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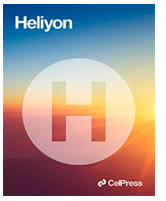

Research article

Ski ameliorates synovial cell inflammation in monosodium iodoacetate-induced knee osteoarthritis

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ABSTRACT

Knee osteoarthritis (KOA) is one of the most common degenerative diseases and is characterized by cartilage degeneration, synovial inflammation, joint stiffness and even loss of motor function. In the clinical treatment of arthritis, conventional analgesic and anti-inflammatory drugs have great side effects. We have evaluated the possibility of the endogenous transcription regulator Ski as an anti-inflammatory alternative in OA through experimental studies in animal models and in vivo and in vitro. Male Sprague–Dawley rats were injected with monosodium iodoacetate (MIA) into the knee joints to induce symptoms identical to those of human OA. We isolated knee synovial tissue under sterile conditions and cultured primary synovial cells. In vitro, Ski inhibits the proinflammatory factors IL-1β, IL-6 and TNF-α mRNA and protein expression in lipopolysaccharide (LPS)-stimulated fibroblast-like synoviocytes (FLSs) and U-937 cells. In addition, Ski attenuates or inhibits OA-induced synovial inflammation by upregulating the protein expression of the anti-inflammatory factor IL-4 and downregulating the protein expression of downstream molecules related to the NF-κB inflammatory signaling pathway. In vivo, Ski downregulated proinflammatory factors and p–NF–κB p65 in KOA synovial tissue and alleviated pain-related behaviors in KOA rats. These experimental data show that Ski has strong anti-inflammatory activity. Ski is an endogenous factor, and if used in the clinical treatment of OA, the side effects are small. However, the anti-inflammatory mechanism of Ski must be further studied.

1. Introduction

Osteoarthritis (OA) is the most prevalent progressive joint disease among the elderly population. Among the total population aged over 65 in the world, 50 % have OA symptoms such as pain, stiffness and motor loss, and 25 % suffer from severe disabling pain [1]. As the clinical symptoms of OA patients are mostly characterized by pain and inflammation, the current therapeutic strategies are analgesia and anti-inflammatory strategies. Although the clinical use of nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids for early nonsurgical treatment has a certain effect, their long-term use in OA patients often has negative health consequences in various areas, including the cardiovascular, kidney and gastrointestinal tract of elderly people [2,3]. Therefore, it is urgent to develop a safe and effective alternative drug for the treatment of OA.

The corepressor cellular Sloan Kettering Institute (c-Ski) protein is a highly conserved proto oncoprotein in vertebrate evolution

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(called the Ski protein in humans and the c-Ski protein in animals); its deletion in the mouse model can lead to the inability of mouse embryos to develop until death [4]. In human Ip36 deletion syndrome (the deletion of the ski gene is one of the important causes of the disease), there are phenomena such as slow development and growth retardation [5], which suggest that the function of the Ski protein is very important. Some studies have confirmed that c-Ski can promote wound healing of skin tissue and reduce scarring [6]. At the same time, many studies have reported that the inflammatory response is one of the important factors affecting wound healing speed and healing quality [7]. The expression of c-Ski increases significantly during the period of reduced wound inflammatory response and increased cell proliferation [8]. Other studies have shown that c-Ski can inhibit acute inflammatory responses, such as wounds, and downregulate the production of proinflammatory factors, but it is still unknown whether chronic inflammatory responses, such as OA, have any effect.

In recent years, OA has been recognized as a systemic chronic low-grade inflammatory response state [9]. Knee osteoarthritis (KOA) is a typical OA disease, and synovitis is an early event that occurs and develops before the degradation of KOA cartilage. Synovitis is a characteristic of OA, and it is closely related to the symptoms of KOA [10]. Fibroblast-like synoviocytes (FLSs) and macrophage-like synovial cells are synovial effector cells. Inflammatory cytokines and mediators play an important role in the pathological process of KOA synovitis. The synovium and chondrocytes of KOA patients produce and release cytokines and inflammatory mediators, which not only cause their own synovitis and pathological changes in cartilage erosion but also enter the articular cavity and affect the occurrence and development of KOA diseases [11,12]. It is an urgent task for researchers to develop a drug without side effects that inhibits the production and release of proinflammatory mediators by synovial cells.

We found that increasing Ski protein levels via transfection of the recombinant adenovirus Adeno-MCMV-SKI-3Flag P2A-EGFP (Ad-Ski) inhibited the inflammation of FLSs in KOA rats. Here, we first used the classical OA animal model induction method to induce KOA in rats by a single intra-articular injection of monosodium iodoacetate (MIA) [10,13,14]. On Day 14, synovial tissue was isolated, and primary FLSs were cultured. We stimulated FLSs and U-937 cells with lipopolysaccharide (LPS) and then detected the changes in the expression of the proinflammatory factors IL-1β, IL-6 and TNF-α. At the same time, ad-Ski was transfected into FLSs and U-937 cells to overexpress the Ski protein, the changes in the expression of proinflammatory factors were detected, and hormone treatment was used as a control to further verify the anti-inflammatory effect of Ski.

2. Materials and methods

2.1. Animal management, MIA injection, and model preparation

Male Sprague–Dawley (SD) rats (4 weeks old) were purchased from the Experimental Animal Center of the Research Institute of Surgery, Daping Hospital, Army Medical University (Chongqing, China). The animals were maintained in a room under standard laboratory conditions with a 12:12 h light-dark cycle with a constant temperature of 22 ± 2 °C and humidity of 55 ± 10 %. The animals were acclimatized for one week before the experiment. Rats had access to ad libitum feed and drink.

The KOA model was established by a single injection of MIA into the knee joint cavity of rats [15–17]. SD rats were randomly divided into two groups: sham (n = 30) and KOA (n = 30). KOA was induced by MIA. Briefly, on Day 1, KOA group rats were anesthetized with pentobarbital sodium and given an intra-articular injection of 2 mg MIA (Sigma, St. Louis, MO, USA) in 50 μL physiologic saline through the infrapatellar ligament of the bilateral knee joint, while sham group rats were treated with 50 μL sterilized physiologic saline. Six SD rats (18 in total) were sacrificed on Days 0, 1, and 7 to harvest synovial tissue. On Day 14, 12 SD rats were sacrificed to harvest synovial tissues.

2.2. Measurement of the right knee joint diameter

The transverse diameters of the knee joint were measured at Days 0, 1, 7, and 14 with a slide caliper (Mitutoyo, Kanagawa, Japan). Each knee was measured three times, and the mean value was calculated [18].

2.3. Weight-bearing capacity of the hind paw

Changes in hind paw weight distribution between the right (osteoarthritic) and left (contralateral control) limbs were utilized as an index of joint discomfort in the osteoarthritic knee [10]. Rats were placed in an angled plexiglass chamber so that each hind paw rested on a separate force plate. The force exerted by each hind limb (measured in grams) is averaged over a 5-s(s) period. Each data point is the mean of three 5-s readings. The change in hind paw weight distribution was calculated by determining the difference in the amount of weight (grams) between the left and right limbs. The percentage of weight distributed on the right hind limb was calculated using the following equation: weight bearing rate (%) = (right hind limb weight/(left hind limb weight + right hind limb weight)) × 100 %.

2.4. Primary FLS culture

We completed the primary culture of synovial cells. The brief description is as follows: After SD rats were anesthetized, they were immersed in 75 % ethanol for aseptic treatment for 10–20 min, and the knee joints of SD rats were taken under aseptic conditions. Synovial tissues were washed 2–3 times with phosphate-buffered saline (0.01 M, pH 7.2 ， PBS) and then minced into 1 mm × 1 mm × 1 mm pieces and digested in 0.2 % collagenase type I (Gibco by Life Tech, Catalog No.: 17,100–017, USA) for 4–6 h. FLSs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10 % fetal bovine serum (FBS) (Gibco), 100 IU/mL

penicillin and 100 μg/mL streptomycin (Invitrogen, CA, USA) in an incubator at 37 °C and 5 % CO_2. The identification of FLSs was performed by immunohistochemistry. In the cell experiment, we used LPS (1 μg/mL) to interfere with synovial cells for 3 h to mimic and amplify a KOA environment.

2.5. U-937 cell culture

U-937 cells were obtained from the Cell Center, Institute of Basic Medicine, Chinese Academy of Sciences (Beijing, China). The cells were maintained at 37 °C and 5 % CO_2 with DMEM containing 10 % FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, CA, USA). Cells in logarithmic growth phase were used for the experiment. U-937 cells were stimulated with LPS (200 ng/mL) for 3 h to establish an in vitro inflammatory model.

