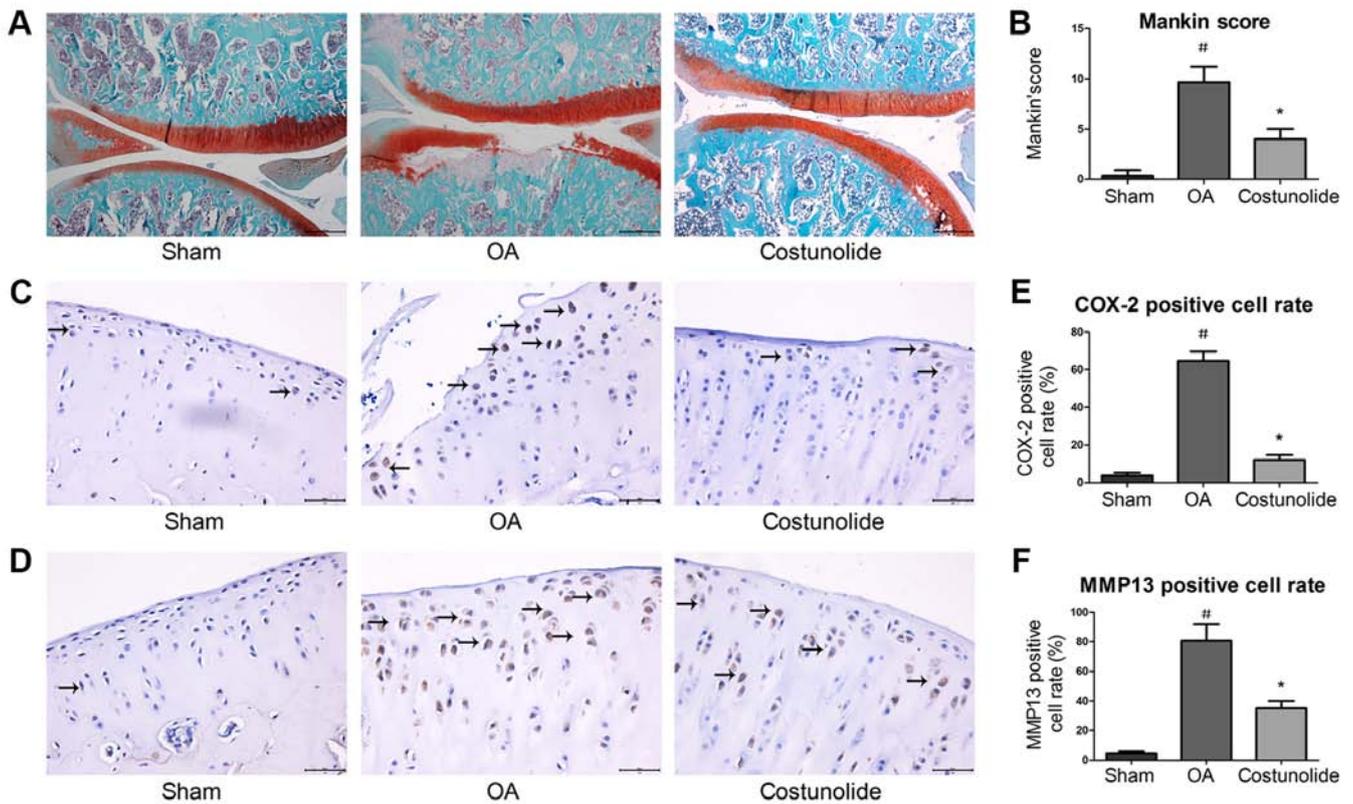
Figure 6. Costunolide ameliorates OA development in a rat destabilization of the medial meniscus model *in vivo*. (A) Histological evaluation of safranin O staining in rat knee joint sections. Scale bar=500 μM. (B) Mankin score of the sham, OA and costunolide-treated groups. (C and D) Analysis of the expression of COX-2 and MMP-13 in each group. Scale bar=50 μM. black arrows indicated the positive cells (E and F) Quantitative analysis of the positive cell rate for COX-2 and MMP-13. The data are typical results from six independent experiments with similar results (n=6). #P<0.05 sham group compared with the OA group; *P<0.05 costunolide-treated group compared with the OA group. OA, osteoarthritis; COX-2, cyclooxygenase-2; MMP, matrix metalloproteinase.

(Fig. 5D). Immunofluorescence staining revealed that the translocation of β-catenin into the nucleus decreased, which was induced by IL-1β stimulation (Fig. 5E and F).

