

Overexpression of miR-146a-5p alleviate SDF1-induced cartilage degradation through repression TRAF6 mediated p38-MAPK signal pathway

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Research

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Abstract

Background : Previous study have revealed that miR-146a-5p has a role in osteoarthritis (OA) development, here, we aim to further explore the underlying mechanism of miR-146a-5P in OA both in vitro and in vitro .

Methods : RT-PCR was used to detect the level of miR-146a-5p in OA patients. Primary chondrocytes were treated with SDF-1 to induce an OA model in vitro , and an IL-1 β mediated OA model in rats were also used. Gain- or loss- of function assays of miR-146a-5p and TRAF6 were conducted to determine their roles in regulating the proliferation, apoptosis and cartilage degradation of chondrocytes.

Results : MiR-146a-5p was overexpressed in OA patients while downregulated in SDF-1 treated chondrocytes. Functionally, miR-146a-5p considerably accelerated the proliferation, inhibited apoptosis and limited ECM degradation of SDF-1 induced chondrocytes. However, TRAF6 upregulation had the opposite effects. Moreover, miR-146a-5p also inhibited OA progression in vivo. Mechanistically, miR-146a-5p targeted at the 3'UTR of TRAF6 and relieved TRAF6 mediated p38-MAPK/NF- κ B activation.

Conclusions : MiR-146a-5p exerted protective effects chondrocytes against SDF-1 or IL-1 β induced OA by regulating the proliferation, apoptosis and ECM degradation of chondrocytes by regulating the TRAF6/ p38-MAPK signaling pathway.

1. Background

OA (osteoarthritis), as an universal joint disease, is an essential cause of joint activity limitation and disability. It is a multifactor joint disease characterized by cartilage degeneration, subchondral sclerosis and osteophyte formation, which severely affects patients' life quality and brings a huge psychological and economic burden to patients and society. In recent decades, some studies have pointed out that the regular function of articular cartilage is highly dependent on the homeostasis of extracellular matrix (ECM), which, as the mechanical structure of chondrocytes, participates in chondrocytes' signal transduction [2–4]. Existing studies have proved anabolic factors' importance in maintaining the homeostasis and regeneration of ECM. Anabolic factors include transformed growth factor β and catabolic factors (including the interaction between matrix metalloproteinases (MMPs) and aggrecanases [5–6]. However, the etiology and pathogenesis of OA are complex and still not very clear, which brings great limitations to the diagnosis and treatment. Therefore, it is imperative to explore new functional genes and molecular intervention mechanisms in OA.

MicroRNAs (miRs), a kind of single non-coding small RNA molecules with 18 ~ 24 bases, the vast majority of which exist in the gene interval zone. They inhibit mRNA translation or cut mRNA, thus regulating gene expression at the transcriptional level. Eventually, they take part in cell growth, differentiation, stem cell function, cell invasion and angiogenesis by affecting cell division, proliferation and apoptosis. A growing body of evidence indicates that miRNA is inextricably linked to the occurrence of OA [7]. Lü Guohua et al. found that LINC00623 competes with HRAS for miR-101 binding, thereby

reducing the inhibitory effect of miR-101 on HRAS expression and regulating the apoptosis, senescence and ECM degradation of OA chondrocytes [8]. Therefore, the regulatory effect of miRNA in OA is complex. Taking miR-146a-5p as an example, it is a common miR, which is reported to have an anti-tumor effect in a number of human tumors [9]. Dong Zhao et al. proved the increase of miR-146a-5p level in OA and the decrease of miRNA-146a-5p level in patients with celecoxib treatment clinical response [10]. However, there is literature reporting that miR-146a-5p targets CXCR4, miR-146a-5p and CXCR4 expressions are negatively related to MMP-3 levels and positively correlated with collagen type II and aggregation, the study says miR-146a-5p has an inhibitory effect on OA [11]. The above results suggest that the specific function and mechanism of miR-146a-5p in OA are still unclear and need to be further explored.

