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miR-93-5p attenuates IL-1β-induced chondrocyte apoptosis and cartilage degradation in osteoarthritis partially by targeting *TCF4*

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ABSTRACT

MicroRNAs (miRNAs, miRs) are frequently dysregulated in osteoarthritis (OA), but the role of specific miRNAs in OA remains unclear. In this study, we found that miR-93-5p is underexpressed in human and rat OA-affected cartilage (compared with normal cartilage) as well as in IL-1β–treated chondrocytes. Overexpression of miR-93-5p promoted chondrocyte viability, suppressed chondrocyte apoptosis, and maintained the balance between anabolic and catabolic factors of the extracellular matrix *in vitro*. Similarly, injection of a miR-93-5p–expressing lentivirus alleviated the destruction of articular cartilage in a rat model of OA (anterior cruciate ligament transection). Furthermore, *TCF4* was identified as a direct target gene of miR-93-5p. miR-93-5p directly targeted the 3′ untranslated region (3′-UTR) of *TCF4* mRNA and repressed TCF4 expression. Overexpression of TCF4 attenuated the effects of miR-93-5p on chondrocyte apoptosis and functions. Finally, analyses of miR-93-5p and TCF4 in OA-affected cartilage tissues revealed that miR-93-5p expression inversely correlated with TCF4 expression. Altogether, these findings indicate that miR-93-5p slows OA progression partially by suppressing TCF4 expression, and this phenomenon may provide novel insights into the function of miRNA in OA.

1. Introduction

Osteoarthritis (OA) is a common age-associated chronic joint disease characterized by articular cartilage degradation, subchondral bone thickening, and synovial inflammation, leading to an imbalance between the anabolism and catabolism of articular cartilage in an articulating joint [1]. Several factors may lead to OA development, including age, adiposity, injury, overuse, and infection [2]. OA is a leading cause of disability in the elderly population and imposes a substantial socioeconomic burden [3]. Nonetheless, the current standard treatment of OA is limited to pain management and joint replacement surgery in the late phase of the disease. Effective disease-modifying therapies for OA are unavailable because of the limited understanding of OA pathogenesis.

microRNAs (miRNAs, miRs) are small, endogenous, noncoding RNAs that negatively regulate gene expression at the post-transcriptional level by binding to the 3′ untranslated region (3′-UTR) of their target mRNAs, leading to degradation of this mRNA or specific suppression of protein synthesis [4]. Recently, the function of miRNAs in OA received much attention. Accumulating evidence indicates that miRNAs are frequently dysregulated in human inflammatory diseases including OA and play a crucial role in the development of arthritis and in joint homeostasis [5–7]. For example, miR-320 regulates matrix metalloproteinase 13 (MMP13) expression in chondrogenesis and IL-1β–induced chondrocyte responses [7]. miR-146a facilitates OA by regulating cartilage homeostasis *via* targeting CAMK2D and PPP3R2 [6]. miR-145 attenuates TNF-α–driven cartilage matrix degradation in OA *via* direct downregulation of MKK4 [8]. Thus, a better understanding of the mechanisms of action of miRNAs underlying the progression of OA may help to improve the efficacy of OA treatment.

miR-93-5p is a member of a miRNA family that is associated with inflammatory diseases. Upregulation of miR-93-5p reduces the injury of cardiac microvascular endothelial cells and the inflammatory response by downregulating SPP1 *via* inactivation of the NF-κB signaling pathway [9] as well as alleviates neuropathic pain development and reduces the expression of inflammatory cytokines, including IL-1β, TNF-α, and IL-6 in CCI rats [10]. miR-93-5p also inhibits inflammatory responses and apoptosis after cerebral ischemia-reperfusion by targeting IRAK4 [11]. Therefore, the involvement of miR-93-5p in OA pathogenesis was investigated in our study.

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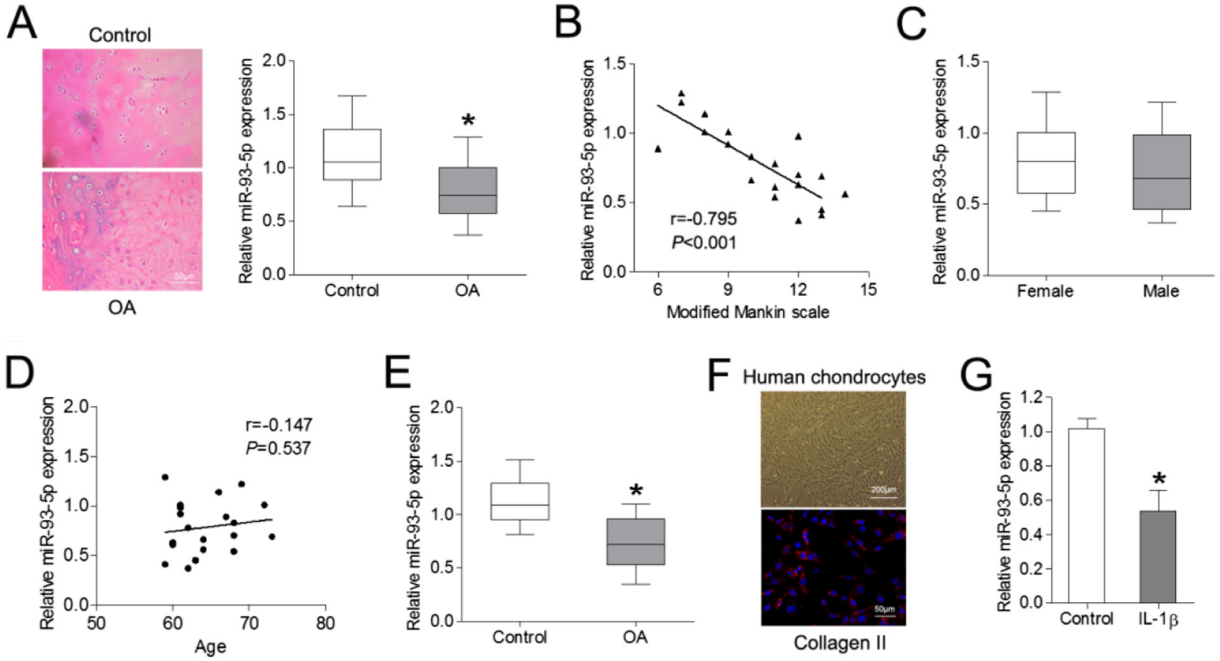

Fig. 1. Expression of miR-93-5p in OA cartilage and IL-1β–treated chondrocytes. (A) The expression of miR-93-5p was measured by qRT-PCR in healthy (control, $n = 20$) and OA ($n = 20$) human cartilage tissues. (B) The expression of miR-93-5p was negatively correlated with a modified Mankin scale ($r = -0.795$, $P < 0.001$). (C) qRT-PCR analysis of miR-93-5p expression in the cartilage samples from female group and male group. (D) No correlation was found between miR-93-5p expression level and age ($r = -0.147$, $P = 0.537$). (E) qRT-PCR analysis of miR-93-5p expression in the cartilage samples from the rat model of OA. Normal cartilage samples served as controls. (F) Morphology of chondrocytes and chondrocyte identification by immunofluorescence staining for type II collagen. (G) qRT-PCR analysis of miR-93-5p expression in chondrocytes treated with 10 ng/ml IL-1β for 12 h. $^{*}P < 0.05$.