2.6. Construction, amplification, purification and transfections of ad-ski

The Ski-overexpressing recombinant adenovirus Adeno-MCMVSKI-3Flag-P2A-EGFP (Ad-ski) and the empty control recombinant adenovirus Adeno-MCMV-3Flag-P2A-EGFP (Ad-EGFP) were purchased from Obio Technology Corp., Ltd. Human Ski cDNA (GenBank accession number NM_003036.3) was packaged into the adenovirus vector. The virus was amplified in human embryo kidney 293 (HEK293) cells, purified by Vivapure Adeno PACK 20 (Sartorius), and titrated as previously described [19]. The efficiency of Ad-Ski infection in FLSs was demonstrated by Western blotting and immunofluorescence. FLSs were transiently transfected with Ad-EGFP or Ad-ski for 48 h, following the manufacturer's instructions. Ski-overexpressing recombinant adenovirus of Ski was obtained from Professor Ping Li of the Chinese Army Characteristic Medical Center.

2.7. EdU incorporation assay

We assessed FLS proliferation using a Cell-Light 5-ethynyl2-deoxyuridine (EdU) Apollo567 In Vitro Kit (Ribobio Co., Ltd). According to the manufacturer's instructions. Briefly, FLSs (2×10^4/well) were cultured in a Millicell ^{EZ} SLIDE 8-Well glass slide (Merck Millipore Ltd.) and then incubated with 50 μmol/L EdU (1:1000) for 24 h. FLSs were fixed with 4 % formaldehyde for 30 min at 37 °C, followed by permeabilization in 0.5 % Triton X-100 at 37 °C. Then, 100 μL of Apollo® reaction cocktail was added to each well and incubated for 30 min under light-shading conditions. After three washes with PBS, the nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) for 30 min at 37 °C, and the EdU-labeled cells were observed by laser scanning confocal microscopy (Leica SP8).

2.8. Immunofluorescence staining

The FLSs were fixed with 4 % paraformaldehyde for 20 min at 37 °C, permeabilized with 0.3 % Triton X-100, and incubated with primary antibodies against vimentin (1:200, Abcam)) at 4 °C overnight. After several washes with PBS, the cells were treated with a Cy3-conjugated goat anti-rabbit antibody (1:500, Abcam) for 1 h at 37 °C. The nuclei were stained with DAPI. The results were captured by laser scanning confocal microscopy and analyzed using Image-Pro Plus 6.0. If more than 98 % of the synovial cells stained vimentin, the FLS purity was more than 98 %.

2.9. Experimental design in vivo

SD rats were randomly divided into six groups: sham (0 mg MIA/knee, n = 5), KOA (2 mg MIA/knee, n = 5), KOA (2 mg MIA/knee) + Ad-EGFP (2×10^8 IU, n = 5), and KOA (2 mg MIA/knee) + Ad-Ski (2×10^8 IU, n = 5). On Day 0, the rats were anesthetized with pentobarbital sodium, and then 50 μL of saline containing 2 mg MIA was injected into the articular cavity through the infrapatellar ligament of the right knee joint, while 50 μL of saline without 2 mg MIA was given to the rats in the Sham group. Seven days after MIA (0 or 2 mg) injections, the rats were treated by injecting 50 μL of sterilized saline (containing 0 IU or 2×10^8 IU adenovirus) into the joint cavity through the right patellar ligament. The rats were anesthetized and euthanized after 7 days of observation. Cartilage and synovial tissue were harvested and stored at −80 °C.

2.10. Immunoprecipitation (IP)

FLSs from each group were lysed for 15 min on ice in IP lysis/wash buffer (Pierce) supplemented with protease and phosphatase inhibitor mini tablets (Thermo Scientific). The following steps were completed according to the instructions of the Crosslink Immunoprecipitation Kit (Thermo Scientific). Briefly, IP of Ski was performed using an anti-Ski antibody as the IP antibody, and Western blotting was performed with an anti-Ski or *anti*-p–NF–κB p65 antibody. Rabbit IgG served as a negative control. Input is the total cell lysate of the control without anti-Ski immunoprecipitation. Each experiment was repeated three times. Western enhanced chemiluminescence (ECL) substrate (Bio Rad, Hercales, CA) was used for detection.

2.11. Western blot

Western blot analysis was performed according to standard procedures as previously described [20]. Total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) and

probed with the indicated antibodies. The information of antibodies were as follows: 1) polyclonal rabbit *anti*-TNF-α (1:1000 , Cell signaling TECH,USA) , 2) polyclonal rabbit *anti*-IL-1β (1:1000 , Cell signaling TECH,USA), 3)polyclonal rabbit *anti*-IL-6 (1:1000 , Cell signaling TECH,USA), 4) monoclonal rabbit anti-Ski (1:1000 , Abcam Ltd , Hong Kong, China) , 5) polyclonal rabbit *anti*-–HIF–1α (1:1000, Abcam), 6) polyclonal rabbit *anti*-IL-4 (0.1 μg/mL , Abcam Ltd , Hong Kong, China) ,7) monoclonal mouse *anti*-VCAM1 (1:2000 , Abcam Ltd , Hong Kong, China) ,8) monoclonal mouse *anti*-VEGFA (6 μg/mL , Abcam Ltd , Hong Kong, China) , 9) monoclonal mouse *anti*-MMP-1 (1:1500 , invitrogen Ltd , USA) ,10) monoclonal mouse *anti*-MMP-2 (1:500 , invitrogen Ltd , USA) ,11) polyclonal rabbit *anti*-MMP-3 (1:1000 , invitrogen Ltd , USA) , 12) polyclonal rabbit *anti*–NF–κB p65 (1:300 , Santa Cruz, CA,USA) , 13) monoclonal mouse anti-Ski (1:500 , Santa Cruz, CA,USA) , 14) polyclonal rabbit anti-Flag (1:1000 , Abcam Ltd , Hong Kong, China) , 15) monoclonal rabbit *anti*-p–NF–κB p65 (phospho S536 , 1:1000 , Abcam Ltd , Hong Kong, China) , 16) monoclonal mouse *anti*-GAPDH (1:10,000 , Abcam Ltd , Hong Kong, China) , 17) monoclonal mouse *anti*-β-actin (1:10,000 , Abcam Ltd , Hong Kong, China) .The membranes were washed with TBST and incubated with a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase and detected with a chemiluminescence substrate (ECL, Amersham Biosciences). GAPDH expression was measured in each sample to verify equal protein loading.

2.12. Real-time quantitative PCR

Total RNA was extracted using TRIzol (Invitrogen, CA, USA). cDNA was generated using a Reverse Transcription Kit (Promega, USA) following the manufacturer's protocol. Quantitative real-time PCR (qRT−PCR) was performed with a CFX Connect™ real-time PCR system (Bio-Rad, CA, USA). cDNA was amplified using Power SYBR® Green PCR 2 × master mix (Promega, CA, USA). The primer sequence was completely correct by Refs. [21,22] and primer blast comparison of NCBI. Primers were synthesized by Beijing Huada Technology Co., Ltd. The sequences for primers were as follows: IL-1β forward, 5′-CCTTGTGCAAGTGTCTGAAG-3′, and reverse, 5′-GGGCTTGGAAGCAATCCTTA-3′; TNF-α forward, 5′-CAAGGAGGAGAAGTTCCCA-3′, and reverse, 5′-TTGGTGGTTTGCTACGACG-3′; IL-6 5′-TCCTACCCCAACTTCCAATGC-3′ and 5′-TAGCACACTAGGTTTGCCGAG-3′; GAPDH forward: 5′-TCTTCCAGGAGCGAGATCCC-3′, and reverse, 5′-TTCAGGTGAGCCCCAGCCTT-3′. The cycling parameters for IL-1β, TNF-α, IL-6 and GAPDH were 5 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 40 s. The mRNA level of individual genes was normalized to GAPDH and calculated by the $2^{-\Delta\Delta CT}$ data analysis method.