Stromal cell-derived factor 1 (SDF-1) is a cytokine associated with inflammation, which has been found in the synovium of OA patients in recent years and is considered to be a vital cytokine related to OA occurrence [12]. Studies have shown that SDF-1 activates the downstream p38-MAPK signaling pathway by binding to a G-protein coupled receptor C-X-C chemokine receptor type 4 (CXCR4), thereby inducing the release of MMPs, increasing extracellular matrix degradation and exacerbating OA [13-14]. Therefore, inhibiting p38-MAPK activation is of great value for maintaining chondrocytes proliferation, inhibiting extracellular matrix degradation, and alleviating OA. TRAF6 is a critical multifunctional signaling molecule existing in cells, which promotes the synthesis and secretion of IL-1 β , IL-6 and TNF α by activating p38-MAPK, participate in the regulation of inflammatory response, lymph node metastasis and other processes, and exert a substantial effect on innate and acquired immunity. He Zhiyong et al. stated in lung cancer that TRAF6 gene knockout inhibited the protein expression of CXCR4 in SPC-A1 cells, thus inhibiting cancer cell migration and invasion and promoting cell apoptosis, but had little effect on cell proliferation and cell cycle. At present, the value of regulating TRAF6 in OA is still elusive. Akhtar Nahid et al. reported that Epigallocatechin-3-gallate stimulated the expression of TRAF-6 in human chondrocytes by inhibiting IL-1 β . However, the role of SDF-1 /TRAF6 in OA is still less understood. In particular, the mechanism of miRNA regulating TRAF6 has not been reported.

Considering that miR-146a-5p is highly expressed in OA and is closely related to the therapeutic effect of patients, but it mainly plays the role of limiting the inflammatory response, we found the targeting relationship between miR-146a-5p and TRAF6 through bioinformatics analysis. Hence, this study aims at investigating the biological effect and mechanism of miR-146a-5p /TRAF6 regulatory axis in OA. Primary chondrocytes were treated with SDF-1 to establish an OA model *in vitro*, and miR-146a-5p /TRAF6 expression was interfered to explore the regulation and related mechanisms of miR-146a-5p /TRAF6 on chondrogenic inflammatory response and ECM.

2. Materials And Methods

2.1 Specimen collection

From December 2016 to December 2016, thirty-one cases of human OA knee cartilage specimens were collected from First Affiliated Hospital of Kunming Medical University. Prior to total articular cartilage replacement, the patienta had met the clinical and radiological diagnostic criteria for OA. Twelve specimens of healthy knee cartilage were from patients with traumatic amputations who had no preoperative history of OA or other joint diseases. The medical ethics committee of First Affiliated Hospital of Kunming Medical University approved the research, and all patients' informed consent were obtained.

2.2 Primary chondrocyte culture

Primary human chondrocytes were isolated and cultured in accordance with a previous study [11]. Firstly, we minced degenerated cartilage tissue samples into pieces (less than 1 mm³) and put them for digestion with 0.15% collagenase II (Invitrogen, Carlsbad, CA, USA), stirring every 20 min after 2 h (37 °C, 5–6 hours). Secondly, after centrifugation, chondrocytes were isolated and cultured in Dulbecco Modified Eagle Medium/Ham Nutrient Mixture F12 (DMEM/F12; Gibco, Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc., Shanghai, China). Then, we added 100 U/ml penicillin G and 100 µg/mL streptomycin solution (Gibco, Life Technologies, Carlsbad, CA) into the medium and incubated the cells in a humidified environment (37 °C, 5% CO₂). The medium was changed every three to four days.

2.3 Cell transfection

Through transfection of sh-TRAF6 or TRAF6 overexpressing vector, TRAF6 expression was achieved. Lipofectamine 3000 (Invitrogen) was used to transfect miRNA mimics or miRNA inhibitors (Genepharma, Shanghai, China) for the modulation of miR-146a-5p. After 48-hour culture, the cells were identified as successfully transfected and went on to the follow-up experiments. OA was simulated with 100 ng/mL SDF-1 (Amyjet Scientific, Wuhan, China) for 12 h *in vitro*.

2.4 Brdu

The cells were inoculated on 12-well plate glass slices (1.0×10⁵ / well) and incubated (37 °C, 5% CO₂, 12 h) until all the cells attached to the wall. All the cells treated with experimental requirements were added to BrdU with a final concentration of 3.0 µmol/L and incubated 4 h at 37 °C. Then, we washed the cells three times with phosphate-buffered saline (PBS), secured them using 4% paraformaldehyde (30 min, room temperature) and washed them three times with PBS again. Next, they went through 30 min acid denaturation in 2 mol/L phosphate-buffered saline containing 0.1% Tweten-20 (PBST). PBST containing 5% bovine serum albumin was applied to block the non-specific antigen 1 h at room temperature. BrdU antibody was diluted with PBS at 1:1000 and underwent a two-hour incubation at room temperature. Later, these slices were PBS rinsed 3 times, diluted 1:1000 fluorescent secondary antibody with PBS, went

through one-hour incubation at room temperature and rinsed with PBS three times again. We sealed it with an anti-fluorescent sealing tablet containing DAPI. Four fields were randomly selected from each glass slide to count the quantity of BrdU staining positive cells, and the video was taken. Cell proliferation rate = BrdU positive cell number / total cell number × 100%. The experiment was repeated three times.