In our study, we showed that miR-93-5p is significantly underexpressed in cartilage tissues of patients with OA as well as in IL-1β–treated normal chondrocytes. Moreover, we demonstrated that overexpression of miR-93-5p significantly alleviates OA through positive effects on chondrocyte proliferation and inhibition of cartilage matrix degradation and chondrocyte apoptosis *via* suppression of transcription factor 4 (TCF4) expression. We next determined the influence of miR-93-5p on OA development *in vivo* in a rat model of OA. These results provide further insight into the participation of miRNA in the pathogenesis of OA and should facilitate the development of novel therapeutic strategies.

2. Materials and methods

2.1. Human OA cartilage

Cartilage tissues of OA were collected from patients with OA who underwent total knee joint replacement surgery ($n = 20$, 12 females and 8 males, 64.3 ± 4.2 years). These tissues were not fibrous or wholly degenerated, and subchondral bone was not included. Undamaged areas in partial patients above were sampled as normal cartilage (control; $n = 10$, 5 females and 5 males, 65.1 ± 4.8 years). We also obtained 10 normal cartilage tissues from patients who were underwent the amputation without OA or from trauma patients (6 females and 4 males, 26.4 ± 4.4 years). Each tissue was measured by Safranin O-fast green staining to assess the extent of cartilage deterioration, and then graded based on a modified Mankin scale; grade I: 0–5, grade II: 6–10, grade III: 11–14 [5,6]. Ethical approval was obtained from the Yangpu District Central Hospital (Shanghai, China). Written informed consent was obtained from each participant.

2.2. Cell culture, transfection, and IL-1β treatment

Chondrocytes were extracted from cartilage tissue samples as previously described [12,13]. In brief, human normal cartilage tissues were dissected into small pieces and digested with 0.1% trypsin and then with 0.2% type II collagenase in Dulbecco's modified Eagle's medium (DMEM). Undigested tissue was separated from cells using a 40 mm filter. Chondrocytes were isolated after centrifugation and were maintained in DMEM/F12 containing 10% of fetal bovine serum at 37 °C and 5% CO_2. IL-1β (10 ng/ml) in the complete medium was used to induce chondrocyte apoptosis.

2.3. Lentivirus infection

The coding sequences of *TCF4* and miR-93-5p precursor sequences were cloned into lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA), separately. Lentiviruses were produced in HEK293T cells and then purified, concentrated, and titered. Stably infected cells were selected with puromycin. Chondrocytes were seeded in 6-well plates and infected with recombinant lentivirus-transducing units plus 8 μg/ml polybrene (Sigma, St. Louis, MO, USA).

2.4. RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed with the PrimeScipt RT Kit (Takara, Kusatsu, Japan). cDNA was subjected to qRT-PCR on a 7500 Sequence Detection System (ABI, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara). Fold changes in expression were calculated by the comparative threshold cycle (C_t) method *via* the formula $2^{-\Delta\Delta Ct}$. *U6* and *GAPDH* served as controls for miRNA and mRNA, respectively.

2.5. Western blotting

Samples were lysed, and their protein contents were measured by means of the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The lysates were centrifuged, subjected to sodium