2.13. Statistical analysis

The data are presented as the mean ± standard error (SEM) for all independent experiments. We adopted analysis of variance (ANOVA) and the least significant difference (LSD) *t*-test to analyze the Western blot and qRT−PCR test results. All data were analyzed

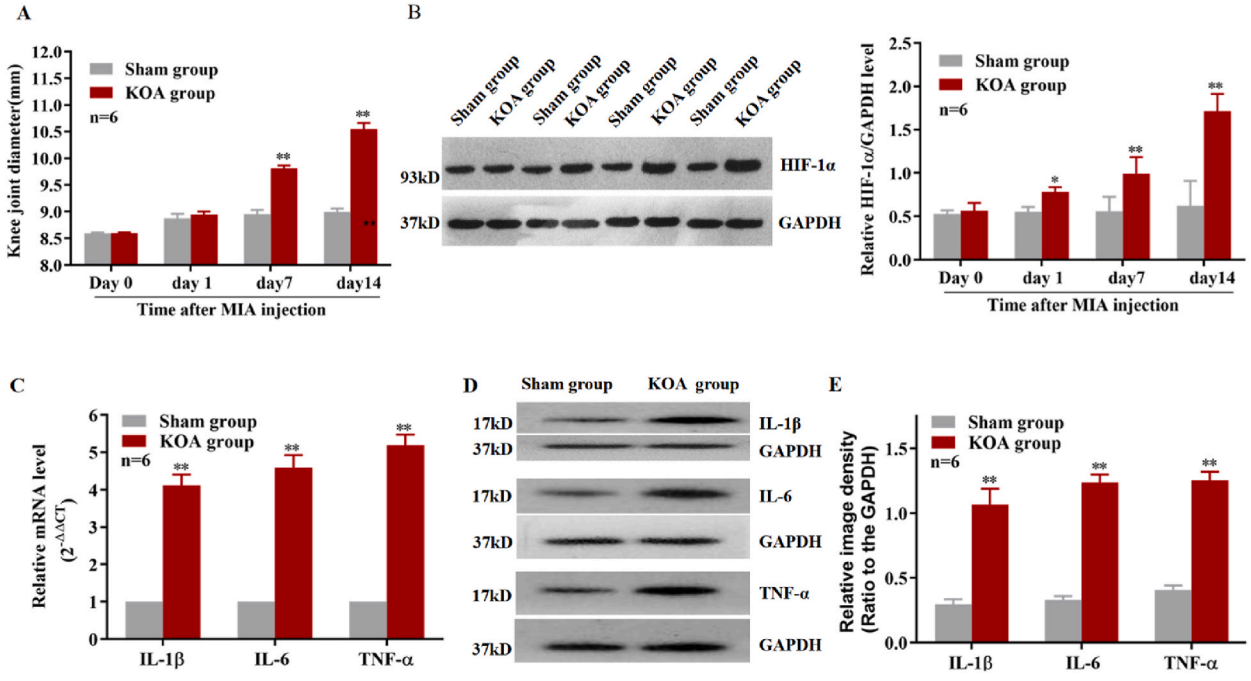

Fig. 1. Transverse diameter of the knee joint in rats, HIF-1α and proinflammatory factor levels in synovial tissue. **(A)** The transverse diameters of the right knees. (B) The levels of HIF-1α in synovial tissue were detected by Western blot. The expression of HIF-1α was significantly increased after MIA induction. (***C*–E**) The levels of proinflammatory factors in synovial tissue were detected by qRT−PCR and Western blot. The expression of IL-1β, IL-6 and TNF-α was significantly increased after MIA induction. Data are expressed as the mean ± SEM, $*P < 0.05$, $**P < 0$ 01 vs. the Sham group.

using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA), Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and LABWORKS software. Independent sample comparison t tests were used for the two groups of data. The Pearson correlation coefficient was used in the correlation analysis of variables. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Rat KOA molecular model

To quantify the degree of knee swelling in rats, transverse diameters of the right knees were measured. The transverse diameter of the right posterior knee joint of SD rats showed an upward trend after a single injection of MIA into the joint cavity, which was significantly higher than that of the sham group on the 7th and 14th day of modeling (Fig. 1A). After MIA modeling, the expression of hypoxia-inducible factor (HIF)-1α in synovial tissue was higher than that in the Sham group, reaching a peak at 14 days (Fig. 1B). On the 14th day after MIA modeling, the mRNA and protein expression levels of IL-1β, IL-6 and TNF-α in synovial tissue were significantly different from those in the sham group (Fig. 1C–E). The hypoxic state of the synovial tissue of SD KOA rats gradually worsened, and the expression of proinflammatory factors in the synovial tissue of SD KOA rats was upregulated. The intraarticular (i.a.) injection of MIA into the rat knee joint produces inflammatory mediator changes representative of those seen in human OA.

3.2. 3rd-to 7th-generation primary cultured FLSs have biological activity

The isolated synovial tissue was cultured to the 6th day, the cell fusion reached 70–80 %, and the cells grew adherently, but in the observation field, there were still very few round and oval cells (Fig. 2A). The cells were passaged to the third generation (Days 12–14), the cell morphology was basically spindle-shaped, the nucleus was in the center, and the cells grew well (Fig. 2B). The cells were passed to the 8th generation, the cell growth was slow, the cell morphology changed from a spindle to an approximate ellipse, the intercellular space was enlarged, and the cells had vacuole necrosis and aging (Fig. 2C). Immunofluorescence chemistry revealed that the third-generation synovial cells had a regular morphology, were spindle-shaped, and had an oval nucleus in the center of the cell; the positive rate of vimentin-stained cells was higher than 98 % (Fig. 2D–F). Vimentin is a characteristic marker protein of FLS, suggesting that the cultured synovial cells are FLS. The EdU proliferation experiment of the 7th generation FLSs showed that FLSs still had the ability to proliferate (Fig. 3A–C). The EdU proliferation experiment of the 8th generation FLSs showed that FLSs had lost their proliferation ability (Fig. 3D–F). Western blot analysis of the PCNA protein of each generation of FLS from the 3rd to 8th generation showed that no significant difference was observed between the PCNA protein expression of each generation of FLS from the 4th to 7th generation and that of the third generation. We observed a significant difference in the expression of PCNA protein between the 8th generation and the 3rd generation (Fig. 3G-L). In summary, FLSs from the 3rd to 7th generation primary cultures were used in our study.

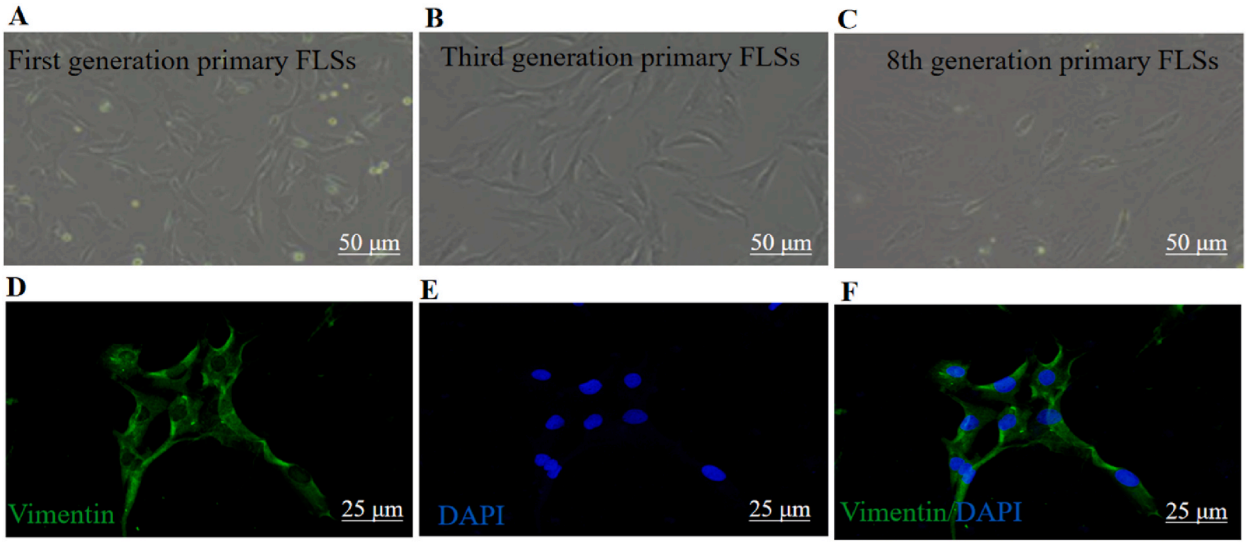

Fig. 2. Primary culture and purity of FLSs. **(A)** Synovial cells were cultured to the first passage (× 10). **(B)** Synovial cells were cultured to the third generation (× 10). **(C)** Synovial cells were cultured to the eighth generation (× 10). **(D–F)** Synovial cells were cultured to the third generation for vimentin fluorescence chemical identification (× 40).