2.5 Apoptosis experiment

First of all, we performed FITC annexin V and propidium iodide (PI) double staining to examine the cell apoptosis. The cells in the logarithmic phase underwent trypsinization, inoculation in a six-well plate (1×10^5 per well) and 24-hour incubation at 37 ° C. The transfected and treated cells were collected, PBS washed, suspended in annexin-binding buffer and stained with 5 µL FITC annexin V and 0.1 µg PI from the FITC annexin V/Dead cell apoptosis kit (Invitrogen) (15 min, room temperature). After dilution in annexin binding buffer, the stained cells were detected by BD flow cytometry (Accuri C6). Flowjo software (TreeStar, SanCarlos, CA, USA) was applied to analyze the percentage of apoptotic cells. The experiment was repeated three times and the measurement was made three times.

2.6 RT-qPCR

According to the manufacturer's protocol, the RNeasy® Plus Micro Kit (QIAGEN, Hilden, Germany) was used to extract total RNA from the treated cells of each group. Next, the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific) was used to reverse the RNA into cDNA according to the agreement of the supplier. Real-time fluorescence quantitative PCR experiment was conducted with U6 and GAPDH as the endogenous control. The LightCycler 480 fluorescent quantitative PCR was applied for analysis, and the $2^{-\Delta\Delta Ct}$ method was employed for result analysis. The experiments were repeated three times. Primers of each gene are as follows:

TRAF6: forward 5'-TGGCCTCGAGATGTGCTTCAG-3'reverse 5'-TCTCCTTGGCGGCCGCACTTCCTTC-3'

miR-146a-5p: forward 5'-CGATGACGGTAAGAGCTCAGGCTC-3'reverse 5'-ATTACGTGCTCAGGGTCATTTTCATCAC-3'

GAPDH: forward 5'-AGGTCGGTGTGAACGGATTTG-3'reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'

U6: forward 5'-CTCGCTTCGGCAGCACA-3' reverse 5'-AACGCTTCACGAATTTGCGT-3'

2.7 Western blot

The culture medium was discarded after cell treatment, and protein lysate (Roche) was added to separate total protein. 50 g total protein was employed to 12% polyacrylamide gel and went through 2 h electrophoresis at 100V. Next, it was electrically transferred to polyvinylidene fluoride (PVDF) membranes. Afterwards, the membranes underwent one-hour sealing with 5% skimmed milk powder at room

temperature, TBST wash (3 times, 10 min each) and incubation with primary antibody (concentration 1:1000; Caspase-3:ab13847; Bax:ab182733; Bcl-2:ab32124; MMP3:ab53015; MMP13:ab39012; ADAMTS4:ab185722; ADAMTS5:ab41037; ACAN:ab3778; Col2a1:ab34712; TRAF6:ab33915; P38:ab170099; p-P38:ab4822; NF-kb:ab16502; p-NF-kb:ab86299; GAPDH:ab181602; abcam group, USA) (overnight, 4 °C). After TBST wash, the membranes were incubated at room temperature with horseradish peroxidase (HRP) -labeled anti-rabbit secondary antibody (concentration 1:300) for 1 h. Later, they were washed three time with TBST, 10 min each. Finally, Western blot reagent (Invitrogen) was used for color imaging, and Image J was applied to analyze the gray values of each protein.

2.8 Dual luciferase activity experiment

A wild-type or mutated TRAF6 was cloned into the pmirGLO reporter plasmid. Wild-type or mutant reporter vectors were co-transfected with miR-146a-5p mimics into primary chondrocytes. TRAF6 3'UTR wild-type and mutant plasmids were constructed by GenePharma (Shanghai, China). Forty-eight hours after transfection, the dual luciferase reporter assay (Promega, Madison, WI, USA) was performed to determine the luciferase activities of Firefly and Renilla. Luciferase activity was presented by the ratio of firefly luciferase intensity to renal luciferase intensity.