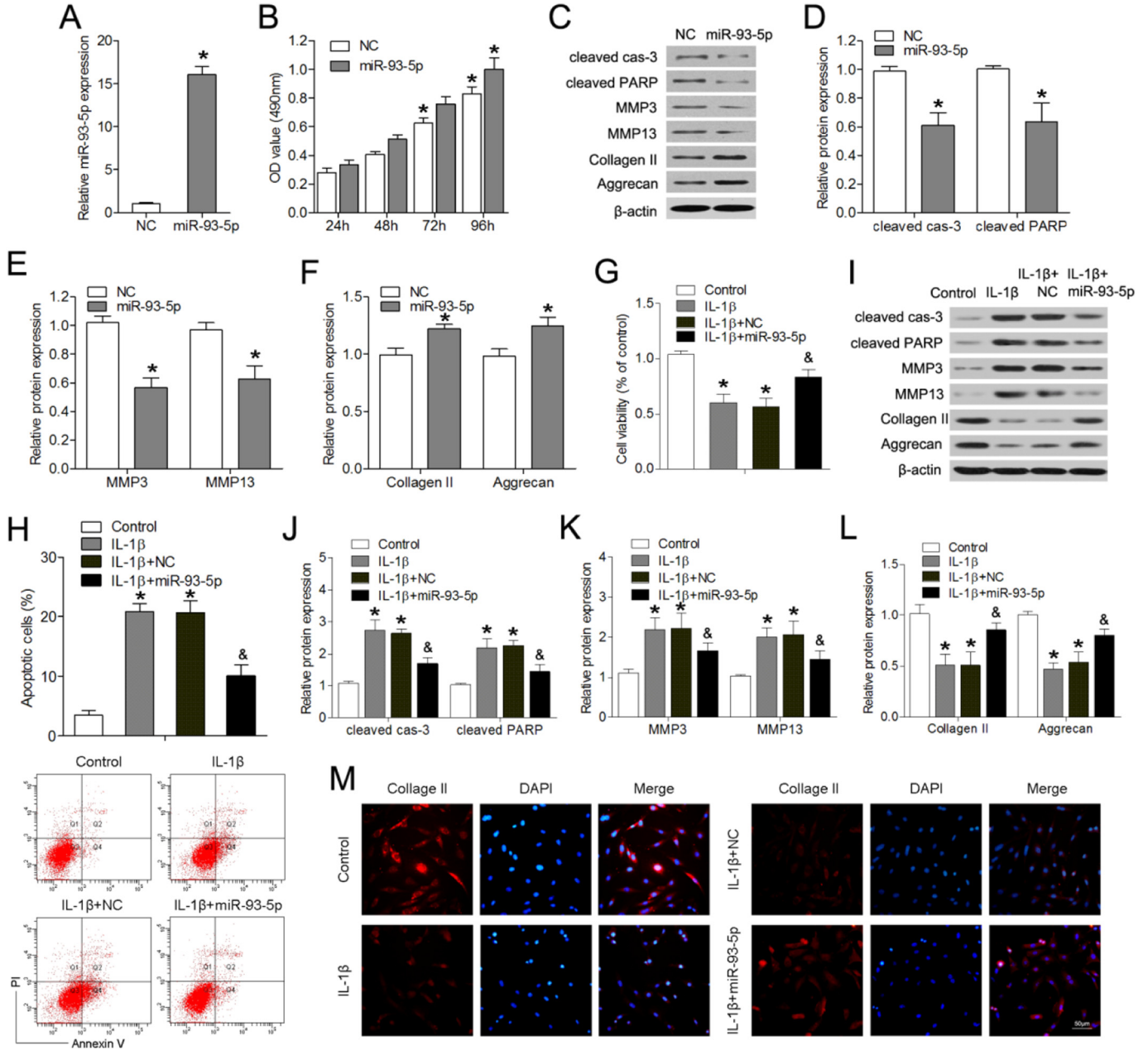

Fig. 2. miR-93-5p participates in the regulation of chondrocyte proliferation, apoptosis, and functions. (A) qRT-PCR analysis of miR-93-5p expression in human chondrocytes infected with a miR-93-5p–expressing or negative control (NC) lentivirus. (B) Chondrocyte viability was determined by the MTT assay at the indicated time points after infection with the miR-93-5p–expressing or NC lentivirus. (C) The expression of apoptotic effectors (cleaved caspase 3 and PARP), catabolic enzymes (MMP3 and MMP13), and ECM components (collagen II and aggrecan) was detected by western blotting. (D-F) Relative densitometric analysis of caspase 3, PARP cleavage products, MMP3, MMP13, collagen II and aggrecan. (G) Chondrocytes infected with the miR-93-5p–expressing or NC lentivirus were treated with 10 ng/ml IL-1β for 12 h. Cell viability was determined by the MTT assay. (H) Apoptosis was analyzed by flow cytometry after dual staining with an annexin V–fluorescein isothiocyanate (FITC) conjugate and propidium iodide (PI). (I) The expression of apoptotic effectors (cleaved caspase 3 and PARP), catabolic enzymes (MMP3 and MMP13), and ECM components (collagen II and aggrecan) was detected by western blotting. (J) Relative densitometric analysis of caspase 3 and PARP cleavage products. (K) Relative densitometric analysis of MMP3 and MMP13. (L) Relative densitometric analysis of collagen II and aggrecan. (M) An immunofluorescent assay of collagen II. $^{*}P < 0.05$ as compared with the NC or control group, $^{\&}P < 0.05$ as compared with the IL-1β or IL-1β + NC group.

dodecyl sulfate polyacrylamide gel electrophoresis, and were transferred to polyvinylidene fluoride membranes. The membranes were blocked and incubated with a specific primary antibody overnight at 4 °C, and then horseradish peroxidase–labelled secondary antibodies were added. Protein expression was visualized with an enhanced chemiluminescence reagent. The following primary antibodies were employed: anti-TCF4 (ab185736, Abcam, Cambridge, MA, USA), anti–cleaved caspase 3 and anti-cleaved PARP (Cell Signaling Technology; Beverly, MA, USA) and anti-MMP3, anti-MMP13, anti-collagen II, and anti-aggrecan (Proteintech Group, Wuhan, China). β-actin served as an internal control.

2.6. Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine cell viability. Lentivirus-infected cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 96 h. Then, 0.5 mg/ml MTT was added and

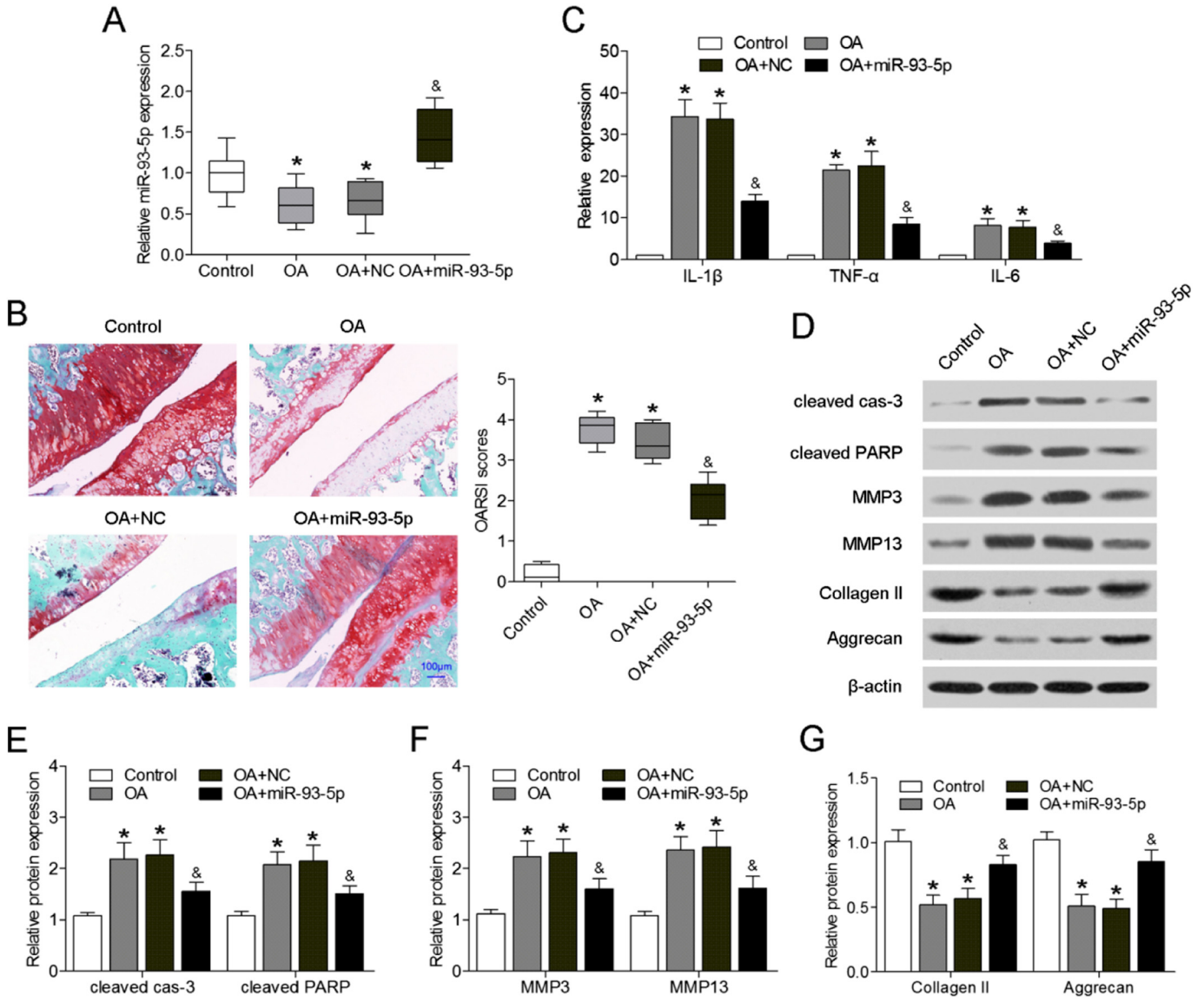

Fig. 3. miR-93-5p alleviates cartilage matrix degradation *in vivo*. (A) miR-93-5p expression was measured in rat cartilage tissue samples from the control group, OA group, and OA rats treated with the miR-93-5p–expressing or NC lentivirus. (B) Safranin O staining and OARSI grades in sham- and ACLT-operated rats intra-articularly injected with a lentivirus expressing miR-93-5p or NC RNA. (C) The mRNA expression of inflammatory cytokines in the rat cartilage from the control group, OA group, OA + NC group, and OA + miR-93-5p group. (D) The amounts of cleaved caspase 3, cleaved PARP, MMP3, MMP13, collagen II, and aggrecan were evaluated by western blotting. (E) Relative densitometric analysis of caspase 3 and PARP cleavage products. (F) Relative densitometric analysis of MMP3 and MMP13. (G) Relative densitometric analysis of collagen II and aggrecan. $^{*}P < 0.05$ as compared with the control group, $^{\&}P < 0.05$ as compared with the OA or OA + NC group.

incubated for 4 h. Finally, the supernatant was removed, and the formazan crystals were dissolved in dimethyl sulfoxide. Absorbance of the resulting colored solution was quantified at 490 nm on a microplate reader.