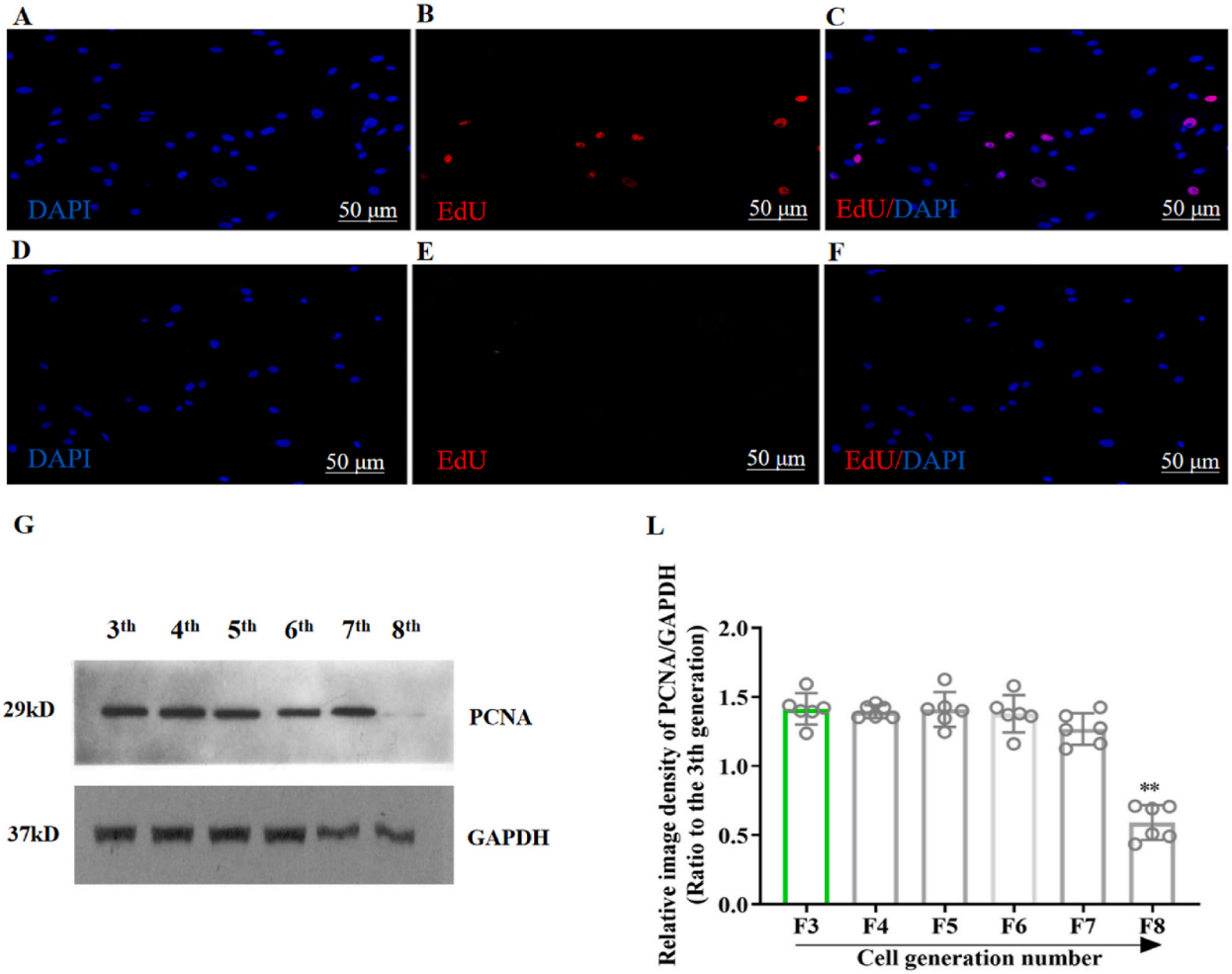

Fig. 3. Proliferation of primary FLSs. **(A–C)** The seventh generation synovial cell EdU proliferation experiment (× 10). **(D–F)** The eighth generation synovial cell EdU proliferation experiment (× 10). **(G)** Western blot results of PCNA protein in primary cultured FLSs (3rd - 8th generation). **(L)** The relative content of PCNA protein in FLSs of each generation. One-way ANOVA, F (6, 28) = 45.936, $P < 0.001$. Compared with third-generation cells, only eighth-generation cells had significantly decreased PCNA protein expression.

3.3. Increasing the level of ski protein can inhibit LPS-induced high expression of proinflammatory factors in OA primary FLSs

To examine the role of increased Ski protein levels in the high expression of OA primary FLS proinflammatory factors caused by LPS induction, we used an adenovirus transfection method. The results showed that Ad-ski transfected FLSs cells with obvious effects; after 1000 MOI of Ad-ski transfected FLSs, the expression of c-Ski protein was the highest (Fig. 4A–C), and the subsequent experiments used 1000 MOI of Ad-ski to transfect FLSs. The basic expression of the FLS c-Ski protein is almost undetectable. After FLSs were transfected with Ad-ski, the relative levels of IL-1β and TNF-α protein expression were closely and negatively correlated with the relative levels of Ski protein expression (Fig. 4B–D-G). Ad-ski transfection significantly increased Ski protein levels. This treatment not only significantly reduced the protein and mRNA expression of IL-1β and TNFα in OA primary FLS but also blocked LPS from inducing high protein and mRNA expression of IL-1β and TNFα (Fig. 5A–E). The anti-inflammatory effect of Ski was the same as that of dexamethasone (Dex, 10 μM, Sigma, USA) (Fig. 5A–E). There was no statistically significant difference between the two groups.

3.4. Increasing the level of ski protein can inhibit LPS-induced high expression of proinflammatory factors in the U-937 cell line

Forty-eight hours after transfection of Ad-EGFP and Ad-ski at different MOIs into U937 cells, triple-label (nuclear, adenovirus, and Ski protein) fluorescence staining was used, and Western blotting was used to analyze the expression of Ski and proinflammatory factor proteins. Ad-ski was used to transfect U-937 cells, and the effect was obvious; 500 MOI Ad-ski was used to transfect U-937 cells, and the expression of Ski protein was the highest (Fig. 6A and B). Follow-up experiments used 500 MOI Ad-ski to transfect U-937 cells. The basic expression of Ski protein in U-937 cells was relatively high (Fig. 6B and C). After U-937 cells were transfected with Ad-ski, the relative levels of IL-1β and TNF-α protein expression were closely and negatively correlated with the relative levels of Ski protein