Histopathological and immunohistochemical changes in articular cartilage. To investigate the protective effects of costunolide on OA development *in vivo*, a surgically-induced rat OA model involving DMM was established. Histopathological changes in cartilage were assessed by safranin O-fast green staining. Fissure in the matrix and loss of safranin O staining in chondrocytes were observed in rats following surgery. Intra-articular injection of costunolide suppressed cartilage degradation, thereby delaying OA progression (Fig. 6A). The Mankin score of the costunolide group was decreased compared with that of the OA group (Fig. 6B). Immunohistochemistry revealed that COX-2 and MMP-13 expression were significantly decreased in the costunolide group compared with the OA group (Fig. 6C-F). These experimental results demonstrated that costunolide ameliorated the progression of OA *in vivo*.

Discussion

OA is a progressive degenerative joint disease characterized by synovial inflammation, destruction of subchondral bone, formation of osteophytes and degradation of articular cartilage. At present, the treatments for OA include pharmacological and non-pharmacological therapies. NSAIDs are commonly used drugs for the treatment of OA to relieve the symptom of patients, but cannot effectively prevent cartilage degeneration, and replacement surgery is usually performed during end-stage disease.

Costunolide, a sesquiterpene lactone, exhibits anti-oxidant and anti-inflammatory properties. A previous study demonstrated that costunolide significantly inhibited RANKL-induced bone marrow-derived macrophage differentiation into osteoclasts in a dose-dependent manner without affecting cytotoxicity (28). Certain studies have hypothesized that osteoclasts serve an important role in the pathogenesis of OA, indicating that agents that can effectively suppress subchondral bone loss and chondrocyte degradation may aid in the treatment of OA (29). However, whether costunolide is able to suppress cartilage degeneration remains unclear at present. The present study indicated that costunolide ameliorated cartilage degeneration via suppression of the NF-κB and Wnt/β-catenin signaling pathways.

The process of matrix degradation in OA is attributed to the release of MMPs, which are primarily responsible for degrading the ECM, particularly MMP-1 and MMP-13 (30). It has been suggested that certain risk factors associated with OA include the activation of catabolic factors, including the pro-inflammatory cytokine IL-1β. Previous studies have revealed that the expression of IL-1β is increased in joints with OA compared with in normal joints (31). IL-1β stimulation may upregulate MMPs expression and aggravate chondrocyte apoptosis, which causes OA (32). Downregulation of MMPs
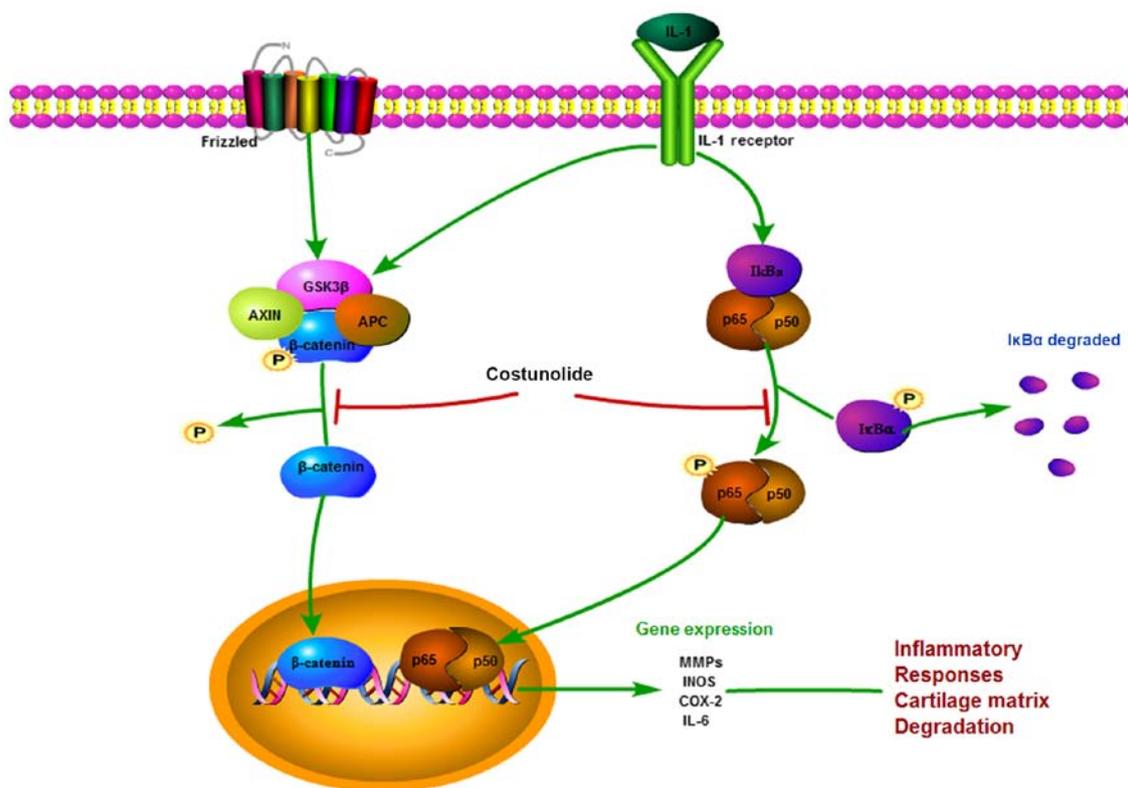
Figure 7. Schematic illustration of the potential protective effects of costunolide in osteoarthritis development. IL, interleukin; GSK3β, glycogen synthase kinase-3β; AXIN, Axin-1; APC, adenomatous polyposis coli; IκB-α, NF-κB inhibitor α; p65, transcription factor p65; p50, NF-κB p105 subunit; MMPs, matrix metalloproteinases, INOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