2.7. Induction of apoptosis in chondrocytes

The apoptotic rate of IL-1β–treated chondrocytes was analyzed with the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen; San Jose, CA, USA) and evaluated by fluorescence-activated cell sorting (BD Biosciences, San Diego, CA, USA).

2.8. An immunofluorescent assay

Immunofluorescence was performed to evaluate expression of collagen II in chondrocytes. Cells were incubated with an antibody to collagen II simultaneously overnight at 4 °C. After washing with PBS, the cells were incubated with a secondary antibody (Alexa Fluor 488–conjugated goat anti-rabbit IgG antibody) for 1 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; KeyGEN Biotech, Nanjing, China) for 10 min, and images were captured by means of an Olympus microscope (Olympus Corporation, Tokyo, Japan).

2.9. A luciferase reporter assay

Either wild-type (WT) or mutant (MUT) *TCF4* 3′-UTR containing a potential binding site for miR-93-5p fragments was inserted into the pmirGLO luciferase vector (Promega, Madison, WI, USA). Chondrocytes were seeded in 96-well plates per well and were cotransfected with the reporter plasmid and a miR-93-5p mimic or negative control (NC) RNA using Lipofectamine 2000 (Invitrogen). The luciferase activity was

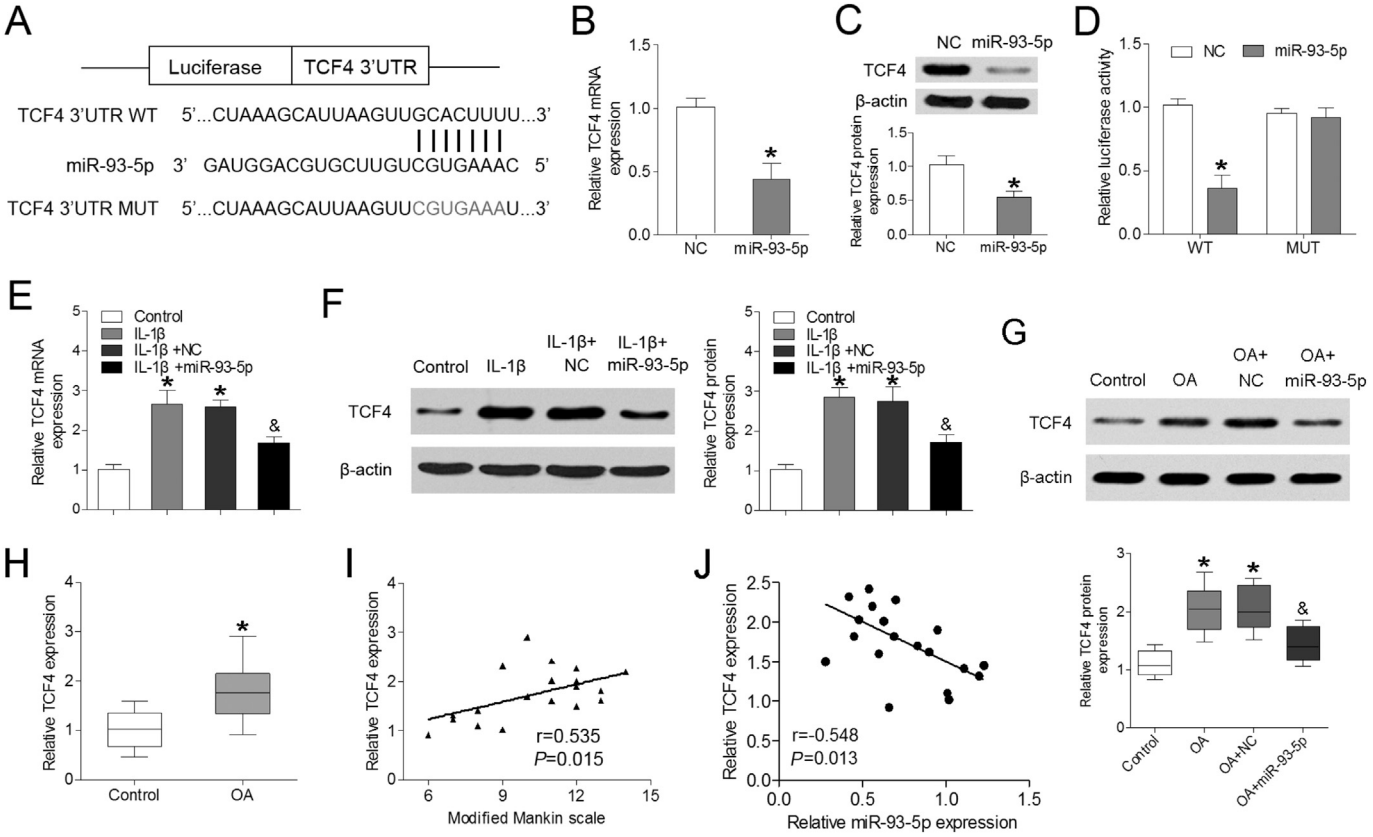

Fig. 4. miR-93-5p suppresses *TCF4* expression by directly targeting the 3′-UTR of its mRNA. (A) The predicted binding site for miR-93-5p in the 3′-UTR of *TCF4* mRNA. (B, C) *TCF4* mRNA and protein expression levels were assessed by qRT-PCR and western blots in chondrocytes transfected with the miR-93-5p–expressing or NC lentivirus. (D) Relative luciferase activity of chondrocytes after cotransfection of the WT or MUT *TCF4* 3′-UTR reporter construct along with a miR-93-5p mimic or NC RNA. (E, F) TCF4 mRNA and protein expression levels were evaluated by qRT-PCR and western blotting in IL-1β–treated chondrocytes transfected with the miR-93-5p–expressing or NC lentivirus. (G) TCF4 protein expression was measured in rat cartilage tissue samples from the control group, OA group, OA + NC group, and OA + miR-93-5p group. (H) The mRNA expression of *TCF4* in healthy and OA-affected human cartilage tissues was measured by qRT-PCR. (I) The correlation between *TCF4* expression and a modified Mankin scale in OA tissues. (J) The correlation between miR-93-5p and *TCF4* mRNA amounts in OA human cartilage tissue samples. $^{*}P < 0.05$ as compared with the control or NC group, $^{\&}P < 0.05$ as compared with the IL-1β, IL-1β + NC, OA, or OA + NC group.

measured *via* a Dual-Luciferase Reporter Assay System (Promega) after 48 h. The ratio of firefly and *Renilla* luciferase activities was calculated.