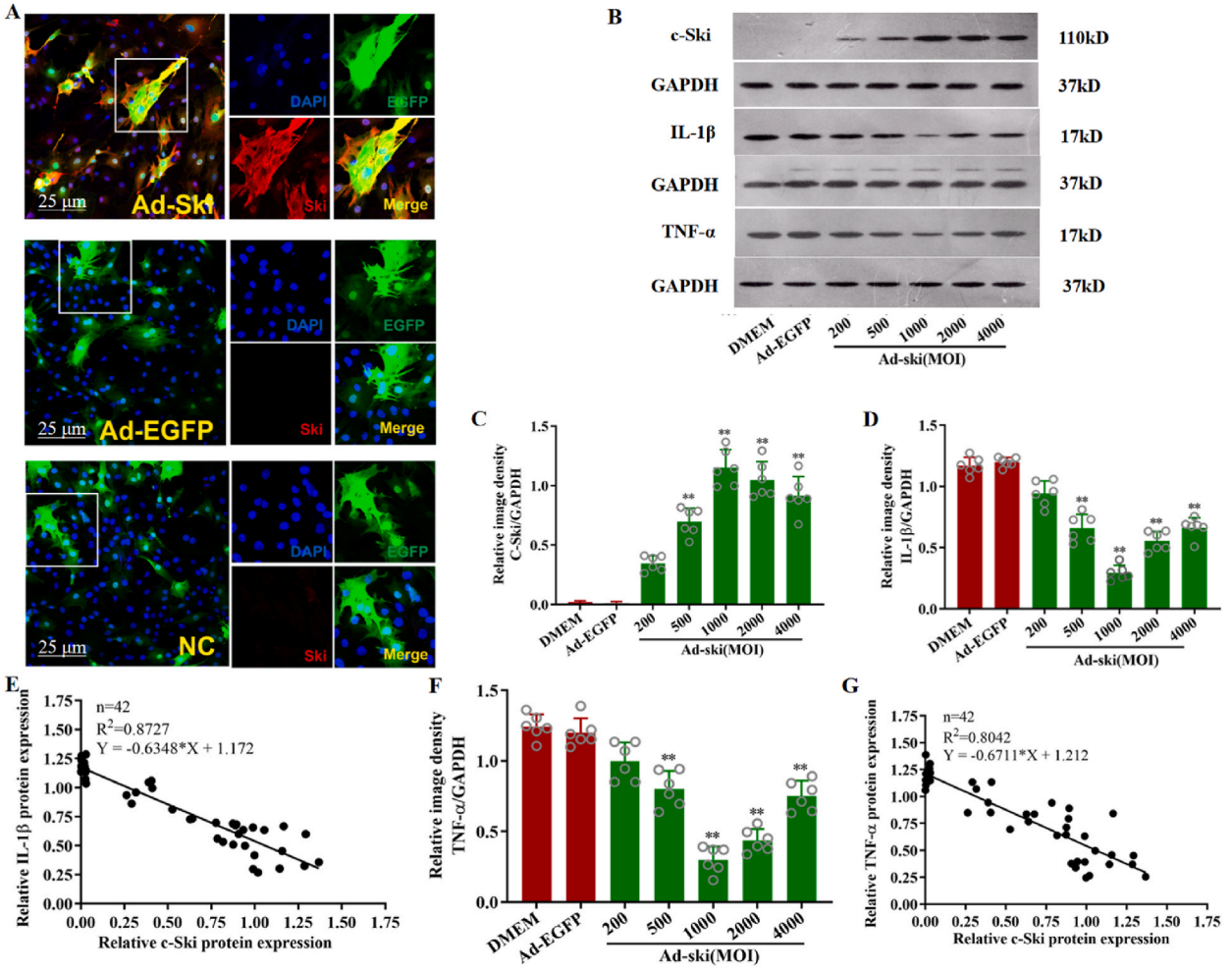

Fig. 4. After FLSs were transfected with Ad-sSki, Ski protein was significantly highly expressed, and Ski protein expression was negatively correlated with the expression of proinflammatory factor proteins. **(A)** Immunofluorescence staining for Ski expression. Nuclei are indicated with DAPI staining (blue), Ski expression is indicated by red fluorescence, and adenovirus has its own green fluorescence. The left panels show high-magnification images of the boxed regions in the right panels (× 200). The incubation antibody for Ad-ski and Ad-EGFP was Ski antibody, and the incubation antibody for the NC control was normal IgG antibody. The results were obtained from four independent experiments. **(B and C)** FLSs were infected with Ad-EGFP or Ad-ski (MOI = 200, 500, 1000, 2000 and 4000) for 48 h. The Ski levels were examined by Western blot analysis. **(D and F)** FLSs were infected with Ad-EGFP or Ad-ski (MOI = 200, 500, 1000.2000 and 4000) for 48 h. The IL-1β (D) and TNF-α (F) levels were examined by Western blot analysis. **(E and G)** FLSs were infected with Ad-EGFP or Ad-ski (MOI = 200, 500, 1000, 2000 and 4000) for 48 h. To confirm the correlation between the expression of c-Ski and proinflammatory cytokines, we performed a correlation analysis. The results showed that Ski was negatively correlated with the expression of IL-1β (E) and TNF-α (G). Data are expressed as the mean ± SEM. $^{**}P < 0.01$ vs. the Ad-EGFP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expression (Fig. 6B–G). Ad-ski transfection can significantly increase the Ski protein level of U-937 cells and can not only significantly reduce the basic expression of IL-1β and TNFα protein and mRNA in U-937 cells but also block LPS from inducing high IL-1β and TNFα protein expression in U-937 cells. While LPS induces U937 cells to produce proinflammatory factors, it also downregulates the basic expression of Ski (Fig. 7). The anti-inflammatory effect of Ski was the same as that of Dex, and there was no statistically significant difference between the two (Fig. 7).

3.5. After transfection with Ad-ski, it was found that the high expression of Ski not only has anti-inflammatory effects but also that the anti-inflammatory effects may be related to the NF-κB p65 signaling pathway

LPS-MIA-FLSs were infected with Ad-ski for 48 h, and the significantly high expression of Ski protein reduced the high expression of the proinflammatory factor IL-6 induced by LPS and significantly increased the expression of the anti-inflammatory factor IL-4 (Fig. 8A-B). At the same time, the protein expression of VEGF, VCAM1, MMP-1, MMP-2, MMP-3, and p–NF–κB p65 was significantly downregulated (Fig. 8B–E), and these protein molecules are related to the NF-κB p65 signaling pathway. IP assays showed that

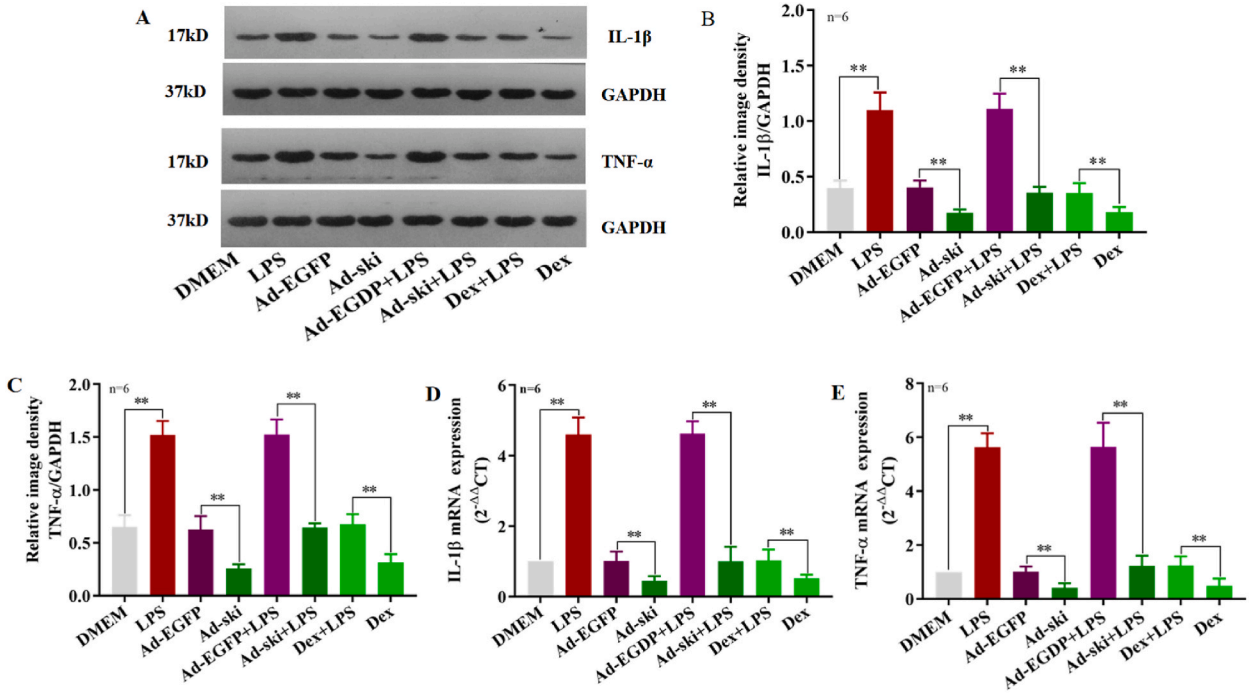

Fig. 5. After FLS are transfected with Ad-Ski, the high expression of Ski protein is increased, and the expression of pro-inflammatory factors that FLS and LPS stimulate FLS is inhibited. (A-E) LPS could induce high expression of IL-1β and TNF-α mRNA and protein in FLS. After Ad-ski transfection, LPS-induced overexpression of IL-1β and TNF-α mRNA and protein in FLS was blocked (**$P < 0.001$). The anti-inflammatory effect of Ski was similar to that of Dex. There was no significant difference between Ski and Dex ($P > 0.609$). Data are expressed as the mean ± SEM.

the Ski protein and p–NF–κB p65 protein formed a complex (Fig. 8F).

3.6. Ski inhibited the inflammation of the knee joint synovium in KOA rats

To investigate whether Ski inhibits knee synovial tissue inflammation in vivo, we analyzed the expression of c-Ski, Flag-*c*-Ski, IL-1β, TNF-α, NF-κB p65, and p–NF–κB p65 proteins in synovial tissue and pain-related behaviors in rats. The adenovirus successfully infected the synovial tissue (Fig. 9A). Compared with the KOA group, the knee transverse diameter of the affected limb was significantly reduced in the KOA + Ad-ski group, which was close to that in the Sham group (Fig. 9B). Moreover, the load-bearing capacity of the affected limb was significantly improved (Fig. 9B). The protein expression levels of IL-1β, TNF-α, and p–NF–κB p65 in the KOA + Ad-ski group were significantly reduced (Fig. 9C-D), while the protein expression levels of NF-κB p65 were not significantly different (Fig. 9D). Protein expression of IL-1β, TNF-α, and p–NF–κB p65 and pain-related behaviors were not statistically significant in the Sham group compared with the KOA + Ad-ski group (Fig. 9B–D).