2.9 RIP

Using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-700, Millipore, MASSACHUSETTS, USA), RIP analysis of transfected cells was carried out 48 h after transfection. Then, the cells were incubated with anti-Ago2 antibody (Millipore) or negative control IgG (Millipore), and the relative enrichment of TRAF6 was evaluated via qRT-PCR.

2.10 OA model and intervention

After satisfactory anesthesia, the mice were dissected. The medial longitudinal incision of the right knee joint was about 4cm long. After exploring the joint cavity without primary disease, the anterior-posterior cruciate ligament and the medial collateral ligament and meniscus were carefully completely removed (without damaging the articular cartilage surface), the wound was sutured layer by layer, the sterile dressing was not fixed, and the mice were fed with standard. Then they were forced to move for several days, and 200,000 units of penicillin were injected intramuscularly for 7 days to prevent infection. To difine whether the model is successful, X-ray and light microscopy was used. PBS was injected into joint cavity as control, and miR-146a-5p mimic (100nm, genepharma, Shanghai, China) was injected for one month as experimental group. The animals in each group were killed and the materials (synovium and articular cartilage) were taken for examination.

2.11 Data analysis

SPSS21.0 statistical software (SPSS Inc., Chicago, IL, USA) was taken for statistical analysis. In this study, continuous data were used as the mean \pm standard deviation (SD), and the t-test method was applied for comparison. Pearson was used for correlation analysis. Five independent samples were prepared for each experiment. $P < 0.05$ was statistically valuable.

3. Results

3.1 Aberrant expression of miR-146a-5p /TRAF6 in OA

To confirm the function of miR-146a-5p /TRAF6 in OA process, RT-qPCR was conducted to examine miR-146a-5p /TRAF6 expression in thirty-one OA cartilage specimens and twelve healthy specimens. As illustrated in FIG. 1, miR-146a-5p expression in patients with OA was notably increased, and TRAF6 expression was also increased (FIG. 1 A-B, $P < 0.01$). The Pearson correlation was taken to analyze the expression correlation of miR-146a-5p /TRAF6 in patients with OA. The miR-146a-5p expression was increased after SDF-1 intervention in the model *in vitro*, while TRAF6 expression was raised, with a negative correlation between the two (FIG. 1 D-F, $P < 0.01$). The above results proved that miR-146a-5p /TRAF6 exerted a vital effect on OA and may have a regulatory relationship.

3.2 MiR-146a-5p inhibited SDF-1-induced chondrocyte apoptosis and ECM degradation

Aiming at detecting the function of miR-146a-5p in primary chondrocytes proliferation and apoptosis, miR-146a-5p mimic and miR-146a-5p inhibitor were transplanted into cells. Meanwhile, cell models with high and low expression of miR-146a-5p were successfully established (FIG. 2A, $P < 0.001$). BrdU outcomes demonstrated that miR-146a-5p mimic improved the viability of chondrocytes. Compared with the anti-NC group, the cell viability of the anti-miR-146a-5p group was considerably reduced (FIG. 2B). Cell apoptosis was measured via flow cytometry. It turned out that miR-146a-5p up-regulation restrained chondrocyte apoptosis, and its knockdown promoted cell apoptosis (FIG. 2C). ELISA detection of inflammatory factors manifested that miR-146a-5p mimic remarkably reduced the expressions of TNF- α , IL-6 and IL-1 β , while anti-miR-146a-5p showed the opposite effect (FIG. 2D). WB statistics of apoptosis-related proteins suggested that cleaved Caspase 3 and Bax expressions decreased remarkably in the miR-146a-5p mimic group, while Bcl-2, ACAN and Col2a1 expressions increased (FIG. 2E). The ECM-related protein detection results suggested that the expressions of MMP3, MMP13, ADAMTS5 and ADAMTS4 in miR-146a-5p mimic group were knocked down, while ACAN and Col2a1 expressions were increased (FIG. 2F). Anti-miR-146a-5p had the opposite effect on apoptosis-related proteins and ECM-related proteins (FIG. 2 E-F). This phenomenon implied that miR-146a-5p promoted the activity of chondrocytes in OA and limited the apoptosis, inflammatory response and MMP activation.