2.10. Experimental OA

Twenty-four male Sprague–Dawley rats (10 weeks old) were assigned into four groups ($n = 6$ each group): Control group, OA group, OA + NC group and OA + miR-93-5p group. Experimental OA in rats was induced by anterior cruciate ligament transection (ACLT) as described elsewhere [14]. Briefly, after induction of anesthesia, the right knees of rats were sterilized and a parapatellar skin incision was made on the medial side of the joint. The incision was made on the medial side of the patellar tendon, the patella was dislocated, and the anterior cruciate ligament was transected. The sham surgery group underwent anesthesia and surgical incision in the joint capsule but without ACLT. For the therapeutic experiment, a lentivirus expressing miR-93-5p, the control lentivirus, or the equivalent volume of vehicle was injected into the operated knee joint of rats 1 week after the surgical procedure (1×10^9 plaque-forming units in a total volume of 20 μl, two times a week for 7 weeks, $n = 6$ per group). The rats were killed 8 weeks after the operation, and right knee joints were processed for experiments.

2.11. Histological analysis and immunostaining

Rat cartilage was fixed in 4% paraformaldehyde and then was embedded in paraffin and sectioned at a thickness of 5 μm. To evaluate the degree of cartilage destruction, safranin O staining was performed as previously described [15]. Histological scoring was conducted in accordance with the Osteoarthritis Research Society International (OARSI) grading system [16] ranging from 0 (normal) to 6 (> 80% loss of cartilage). The score was determined in multiple serial sections from each murine knee.

2.12. Statistical analyses

All these analyses were performed in GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Experiments were repeated independently at least three times (each biological replicate the mean of three technical replicates for that sample). Data are expressed as mean ± SD. Student's *t*-test was carried out to compare the data between two groups, and one-way ANOVA for comparisons involving more than two groups. Pearson's correlation analysis was conducted to evaluate the correlation between miR-93-5p and TCF4 expression levels. Data with $P < 0.05$ were considered statistically significant.

3. Results

3.1. miR-93-5p was downregulated in OA cartilage and IL-1β–treated chondrocytes

H&E staining showed normal morphology of joints in the control group, whereas the surface of the joint cartilage exhibited defects,

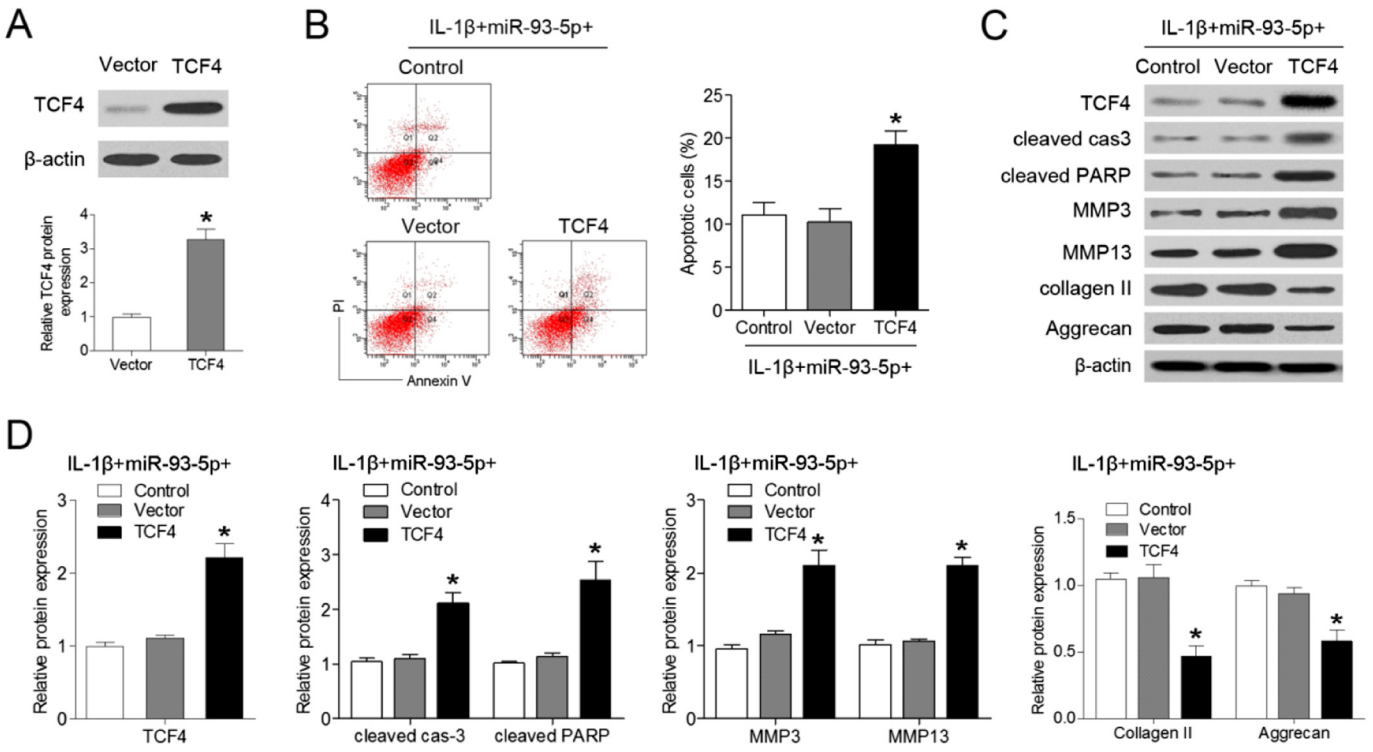

Fig. 5. The inhibitory effects of miR-93-5p on IL-1β–induced chondrocyte apoptosis and ECM degradation are attenuated by TCF4. (A) Western blot analysis of TCF4 expression in human chondrocytes infected with a TCF4-expressing or control lentivirus (indicated as "Vector"). (B) Chondrocytes overexpressing miR-93-5p were infected with the TCF4-expressing or control lentivirus and then treated with IL-1β. Apoptosis was analyzed by flow cytometry after dual staining with the annexin V–FITC conjugate and propidium iodide (PI). (C) The protein amounts of TCF4, cleaved caspase 3, cleaved PARP, MMP3, MMP13, collagen II, and aggrecan were assessed by western blotting. (D) Relative densitometric analysis of TCF4, cleaved caspase 3, cleaved PARP, MMP3, MMP13, collagen II, and aggrecan. $^{*}P < 0.05$ as compared with the IL-1β + miR-93-5p or IL-1β + miR-93-5p + vector group.

damage and structural breakage with a decreased number of cartilage cells in the articular cartilage tissue of the OA group (Fig. 1A). To determine the function of miR-93-5p in OA, we first examined the expression levels of miR-93-5p in 20 OA-affected cartilage tissue samples and 20 normal cartilage tissue samples. qRT-PCR results showed that miR-93-5p expression was significantly lower in OA cartilage tissue than in normal cartilage tissue (Fig. 1A). The expression of miR-93-5p was negatively correlated with a modified Mankin scale (Fig. 1B). There was no statistical significance between female group and male group (Fig. 1C), and no correlation was found between miR-93-5p expression level and age (Fig. 1D). Additionally, the expression pattern of miR-93-5p in the rat model of OA was consistent with that in the patients' samples (Fig. 1E). The chondrocytes exhibited a morphology which is considered typical of chondrocytes, with a spherical, fusiform and slabstone shape. Chondrocyte were identificated by immunofluorescence staining for type II collagen, had the typical morphology of chondrocytes (Fig. 1F). Moreover, the treatment with IL-1β significantly decreased the expression of miR-93-5p in chondrocytes (Fig. 1G). Taken together, these results suggested that downregulation of miR-93-5p might be involved in OA progression.