4. Discussions

After the right knee joint cavity of rats was injected with MIA, the transverse diameter of the right knee was significantly increased, and the right knee joint was swollen. Moreover, the protein expression of HIF-1α in synovial tissue gradually increased, and hypoxia gradually increased. The expression of the proinflammatory factors IL-1β, IL-6 and TNF-α in KOA synovial tissue was significantly higher than that of the control group, and the synovium had inflammatory changes. Subsequently, in vitro experiments of cultured primary synovial cells and U-937 cells proved that adenovirus transfection could significantly increase the expression of c-Ski (Ski) protein and significantly reduce the expression of proinflammatory factors, and their protein expression was closely and negatively correlated. Further experiments showed that c-Ski (Ski) can not only significantly reduce IL-1 β, IL-6 and TNF-α basal expression in FLS and U-937 cells but can also reverse LPS-induced IL-1 β, IL-6 and TNF-α high expression in FLS and U-937 cells. These data suggest that c-Ski (Ski) has an anti-inflammatory effect on synovitis, which is similar to that of dexamethasone. In addition, in vitro experiments showed that the increased c-Ski protein transfected by adenovirus could not only inhibit the expression of proinflammatory factors but also upregulate the protein expression of the anti-inflammatory factor IL-4. At the same time, the protein expression level of p–NF–κB p65 was downregulated by c-Ski. The IP experiment results showed that the combination of Ski and p–NF–κB p65 protein and the protein expression of VEGF, VCAM1 and matrix metalloproteinases (MMPs) related to the NF-κB signaling pathway were also downregulated by the c-Ski protein. In vivo, Ski downregulated proinflammatory factors and p–NF–κB p65 in KOA synovial tissue and alleviated pain-related behaviors in KOA rats. The results of this study show that c-Ski (Ski), as an endogenous factor, can complete the

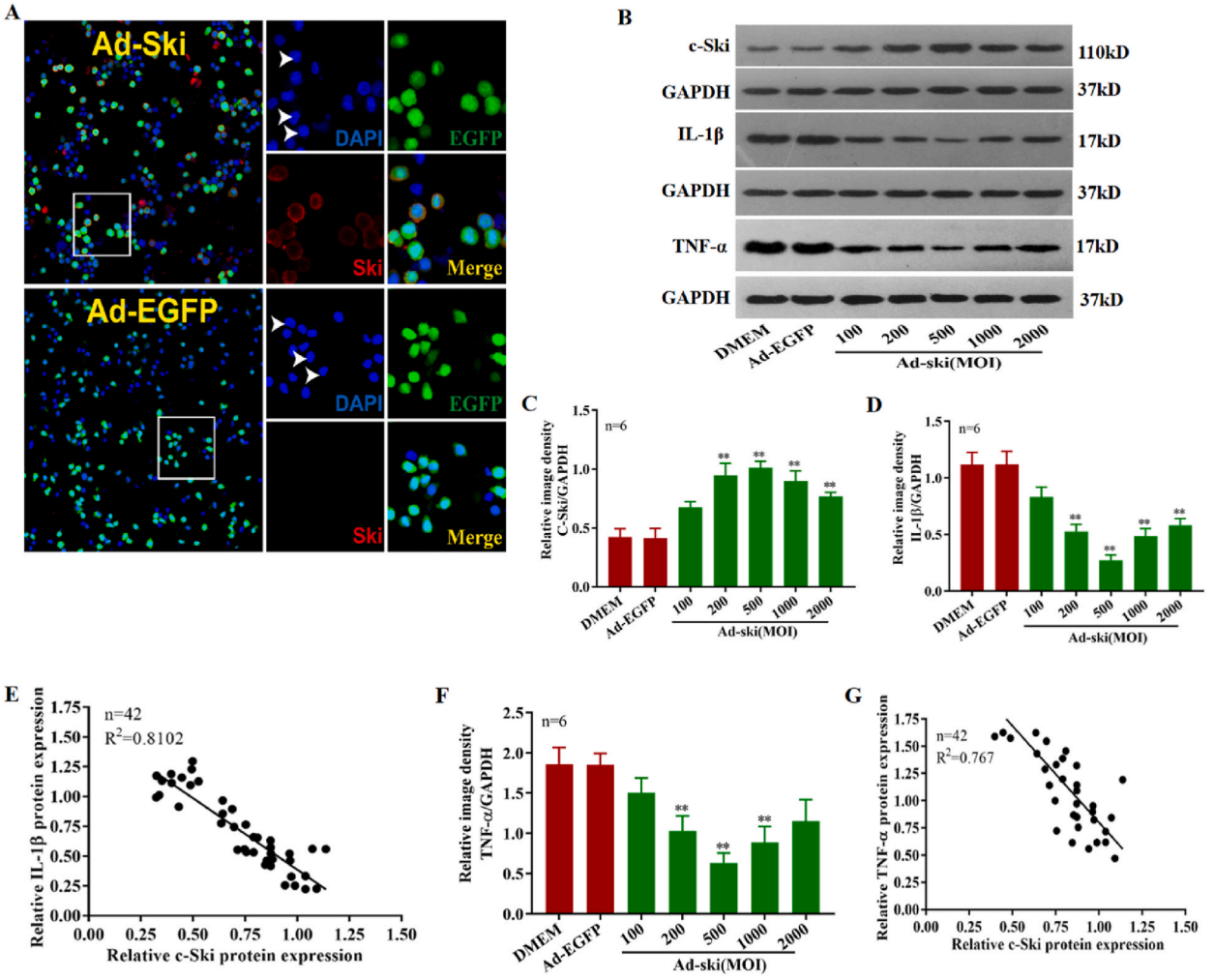

Fig. 6. After U-937 cells were transfected with Ad-Ski, Ski protein was significantly highly expressed, and its protein expression was negatively correlated with the expression of proinflammatory factor proteins. (A) Immunofluorescence staining for Ski expression. **(B-D and** F) Western blot analysis of the protein expression of Ski, IL-1β and TNF-α after U-937 cells were transfected with different MOIs. $^{**}P < 0.01$ vs. the Ad-EGFP group. (B–G) The expression of TNF-α and IL-1β inflammatory factors and its correlation with the expression trend of Ski protein. Data are expressed as the mean ± SEM. $^{**}P < 0.01$ vs. the Ad-EGFP group. The statistical analysis method is the same as in Fig. 4.

anti-inflammatory effect by inhibiting proinflammatory factors and upregulating anti-inflammatory factors, and the anti-inflammatory effect may be related to the NF-κB signaling pathway and has few side effects.

At present, in animal experimental studies of OA, a single injection of MIA (2 mg) into the rat articular cavity is a widely accepted and archetypical model of OA, as it can display many harmful behaviors, including pathological properties, such as synovial tissue inflammation and cartilage degradation [10]. HIF-1α is an essential transcription factor responsible for the induction of a series of genes that facilitate adaptation under hypoxic conditions [23,24]. The higher the expression of HIF-1α is, the more severe the hypoxia. MIA is a glycolysis inhibitor that inhibits glycolysis in chondrocytes, leading to cell death and triggering inflammation [25]. In the detection of synovial tissue proteins in OA rats induced by MIA, we found that HIF-1α was significantly increased in protein expression and that IL-1 β, IL-6 and TNF-α were significantly increased in mRNA and protein expression, severe hypoxia and inflammatory changes in the synovium. Hypoxia and inflammation may be mutual causes and effects and persist in the pathological process of KOA, leading to the increasingly serious condition of KOA. Therefore, we believe that the discussion of anti-inflammatory measures for OA is the key to the treatment of OA, and the control of inflammation is also an urgent problem to be solved in the clinical treatment of OA.

The early stage of MIA-induced experimental OA is characterized by macrophage infiltration and synovial inflammation [10]. Monocyte-macrophages and FLS are the two major functional cells of synovial tissue. The molecular characteristics of synovial inflammation in MIA-induced experimental OA are mainly macrophage infiltration and FLS proinflammatory factor secretion. To investigate the in vitro therapeutic effect of ski on OA synovitis, we isolated synovial tissue from KOA rats induced by MIA and successfully cultured synovial cells. As an inflammatory antigen, LPS can activate monocyte macrophages, FLSs, endothelial cells and epithelial cells to synthesize and release a variety of cytokines and inflammatory mediators through the cell signal transduction system