expression and chondrocyte inflammation leads to a therapeutic effect in OA (33). In the cell viability assay performed in the present study, it was observed that costunolide treatment at ≤6 μM had no effect on the viability of rat chondrocytes, and did not alter gene expression under nonpathological conditions, which indicates that a low concentration of costunolide is not harmful to normal cartilage. The data from the present study also indicated that IL-1β promoted the levels of IL-6, INOS, COX-2, MMP-3, MMP-9 and MMP-13, and decreased the levels of collagen II and SOX9 in chondrocytes. Costunolide reversed IL-1β-induced inflammatory and matrix-degrading gene expression and maintained cartilage phenotype. In addition, histological evaluation of safranin O staining revealed that the OA rat model resulted in cartilage degradation, while injection of costunolide for 8 weeks markedly improved the structure of the cartilage, with a lower Makin score in the costunolide treatment group compared with the OA group. Specifically, the effect of costunolide on the treatment of OA manifested through a decrease in OA-specific gene expression, including COX-2 and MMP-13, as analyzed by immunohistochemistry. The results from these analyses confirmed the protective role of costunolide in ameliorating cartilage erosion and decreasing matrix degeneration *in vitro* and *in vivo*.

There are multiple signaling pathways involved in the progression of OA. As shown in Fig. 7, the present study elucidated the mechanism by which costunolide exhibits anti-inflammatory and anti-catabolic effects in the ECM of chondrocytes via the Wnt and NF-κB signaling pathways, which have been reported to be involved in the progression

of OA (34,35). In the classic sequence of NF-κB activation, IL-1β activates the NF-κB signaling pathway by triggering the phosphorylation of members of the inhibitor of κB family, which are ubiquitinated upon phosphorylation by IκB kinase, and p65 heterodimers are subsequently released (36,37). p-p65 is translocated from the cytosol to the nucleus, which results in the expression of inflammatory genes including MMPs, INOS and IL-6 (38). According to the results of immunofluorescence microscopy and western blot analysis, costunolide significantly suppressed the phosphorylation of IκB and p65 in chondrocytes and decreased the nuclear translocation of p65 upon treatment with IL-1β stimulation.

The Wnt/β-catenin signaling pathway regulates crucial aspects of bone metabolism and formation, and the reconstruction and development of cartilage tissue, which has been acknowledged as important in the progression of OA (39,40). β-catenin is the most important component of the Wnt signaling pathway. During the basal status, β-catenin is steadily phosphorylated by a destructive complex composed of casein kinase 1, glycogen synthase kinase-3β (GSK-3β), Axin-1 and adenomatous polyposis coli. GSK-3β phosphorylates β-catenin to cause its degradation, ultimately inhibiting the activation of the Wnt signaling pathway. Due to the stimulation of IL-1β, β-catenin is stabilized and translocated into the nucleus to activate target genes (41,42). The results from the present study revealed that costunolide promoted total β-catenin degradation while inhibiting the production of non-p-β-catenin (active), which was translocated into the nucleus.

To the best of our knowledge, the present study was the first to examine the effect of costunolide in preventing cartilage degeneration. The underlying mechanism of this effect is associated with the inhibition of the NF-κB and Wnt/β-catenin signaling pathways induced by IL-1β. Additional studies are required to elucidate the exact mechanism by which costunolide regulates the NF-κB and Wnt/β-catenin signaling pathways.

Availability of data and materials

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

Authors' contributions

All authors made a significant contribution to the study and were in agreement with the content of the article. YH and SM conceived and designed the experiments. YH and CM performed the experiments, and JR and KX analyzed the data. LX, JB, WC and LJ interpreted the data, and contributed reagents and materials. YH, YX and LW interpreted the data and drafted the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University (approval no. 2015-107).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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