3.2. miR-93-5p was involved in the regulation of chondrocyte viability and functions

To better understand the role of miR-93-5p in the pathogenesis of OA, we first conducted *in vitro* gain-of-function experiments by overexpressing miR-93-5p through lentivirus infection in human chondrocytes (Fig. 2A). The MTT assay revealed that miR-93-5p overexpression promoted the proliferation of normal and IL-1β–treated chondrocytes (Fig. 2B and G). Western blot analysis showed that miR-93-5p overexpression decreased cleaved caspase 3, cleaved PPAR, MMP3 and MMP13 expression, but increased collagen II and aggrecan in normal chondrocytes (Fig. 2C–F). With respect to chondrocyte apoptosis, the percentage of apoptotic cells markedly increased in IL-1β–treated chondrocytes, and this change was reversed by miR-93-5p overexpression (Fig. 2H). Likewise, western blot analysis showed that miR-93-5p overexpression significantly attenuated the upregulation of cleaved caspase 3 and PPAR induced by IL-1β (Fig. 2I and J). Next, we analyzed the effects of miR-93-5p on matrix synthesis biomarkers. The results indicated that the increase in the expression of catabolic enzymes (MMP3 and MMP13) and the decrease in the expression of ECM components (collagen II and aggrecan) in response to IL-1β treatment were strongly attenuated after miR-93-5p overexpression (Fig. 2I, K, and L). This effect was next confirmed by immunofluorescent staining of collagen II in the lentivirus-infected chondrocytes (Fig. 2M). Taken together, these data suggested that miR-93-5p promoted cell proliferation and matrix synthesis and inhibited apoptosis in chondrocytes.

3.3. miR-93-5p counteracted cartilage matrix degradation in the rat OA model

Considering that miR-93-5p has an inhibitory effect on IL-1β–induced chondrocyte apoptosis and on the induction of matrix-degrading enzymes *in vitro*, we then evaluated the influence of miR-93-5p on OA development *in vivo* in the rat model of OA. Results of qRT-PCR validation showed that the expression of miR-93-5p in cartilage tissue samples was remarkably elevated after the injection of the miR-93-5p–expressing lentivirus (Fig. 3A). Fig. 3B indicates that this injection effectively alleviated cartilage destruction after ACLT surgery, protected cartilage from degradation, and reduced proteoglycan loss and the loss of articular chondrocyte cellularity. In addition, inflammatory cytokines in the cartilage of the OA group were significantly enhanced and this change was attenuated by miR-93-5p treatment (Fig. 3C). Moreover, miR-93-5p overexpression suppressed the enhancement of

apoptotic and catabolic responses and the reduced ECM components in the rat model of OA (Fig. 3D). These results confirmed that miR-93-5p alleviated cartilage degeneration in OA.

3.4. *TCF4 is a direct target of miR-93-5p*

We used computational algorithms (miRanda and TargetScan) to predict the targets of miR-93-5p, and the results revealed that there is a specific binding site in the *TCF4* 3′-UTR for miR-93-5p sequence as depicted in Fig. 4A. qRT-PCR and western blot results revealed that overexpression of miR-93-5p significantly suppressed the mRNA and protein expression of TCF4 in chondrocytes (Fig. 4B and C). To determine whether *TCF4* is a target of miR-93-5p, a luciferase reporter plasmid carrying the wild-type (WT) or mutant (MUT) *TCF4* 3′-UTR was cotransfected with the miR-93-5p–expressing or NC lentivirus. The results showed that overexpression of miR-93-5p statistically significantly reduced the activity of the luciferase reporter fused to the WT *TCF4* 3′-UTR but not the activity of the reporter fused to the MUT *TCF4* 3′-UTR (Fig. 4D). IL-1β increased TCF4 expression at mRNA and protein levels in chondrocytes, and these changes were reduced by miR-93-5p (Fig. 4E and F). Moreover, the protein expression of TCF4 was higher in the cartilage tissue samples from OA rats (relative to sham control), and this effect was attenuated in the OA + miR-93-5p group (Fig. 4G). The expression of TCF4 was also higher in human OA-affected cartilage tissues (relative to normal control) and positively correlated with a modified Mankin scale (Fig. 4H and I). An inverse correlation was also observed between TCF4 and miR-93-5p expression in these OA tissues (Fig. 4J). These findings confirmed that *TCF4* is a direct target gene of miR-93-5p and that TCF4 expression is suppressed by miR-93-5p in chondrocytes during IL-1β treatment or in the course of OA.

3.5. *miR-93-5p attenuated chondrocyte apoptosis and was found to regulate their functions by inhibiting TCF4 expression in IL-1β–treated chondrocytes*

To test whether TCF4 is involved in the process of miR-93-5p–regulated chondrocyte apoptosis and in chondrocyte functions, we constructed a lentiviral vector carrying the *TCF4* gene. Increased expression of TCF4 after lentiviral infection of chondrocytes was confirmed by a western blot (Fig. 5A). As shown in Fig. S1, TCF4 overexpression inhibited cell proliferation and induced cell apoptosis. We then co-overexpressed miR-93-5p and TCF4 in chondrocytes, and functional assays revealed that miR-93-5p's negative effect on apoptosis and miR-93-5p's positive effects on chondrocyte functions were attenuated in the presence of TCF4 (Fig. 5B–D). These data indicated that miR-93-5p exerted its actions in chondrocytes partially through suppression of TCF4 expression.

4. Discussion

Lately, the involvement of miRNAs in OA received much attention. Thus, in the present study, we demonstrated that miR-93-5p is significantly underexpressed in cartilage tissues of patients with OA as well as in IL-1β–treated normal chondrocytes. Overexpression of miR-93-5p alleviated OA in the rat model by increasing chondrocyte activity and by inhibiting cartilage matrix degradation and chondrocyte apoptosis. Moreover, we found that *TCF4* is a direct target of miR-93-5p, and its expression negatively correlated with miR-93-5p expression among human OA-affected cartilage tissue samples. Enhanced TCF4 expression strongly inhibited the effects of miR-93-5p on the chondrocyte apoptosis and on the expression of anabolic and catabolic factors.