3.3 TRAF6 was the target of miR-146a-5p

Papers have stated that miR-146a-5p acts as a competitive endogenous RNA (ceRNA) competing for specific mRNAs. RT-PCR results manifested that miR-146a-5p was mainly expressed in the cytoplasm (FIG. 3A). Through Starbase, we searched for the target mRNA of miR-146a-5p and found that miR-146a-5p contained a binding sequence complementary to TRAF6 (FIG. 3B). This prediction was confirmed by luciferase, and the results revealed that miR-146a-5p mimics reduced luciferase activity of the luciferase reporter containing TRAF6-wt, but not that of the TRAF6-mut vector (FIG. 3C, $P < 0.001$). The RIP experiment also confirmed the targeted binding relationship between miR-146a-5p and TRAF6 (FIG. 3D, $P < 0.001$). Besides, RT-PCR and WB results suggested that TRAF6 expression in the miR-146a-5p mimic group was down-regulated, while TRAF6 expressions in the anti-miR-146a-5p group was up-regulated (FIG. 3E). This suggests that TRAF6 is the target of miR-146a-5p.

3.4 TRAF6 promoted SDF-1-induced chondrocyte apoptosis and ECM degradation

For the purpose of examining the effect of TRAF6 on the proliferation and apoptosis of primary chondrocytes, TRAF6 and sh-TRAF6 were transferred into cells, and the cell models with high and low expression of TRAF6 were successfully established (FIG. 4A, $P < 0.001$). After treated with 100 ng/mL SDF-1, the cells showed a decrease in the TRAF6 group and an increase in the sh-TRAF6 group in viability through BrdU assay (FIG. 4B). Cell apoptosis was measured by flow cytometry. It turned out that TRAF6 promoted chondrocyte apoptosis, and down-regulated TRAF6 limited cell apoptosis (FIG. 4C). ELISA results represented that over-expressed TRAF6 promoted the expressions of TNF- α , IL-6 and IL-1 β , while sh-TRAF6 had the opposite effect (FIG. 4D). WB detection statistics suggested that the expressions of cleaved Caspase 3, Bax and MMP3, MMP13, ADAMTS5 and ADAMTS4 were raised in the TRAF6 group, while the expressions of Bcl-2, ACAN and Col2a1 were down-regulated, and the opposite effect was observed in the sh-TRAF6 group (FIG. 4E-F). The above results indicated that TRAF6 promoted chondrocyte apoptosis and ECM degradation in OA, and inhibited the expressions of ACAN and Col2a1.

3.5 miR-146a-5p regulated SDF-1-induced chondrocyte apoptosis by TRAF6

For more profound study of the targeting regulation of miR-146a-5p on TRAF6 in an *in vitro* model of OA simulated by SDF-1, over-expressed TRAF6 plasmids were supplemented with a miR-146a-5p mimic (FIG. 7A). Brdu experimental results confirmed that supplementation with TRAF6 reversed the promoting effect of miR-146a-5p on cell proliferation ($P < 0.05$, FIG. 5B). Flow detection results illustrated that TRAF6 reversed the inhibitory effect of miR-146a-5p on apoptosis ($P < 0.05$, FIG. 5C). ELISA results confirmed that TRAF6 reversed the inhibition of miR-146a-5p on the inflammatory cytokines IL-6, TNF- α and IL-1 β ($P < 0.05$, FIG. 5D). WB detection results suggested that supplementation of TRAF6 over-expressed plasmids could reverse the regulation of miR-146a-5p on cleaved Caspase 3,

Bax, MMPs, ADAMTS5, ADAMTS4, Bcl-2, ACAN, and Col2a1 ($P < 0.05$, FIG. 5E). The above statistics indicated that miR-146a-5p regulated SDF-1-induced chondrocyte apoptosis and ECM degradation by TRAF6.

3.6 miR-146a-5p regulated the p38 and NF-kB signaling pathways by TRAF6

To further study the downstream specific signaling pathways modulated by miR-146a-5p in the targeted regulation of TRAF6, WB was applied to detect the regulation of miR-146a-5p and the altered p38 and NF-kB after TRAF6. The results suggested that p38 and NF-kB activation of miR-146a-5p mimic considerably decreased, while that of anti-miR-146a-5p group increased (FIG. 6A). P38 and NF-kB activation increased in TRAF6 over-expressed cells, while p38 and NF-kB activation decreased in sh-TRAF6 group (FIG. 6B). Moreover, supplementation of TRAF6 reversed the regulatory effects of miR-146a-5p on p38 and NF-kB (FIG. 6C). These results manifested that miR-146a-5p contributes to the regulation of p38 and NF-kB signaling pathways by TRAF6.