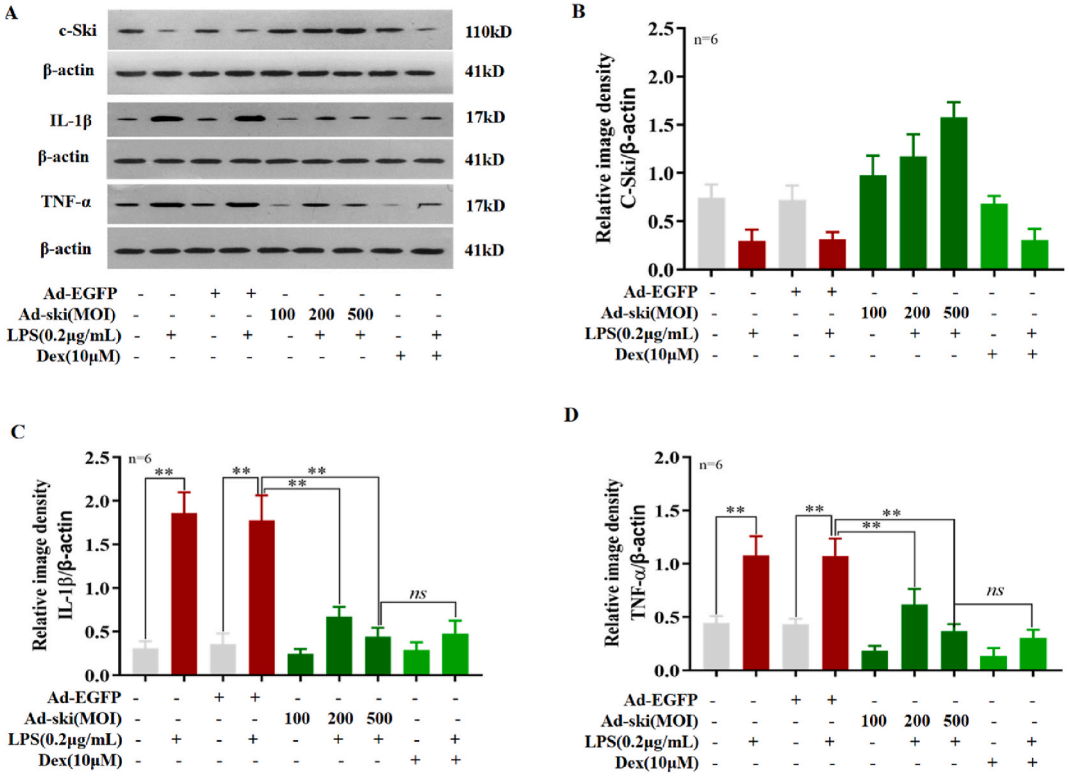

Fig. 7. After U-937 cells were transfected with Ad-Ski, Ski protein was significantly highly expressed, which inhibited the expression of proinflammatory factors in U-937 cells and LPS-induced U-937 cells. (A-D) After U-937 cells were transfected with Ad-Ski, they had significantly high expression of Ski. In addition to blocking the high expression of TNF-α and IL-1β in U-937 cells induced by LPS, Ski can also inhibit the basic expression of proinflammatory factors in U-937 cells. While LPS induces U-937 cells to produce proinflammatory factors, it also downregulates the basic expression of Ski. Ski has the same anti-inflammatory effect as DEX. Data are expressed as the mean ± SEM. $^{**}P < 0.01$. The statistical analysis method is the same as in Fig. 5.

and has good consistency and repeatability [26]. We used LPS to excite FLSs in vitro and determined that the optimal excitation concentration of LPS was 1 μg/mL, and the most suitable excitation time was 3 h, simulating the stable and high expression of proinflammatory factors in primary FLSs. The U-937 cell line is derived from histiocytic lymphoma and is a monocyte. Although U-937 cells themselves release ski protein, transfection with Ad-Ski can effectively increase the high expression of ski protein, which can be used to observe whether ski has an inhibitory effect on proinflammatory factors.

Ski is an intracellular homolog of the viral oncogene v-ski. Its protein product Ski is a multifunctional transcription regulator that participates in many physiological and pathological processes, such as hematopoietic cell proliferation, muscle regeneration, bone and nervous system development, synaptic projection, wound healing, fibrosis, tumorigenesis and proliferation [27,28]. Li [6] et al. found in animal experiments that increasing the expression of c-Ski in wound tissue can significantly reduce the level of inflammatory factors. In vitro experiments showed for the first time that transfection of Ad-ski increased Ski levels in OA primary FLSs and U-936 cell lines, and it not only significantly reduced FLSs and U-937 cell line IL-1β and TNF-α levels in the OA state. It can also reverse the upregulation of IL-1β and TNF-α levels under LPS stimulation. The effect of Ski on inhibiting the level of proinflammatory factors is consistent with the effect of hormones.

Although glucocorticoids not only inhibit the secretion of inflammatory factors but also inhibit the adhesion and phagocytosis of inflammatory cells through nongenomic and genomic effects [29], by inhibiting TNF-α and IL-1β at the protein level, Ski and glucocorticoids have similar inhibitory effects on the secretion of proinflammatory factors. Glucocorticoids have many side effects, and Ski is an endogenous factor. If it can be used in the clinic, the side effects are very small. Although the research results of Ski in the treatment of OA have not been reported, small molecule RNA (miR-155) is considered to directly downregulate Ski expression [30] and promote inflammation [31,32]. It is the main regulator of inflammation and an important target of inflammation regulation. This suggests that regulating Ski may be an important way for miR-155 to regulate inflammation, indicating that the anti-inflammatory effect of Ski is possible. Xie [9] et al. found that miR-1908 can downregulate Ski expression and promote TNF-α and IL-1β expression. Feld [33] et al. used acute myeloid leukemia cells and found that knocking out ski can improve the differentiation-related and inflammatory response-related genes of the cell, which also inversely indicates that Ski has an anti-inflammatory effect. However, the effect of Ski on OA and synovial FLSs remains uncertain. This experiment confirmed for the first time that the increased expression of Ski transfected with Ad-Ski can inhibit the high expression of FLSs of KOA induced by MIA and FLSs of KOA synovium induced by LPS

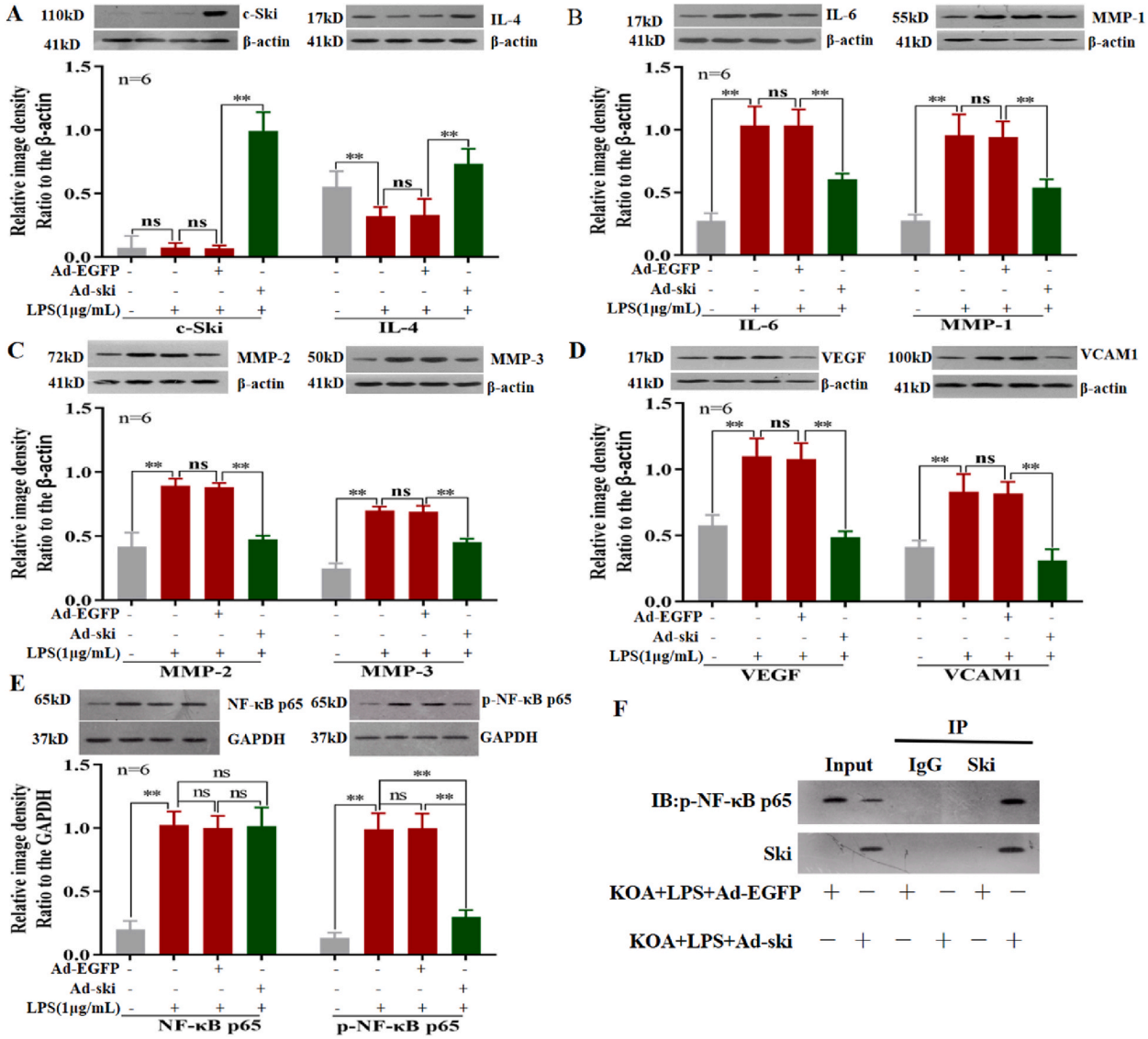

Fig. 8. The expression levels of IL-6, IL-4, VEGF, VCAM, NF-κB p65, p–NF–κB p65, and a variety of matrix-degrading enzymes were detected by Western blot. **(A)** After LPS-stimulated MIA-FLSs, the relative content of IL-4 protein was significantly downregulated, and the relative content of Ski protein did not change significantly. After Ad-ski transfection with LPS-MIA-FLS, the relative content of IL-4 protein was significantly increased, and Ski protein showed high expression. **(B)** After LPS-stimulated MIA-FLS, the relative contents of IL-6 and MMP-1 protein were significantly increased. After Ad-ski transfection with LPS-MIA-FLS, the relative contents of IL-6 and MMP-1 protein were significantly reduced. (***C*-D)** After LPS stimulation of MIA-FLS, the relative contents of MMP-2, MMP-3, VEGF and VCAM1 protein all increased significantly, and after Ad-ski transfection with LPS-MIA-FLS, they all decreased significantly. **(E)** Western blot analysis of the effects of Ski on NF-κB p65 and p–NF–κB p65 in FLSs. Data are expressed as the mean ± SEM. $^{**}P < 0.001$. ns. nonsignificant. **(F)** Analysis of results using anti-Ski antibodies as IP antibodies.