OA is characterized by a change in chondrocyte behavior that leads to elevated production of proteolytic enzymes and consequently to cartilage damage and loss of joint function. Accordingly, a better understanding of the molecular mechanisms underlying the reductions in chondrocyte numbers and degradation of the ECM in cartilage in the course of OA may help to develop new treatments of OA [17]. Some studies have uncovered several miRNAs involved in the pathogenesis of OA, *e.g.*, miR-149, miR-193b, and miR-483-5p [18–20]. It has been reported that miR-93-5p was associated with inflammatory response and ECM deposition/degradation. Xu et al. have showed that miR-93-5p inhibits inflammatory cytokine production in LPS-stimulated murine macrophages by targeting IRAK4 [21]. Zhang et al. have reported that miR-93-5p affected TGF-β1-induced fibroblasts' proliferation and ECM deposition through c-Ski [22]. miR-93-5p also has been reported to reduce collagen loss by downregulating MMP3 [23]. Therefore, the role of miR-93-5p in OA was investigated in our study. Here, we determined that miR-93-5p expression is lower in chondrocytes and cartilage tissues of patients with OA. IL-1β is a cytokine that can induce a range of pathogenic responses in chondrocytes because it plays a vital part in the progression of OA [24,25]. Here, we report that miR-93-5p is also downregulated in IL-1β–treated normal chondrocytes. Overexpression of miR-93-5p was found to significantly inhibit IL-1β–induced chondrocyte apoptosis. In general, IL-1β enhances expression of matrix-degrading enzymes (MMP3 and MMP13), which in turn degrade the ECM, then facilitate synovial inflammation and production of proinflammatory cytokines, and eventually lead to cartilage destruction [26,27]. Our results indicate that overexpression of miR-93-5p corrects the imbalance between the anabolism and catabolism of articular cartilage in an articulating joint.

Transcription factor 4 (TCF4, also called ITF2, SEF2 and E2-2) is a basic helix-loop-helix (bHLH) protein that is involved in various physiological processes [28,29]. TCF4 is a dosage-sensitive gene with emerging functions in the nervous system that associated with Pitt-Hopkins syndrome, intellectual disability, and schizophrenia [30]. Additionally, TCF4 functions as either an oncogene or a tumor suppressor to regulate tumor growth and metastasis [31,32]. In non-tumor areas, TCF4 is expressed specifically in plasmacytoid dendritic cells and is required for their development [33]; overexpression of TCF4 could inhibit endothelial progenitor cell proliferation and induce an increase in p27 expression [34]. Nevertheless, the role of TCF4 in OA remains unclear. In our study, computational algorithms were utilized to predict the targets of miR-93-5p, and *TCF4* was selected for further research. After that, we confirmed that *TCF4* mRNA is a direct target of miR-93-5p and is significantly downregulated by miR-93-5p overexpression in chondrocytes. Moreover, TCF4 turned out to be upregulated in the human and rat cartilage tissues affected by OA, and TCF4 expression inversely correlated with miR-93-5p expression. Overexpression of TCF4 remarkably inhibited the effects of miR-93-5p on chondrocyte apoptosis and on the expression of anabolic and catabolic factors relevant for the cartilage ECM.

In conclusion, we demonstrated that miR-93-5p is downregulated in rat and human OA-affected cartilage tissues as well as in IL-1β–treated normal chondrocytes. Overexpression of miR-93-5p alleviates OA *via* suppression of cartilage matrix degradation and of chondrocyte apoptosis partially by repressing *TCF4* expression *in vitro* and *in vivo*. These findings provide further insight into the participation of miRNA in the pathogenesis of OA and should be useful for the development of novel therapeutic strategies.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] W. Tong, Y. Geng, Y. Huang, Y. Shi, S. Xiang, N. Zhang, L. Qin, Q. Shi, Q. Chen, K. Dai, X. Zhang, In vivo identification and induction of articular cartilage stem cells by inhibiting NF-kappaB signaling in osteoarthritis, Stem Cells 33 (10) (2015) 3125–3137.

[2] C.J. Wang, J.H. Cheng, W.Y. Chou, S.L. Hsu, J.H. Chen, C.Y. Huang, Changes of articular cartilage and subchondral bone after extracorporeal shockwave therapy in osteoarthritis of the knee, Int. J. Med. Sci. 14 (3) (2017) 213–223.

[3] M.S. Makki, A. Haseeb, T.M. Haqqi, MicroRNA-9 promotion of interleukin-6 expression by inhibiting monocyte chemoattractant protein-induced protein 1 expression in interleukin-1beta-stimulated human chondrocytes, Arthritis Rheumatol. 67 (8) (2015) 2117–2128.

[4] E. Huntzinger, E. Izaurralde, Gene silencing by microRNAs: contributions of translational repression and mRNA decay, Nat. Rev. Genet. 12 (2) (2011) 99–110.

[5] J. Lu, M.L. Ji, X.J. Zhang, P.L. Shi, H. Wu, C. Wang, H.J. Im, MicroRNA-218-5p as a potential target for the treatment of human osteoarthritis, Mol. Ther. 25 (12) (2017) 2676–2688.

[6] X. Zhang, C. Wang, J. Zhao, J. Xu, Y. Geng, L. Dai, Y. Huang, S.C. Fu, K. Dai, X. Zhang, miR-146a facilitates osteoarthritis by regulating cartilage homeostasis via targeting Camk2d and Ppp3r2, Cell Death Dis. 8 (4) (2017) e2734.

[7] F. Meng, Z. Zhang, W. Chen, G. Huang, A. He, C. Hou, Y. Long, Z. Yang, Z. Zhang, W. Liao, MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1beta-induced chondrocyte responses, Osteoarthr. Cartil. 24 (5) (2016) 932–941.

[8] G. Hu, X. Zhao, C. Wang, Y. Geng, J. Zhao, J. Xu, B. Zuo, C. Zhao, C. Wang, X. Zhang, MicroRNA-145 attenuates TNF-alpha-driven cartilage matrix degradation in osteoarthritis via direct suppression of MKK4, Cell Death Dis. 8 (10) (2017) e3140.

[9] S.X. Ma, Z.F. Bai, W. Wang, H.Y. Wu, Effects of Microrna-93 on mouse cardiac microvascular endothelial cells injury and inflammatory response by mediating SPP1 through the NF-KappaB pathway, J. Cell. Biochem. 120 (3) (2019) 2847–2858.

[10] X.T. Yan, L.J. Ji, Z. Wang, X. Wu, Q. Wang, S. Sun, J.M. Lu, Y. Zhang, MicroRNA-93 alleviates neuropathic pain through targeting signal transducer and activator of transcription 3, Int. Immunopharmacol. 46 (2017) 156–162.

[11] F. Tian, C. Yuan, L. Hu, S. Shan, MicroRNA-93 inhibits inflammatory responses and cell apoptosis after cerebral ischemia reperfusion by targeting interleukin-1 receptor-associated kinase 4, Exp. Ther. Med. 14 (14) (2017) 2903–2910.

[12] Z. Wang, J. Hu, Y. Pan, Y. Shan, L. Jiang, X. Qi, L. Jia, miR-140-5p/miR-149 affects chondrocyte proliferation, apoptosis, and autophagy by targeting FUT1 in osteoarthritis, Inflammation 41 (3) (2018) 959–971.

[13] J. Hu, Z. Wang, Y. Shan, Y. Pan, J. Ma, L. Jia, Long non-coding RNA HOTAIR promotes osteoarthritis progression via miR-17-5p/FUT2/beta-catenin axis, Cell Death Dis. 9 (7) (2018) 711.