3.7 miR-146a-5p alleviates cartilage degradation by regulating TRAF6 *in vivo*

In order to further confirm that miR-146a-5p / TRAF6 plays a certain role in the process of OA, the OA model was constructed, and miR-146a-5p mimic intervention was given to improve the level of miR-146a-5p in the tissues around the joint (Fig. 7A). He staining showed that miR-146a-5p mimic could significantly reduce the pathological changes of OA (Fig. 7B). WB results showed that after miR-146a-5p intervention, the expression of cleaved caspase 3 and Bax decreased significantly, while the expression of Bcl-2 increased (Fig. 7C); the detection results of ECM related proteins indicated that the expression of MMP3, MMP13, adamts5 and ADAMTS4 decreased significantly in miR-146a-5p mimic group, while the expression of acan and COL2A1 increased (Fig. 7C). The mechanism study showed that the expression of TRAF6 was down regulated and the activation of p38 and NF-KB was also significantly reduced after miR-146a-5p intervention (Fig. 7C). The above results confirmed that miR-146a-5p played a protective role in OA and inhibited cartilage degradation.

4. Discussion

The root cause of OA is the degeneration of articular cartilage, which is directly caused by the dynamic balance disruption of chondrocytes and extracellular matrix (ECM), that is, the reduction of chondrocytes and faster degradation of extracellular matrix (ECM). Therefore, damaging the function and survival of chondrocytes and promoting the degradation of ECM could lead to articular cartilage damages [1-2]. To investigate the function of miR-146a-5p / TRAF6 in OA chondrocytes and its mechanism, we constructed an OA model of primary chondrocytes with 100 ng/mL SDF-1 *in vitro*. The results showed that miR-146a-5p and TRAF expressions were both raised in OA patients, but were negatively correlated

in the two. Up-regulation of miR-146a-5p promoted the proliferation of OA chondrocytes and restrained ECM degradation. The mechanism of miR-146a-5p up-regulation mainly inhibited TRAF6 signaling pathway activation, thereby reducing SDF-1 /CXCR4 activation chondrocyte apoptosis and ECM degradation.

Studies have found that aberrant expression of microRNAs(miRs) accelerate or delay the course of OA by regulating its downstream target factors. In the complex regulatory factor network, the mutual synergy and antagonism between miR and cytokines and between various cytokines play different roles in the pathophysiological process of OA, thus making a great contribution to OA occurrence and development. An Yongbo et al. reported that miR-203a level was raised respectively in OA tissues and OA models *in vitro*. Inhibition of miR-203a significantly reduced interleukin-1 (IL-1) β induced chondrogenic inflammatory response and extracellular matrix degradation [19].

In interleukin-1 β (IL-1) induction of OA environment, melatonin can increase miR-140 expression, promote cartilage cell proliferation, promote cartilage ECM protein (such as collagen type α and acrasin) expressions and inhibit the levels of IL-1 β induced metalloproteinase 9 (MMP9) and 13 (MMP13), ADAMTS4 (a kind of platelet response protein 4 motifs to integrin and metalloproteinases) and ADAMTS5 [20]. IL-1 β stimulation induces miR203 expression, which promotes cell inflammation and cell damage by targeting the estrogen receptor and down-regulates the expressions of proteoglycan and COL2A1 [21]. MiR-193a-3p expression was knocked down in OA cartilage tissues and chondrocytes, and its up-regulation limited the inflammation and apoptosis of chondrocytes, reduced the protein levels of MMP-3, MMP-13 and ADAMTS-5, and up-regulated the expressions of Acan and Col2a1 [22]. Hence, miR plays a role in promoting or inhibiting OA progression in chondrocytes through a variety of mechanisms.