and U-937 cells in the proinflammatory factor, but it is still unknown how ski plays an anti-inflammatory role. It is well established that the NF-κB pathway is the main inflammatory pathway. It is assumed that Ski plays a role through the NF-κB pathway. If this hypothesis holds true, Ski will affect downstream molecules related to the NF-κB signaling pathway. For this, we detected downstream molecules related to the NF-κB signaling pathway with Western blotting.

In this study, we demonstrated that after transfection with Ad-Ski, the transcription of downstream factors related to NF-κB was downregulated, resulting in decreased secretion of VEGF, VCAM1 and a variety of matrix protein-degrading enzymes. VEGF and VCAM1 are related to joint congestion and the growth of neocapillaries in the affected part, and congestion and the increase in neocapillaries in the affected part are important factors of joint pain. The NF-κB signaling pathway is involved in the expression of VEGF and vascular endothelial cell adhesion molecules and is closely related to the congestion state in patients with KOA [34]. MMP-1, MMP-2 and MMP-3 depend on the NF-κB signaling pathway, which releases and secretes key molecules that promote cartilage degradation and aggravate the apoptosis of arthritic cartilage and the inflammatory response of cartilage [35]. Further experimental

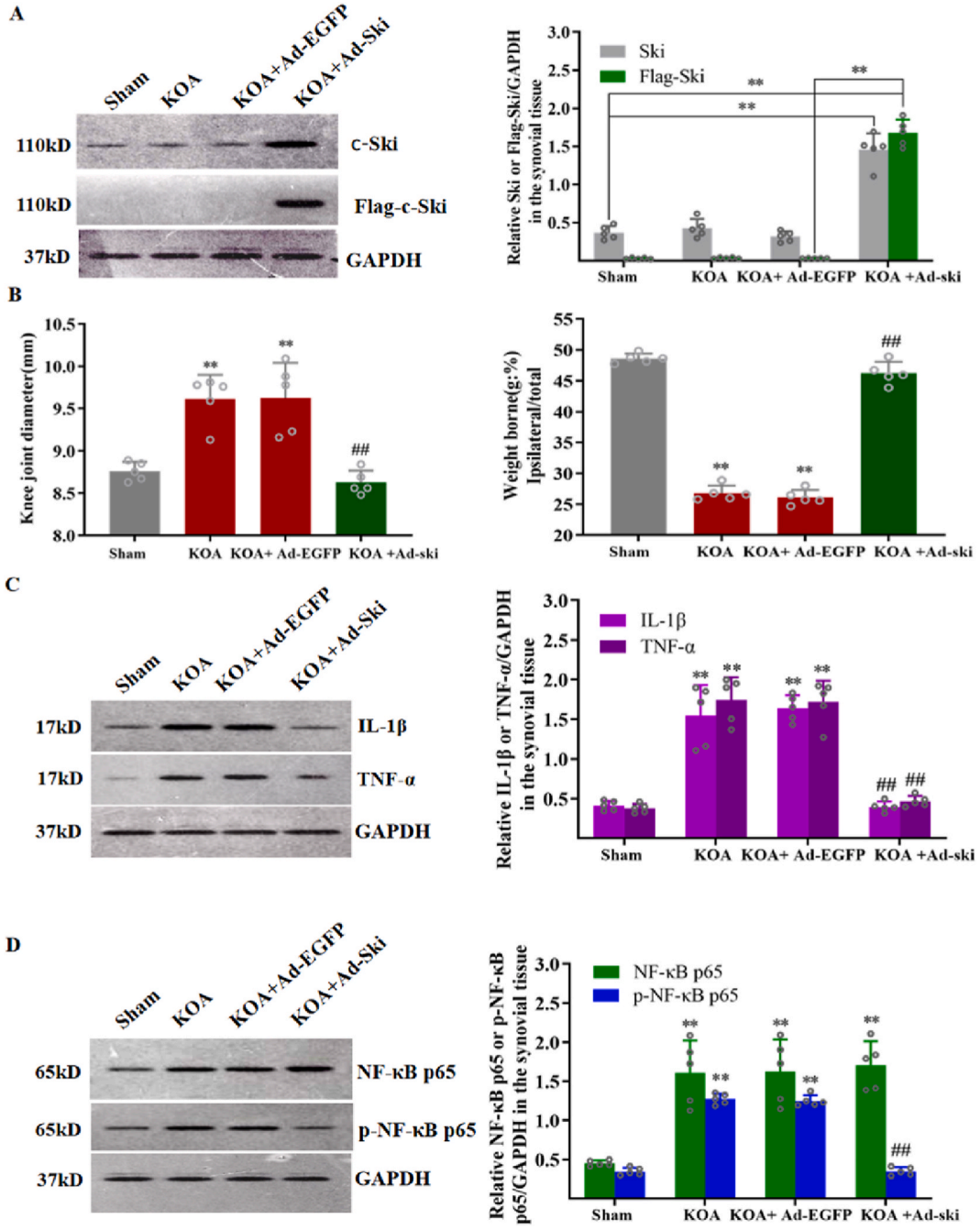

Fig. 9. Ski inhibited synovial tissue inflammation in KOA knees in vivo. **(A)** Expression of Ski and Flag-Ski proteins in synovial tissue after adenovirus transfection. **(B)** The transverse diameter of the right knee joint was measured with a Vernier caliper. Changes in the weight-bearing ratio of the left and right hindlimbs of rats. $^{**}p < 0.01$. **(C)** Western blot analysis of the effects of Ski on IL-1β and TNF-α in rat synovial tissue. **(D)** Western blot analysis of the effects of Ski on NF-κB p65 and p–NF–κB p65 in rat synovial tissue. Compared with the KOA group, $^{**}p < 0.01$. Compared with the Sham group, $^{\#\#}p < 0.01$. n = 5.

results suggest that transfection of ad-Ski can increase the high expression of Ski, not only reducing the expression of proinflammatory factors, VEGF and MMPs but also upregulating the expression of the anti-inflammatory factor IL-4. This result further supports the anti-inflammatory effect of Ski. Moreover, c-Ski inhibited the expression of the p–NF–kB-P65 protein, and Ski bound to the p–NF–kB p65 protein. This also indicates that Ski's anti-inflammatory function may occur through interference with the NF-κB signaling pathway to weaken or inhibit KOA-induced pain and inflammation, delay or prevent joint degeneration, and finally achieve the purpose of treatment. As an endogenous anti-inflammatory factor, Ski has low immunogenicity and few side effects. Ski, as a drug

development for the treatment of OA, should have great potential. However, the anti-inflammatory mechanism of increasing the level of Ski protein in OA needs to be further studied.

5. Conclusion

We concluded that increasing the level of Ski protein has an anti-inflammatory effect on MIA-induced KOA synovial FLSs and LPS-induced KOA synovial FLS inflammation. At the same time, it was also found that increasing the level of Ski protein had a down-regulation effect on the proinflammatory factors of LPS-induced U-937 cells. Ski's anti-inflammatory function may occur through interference with the NF-κB signaling pathway.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Ethics statement

All animal procedures were approved by the Administration of Affairs Concerning Experimental Animals Guidelines of Army Medical University (No. AMUWEC20181430 , June 30, 2018). The work has been carried out in accordance with either the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive 2010/63/EU or the National.

Institutes of Health – Office of Laboratory Animal Welfare policies and laws. All animal studies comply with the ARRIVE guidelines.

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Consent for publication

We declare that the publisher has the authors' permission to publish the relevant contribution.

CRediT authorship contribution statement

Ao Xiong: mainly performed the experiments, Writing – original draft. **Renping Xiong:** Writing – review & editing, Validation, Conceptualization. **Fei Luo:** Writing – review & editing, Validation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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