[14] G. Zhen, C. Wen, X. Jia, Y. Li, J.L. Crane, S.C. Mears, F.B. Askin, F.J. Frassica, W. Chang, J. Yao, J.A. Carrino, A. Cosgarea, D. Artemov, Q. Chen, Z. Zhao, X. Zhou, L. Riley, P. Sponseller, M. Wan, W.W. Lu, X. Cao, Inhibition of TGF-beta signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis, Nat. Med. 19 (6) (2013) 704–712.

[15] K.P. Pritzker, S. Gay, S.A. Jimenez, K. Ostergaard, J.P. Pelletier, P.A. Revell, D. Salter, W.B. van den Berg, Osteoarthritis cartilage histopathology: grading and staging, Osteoarthr. Cartil. 14 (1) (2006) 13–29.

[16] C. Huesa, A.C. Ortiz, L. Dunning, L. McGavin, L. Bennett, K. McIntosh, A. Crilly, M. Kurowska-Stolarska, R. Plevin, R.J. van 't Hof, A.D. Rowan, I.B. McInnes, C.S. Goodyear, J.C. Lockhart, W.R. Ferrell, Proteinase-activated receptor 2 modulates OA-related pain, cartilage and bone pathology, Ann. Rheum. Dis. 75 (11) (2016) 1989–1997.

[17] P.M. van der Kraan, W.B. van den Berg, Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? Osteoarthr. Cartil. 20 (3) (2012) 223–232.

[18] Q. Chen, S. Wu, Y. Wu, L. Chen, Q. Pang, MiR-149 suppresses the inflammatory response of chondrocytes in osteoarthritis by down-regulating the activation of TAK1/NF-kappaB, Biomed. Pharmacother. 101 (2018) 763–768.

[19] Z.K. Chang, F.G. Meng, Z.Q. Zhang, G.P. Mao, Z.Y. Huang, W.M. Liao, A.S. He, MicroRNA-193b-3p regulates matrix metalloproteinase 19 expression in interleukin-1beta-induced human chondrocytes, J. Cell. Biochem. 119 (6) (2018) 4775–4782.

[20] H. Wang, H. Zhang, Q. Sun, Y. Wang, J. Yang, J. Yang, T. Zhang, S. Luo, L. Wang, Y. Jiang, C. Zeng, D. Cai, X. Bai, Intra-articular delivery of Antago-miR-483-5p inhibits osteoarthritis by modulating Matrilin 3 and tissue inhibitor of metalloproteinase 2, Mol. Ther. 25 (3) (2017) 715–727.

[21] Y. Xu, H. Jin, X. Yang, L. Wang, L. Su, K. Liu, Q. Gu, X. Xu, MicroRNA-93 inhibits inflammatory cytokine production in LPS-stimulated murine macrophages by targeting IRAK4, FEBS Lett. 588 (9) (2014) 1692–1698.

[22] C. Zhang, Y. Zhang, H. Zhu, J. Hu, Z. Xie, MiR-34a/miR-93 target c-ski to modulate the proliferaton of rat cardiac fibroblasts and extracellular matrix deposition in vivo and in vitro, Cell. Signal. 46 (2018) 145–153.

[23] W. Jing, W. Jiang, MicroRNA-93 regulates collagen loss by targeting MMP3 in human nucleus pulposus cells, Cell Prolif. 48 (3) (2015) 284–292.

[24] F. Morrovati, N. Karimian Fathi, J. Soleimani Rad, A. Montaseri, Mummy prevents IL-1beta-induced inflammatory responses and cartilage matrix degradation via inhibition of NF-B subunits gene expression in pellet culture system, Adv. Pharm. Bull. 8 (2) (2018) 283–289.

[25] T. Pan, R. Chen, D. Wu, N. Cai, X. Shi, B. Li, J. Pan, Alpha-Mangostin suppresses interleukin-1beta-induced apoptosis in rat chondrocytes by inhibiting the NF-kappaB signaling pathway and delays the progression of osteoarthritis in a rat model, Int. Immunopharmacol. 52 (2017) 156–162.

[26] M. Kapoor, J. Martel-Pelletier, D. Lajeunesse, J.P. Pelletier, H. Fahmi, Role of proinflammatory cytokines in the pathophysiology of osteoarthritis, Nat. Rev. Rheumatol. 7 (1) (2011) 33–42.

[27] M.J. Lopez-Armada, B. Carames, M. Lires-Dean, B. Cillero-Pastor, C. Ruiz-Romero, F. Galdo, F.J. Blanco, Cytokines, tumor necrosis factor-alpha and interleukin-1beta, differentially regulate apoptosis in osteoarthritis cultured human chondrocytes, Osteoarthr. Cartil. 14 (7) (2006) 660–669.

[28] A. Flora, J.J. Garcia, C. Thaller, H.Y. Zoghbi, The E-protein Tcf4 interacts with Math1 to regulate differentiation of a specific subset of neuronal progenitors, Proc. Natl. Acad. Sci. U. S. A. 104 (39) (2007) 15382–15387.

[29] M.P. Forrest, M.J. Hill, A.J. Quantock, E. Martin-Rendon, D.J. Blake, The emerging roles of TCF4 in disease and development, Trends Mol. Med. 20 (6) (2014) 322–331.

[30] M. Sepp, H. Vihma, K. Nurm, M. Urb, S.C. Page, K. Roots, A. Hark, B.J. Maher, P. Pruunsild, T. Timmusk, The intellectual disability and schizophrenia associated transcription factor TCF4 is regulated by neuronal activity and protein kinase a, J. Neurosci. 37 (43) (2017) 10516–10527.

[31] H.W. Shin, H. Choi, D. So, Y.I. Kim, K. Cho, H.J. Chung, K.H. Lee, Y.S. Chun, C.H. Cho, G.H. Kang, W.H. Kim, J.W. Park, ITF2 prevents activation of the beta-catenin-TCF4 complex in colon cancer cells and levels decrease with tumor progression, Gastroenterology 147 (2) (2014) 430–442 (e438).

[32] H. Appaiah, P. Bhat-Nakshatri, R. Mehta, M. Thorat, S. Badve, H. Nakshatri, ITF2 is a target of CXCR4 in MDA-MB-231 breast cancer cells and is associated with reduced survival in estrogen receptor-negative breast cancer, Cancer Biol. Ther. 10 (6) (2010) 600–614.

[33] L.T. Grajkowska, M. Ceribelli, C.M. Lau, M.E. Warren, I. Tiniakou, S. Nakandakari Higa, A. Bunin, H. Haecker, L.A. Mirny, L.M. Staudt, B. Reizis, Isoform-specific expression and feedback regulation of E protein TCF4 control dendritic cell lineage specification, Immunity 46 (1) (2017) 65–77.

[34] Y. Yu, Y. Liang, C. Yin, X. Liu, Y. Su, L. Zhang, H. Wang, Inhibitor of DNA-binding 1 promotes endothelial progenitor cell proliferation and migration by suppressing E2-2 through the helix-loop-helix domain, Int. J. Mol. Med. 38 (5) (2016) 1549–1557.