Currently, reports on miR-146a-5p in OA suggest that the possible effect of miR-146a-5p on OA is still controversial. For example, Skrzypa Marzena et al. proved that miR-146a-5p was over-expressed in both articular cartilage tissues and serum of OA patients, and their levels were positively correlated [23]. Furthermore, the miR-146a-5p level remained high in patients with inadequate treatment response, while the miRNA-146a-5p level decreased in clinical respondents to caixoxib treatment [10]. However, some literature stated that miR-146a-5p targeted CXCR4, and miR-146a-5p was negatively related to the expression levels of CXCR4 and MMP-3 and was positively correlated with collagen type α and acrasin, the study identified the inhibitive effect of miR-146a-5p on OA [11]. The results of this study, consistent with previous reports, confirmed that miR-146a-5p was highly expressed in OA in human specimens, but its expression was decreased in the SDF-1 intervention model *in vitro*. More importantly, this study demonstrated that miR-146a-5p could alleviate SDF-1-induced chondrocyte apoptosis and ECM degradation in the model *in vitro* and in OA vivo model, which provides a credible basis for exploring the specific mechanism of miR-146a-5p in OA. However, the differences between *in vitro* and *in vivo* remain to be further explored, and the possible mechanism is that cross-communication between other miR or related signaling pathways plays a regulatory role.

In addition, miR targets SDF-1 /CXCR4, and its signaling pathway works as a regulator in OA. MiR-221-3p targets SDF1 to inhibit the expressions of catabolic genes MMP13 and ADAMTS5 and prevent the degradation of chondrocyte extracellular matrix induced by IL-1 β [24]. However, there are still few studies on the intervention of other miR-regulated pathways in SDF-1 / CXCR4-related pathways. As a common miR, miR-146a-5p inhibits inflammation in various inflammatory diseases. Ge yu-ting et al. reported that after LPS treatment, miR-146a-5p expression was notably increased, and over-expressed miR-146a-5p knocked down the levels of LPS BV2 cells (TNF- α , IL-1mRNA and IL-6mRNA) and inhibits the protein expression of pNF- κ B by targeting TRAF6 [25]. What's more, numerous studies in the cementoblast-derived cell line [26], Human Olfactory line - and bone-marrow-derived Mesenchymal Stromal Cells [27], et al have confirmed that miR-146a-5p has a significant inhibitory effect on the reaction. The results of this study, consistent with other studies, illustrated that miR-146a-5p notably inhibits SDF-1-induced inflammatory response.

TRAF6 is an essential inflammation-related signaling molecule that activates p38, ERK, ultimately NF- κ B. Studies in clinical specimens have confirmed that the up-regulation of TRAF6 is closely related to the severity of OA. Further studies confirmed that inhibition of TRAF6 limited NF- κ B activation and alleviated OA development [29-30]. The literature on whether the downstream target of miR-146a-5p is related to TRAF6 is mostly limited to studies in the central nervous system. Lu Ying et al. initially reported that microRNA-146a-5p could target TRAF6 and block the up-regulation of LPS-induced astrocyte TRAF6, the activation of c-jun amino-terminal kinase (JNK) and the expression of chemokine CCL2 induced by LPS, thus reducing the expression of tumor necrosis factor α and mechanical hyperalgesia [31]. Chen Yuhan et al. reported that miR-146a-5p overexpression limited the secretion of LPS-induced proinflammatory cytokines by decreasing the phosphorylation levels of TLR-4, IL-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor-6 (TRAF6) and NF- κ B. Thus, it is inevitable that miR-146a-5p can target TRAF6 to regulate NF- κ B activation. In recent decades, microRNA-146a has also been reported to regulate chondrocyte apoptosis and proliferation by targeting TRAF6 [33], our previous study also confirmed the miR-146a-5p *in vitro*[11]. However, all of this studies was limited to the discussion of proliferation and apoptosis, and did not further explore the regulatory effect of miR-146a-5p on the inflammatory response of chondrocytes and the degradation of ECM in OA. The results of this study initially proved that miR-146a-5p alleviates the inflammatory response and ECM degradation in OA by targeting TRAF6.

Collectively, the researches here found that miR-146a-5p can alleviate SDF-1-induced chondrocyte apoptosis, inflammatory response and ECM degradation. Hence, this study further explored the targeted gene of miR-146a-5p and found that miR-146a-5p regulates p38 signaling pathway by targeting TRAF6, thus affecting OA progression.

Abbreviations

chemokine receptor type 4 (CXCR4); c-jun amino-terminal kinase (JNK); extracellular matrix (ECM); microRNAs(miRs); metalloproteinases (MMPs); osteoarthritis (OA); propidium iodide (PI); polyvinylidene

fluoride (PVDF); receptor-associated kinase 1 (IRAK1); Stromal cell-derived factor 1 (SDF-1).

Declarations

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Availability of data and materials

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

Authors' contribution

Yanlin Li, conceived and designed the experiments. Guoliang Wang, Xiao Yang, wrote and performed the experiments. Yaoyu Yang, Lu He, analyzed statistics. Di Jia, investigation and funding acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Our study was approved by the Ethics Review Board of First Affiliated Hospital of Kunming Medical University.

Competing interests

The authors declare that they have no competing interests.

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Figures

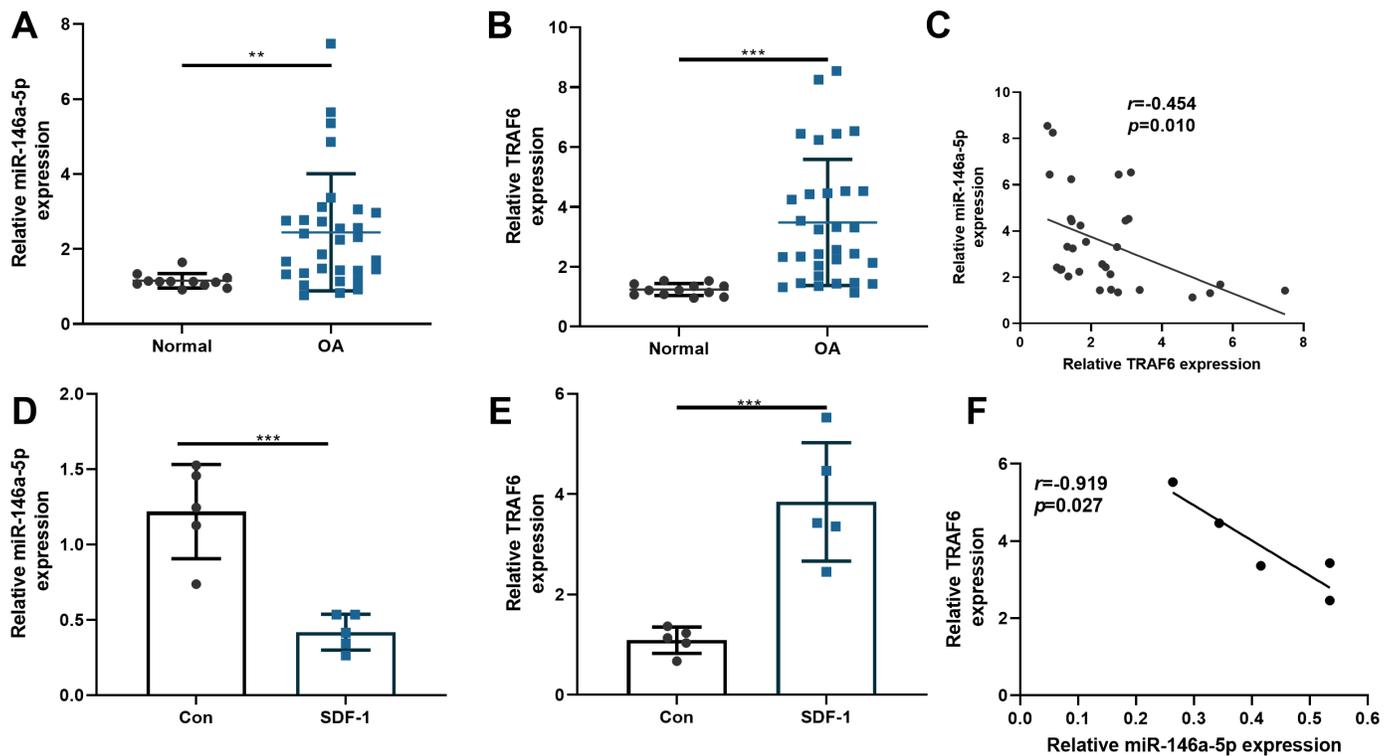


Figure 1

miR-146a-5p / TRAF6 was abnormally expressed in OA. A-B: The expressions of miR-146a-5p / TRAF6 in thirty-one OA cartilage specimens and twelve healthy specimens were detected via RT-qPCR. The miR-146a-5p expressions in OA patients were shown in Figure 1. C: Pearson correlation was taken to analyze the expression correlation of miR-146a-5p / TRAF6 in OA patients, and the correlation coefficient was -0.454. D-E: After SDF-1 intervention, the expressions of miR-146a-5p and TRAF6 in the model in vitro were measured via RT-PCR. F: Pearson correlation analysis of miR-146a-5p and TRAF6 in the model in vitro showed a negative correlation. * P < 0.05, ** P < 0.01, *** P < 0.001.

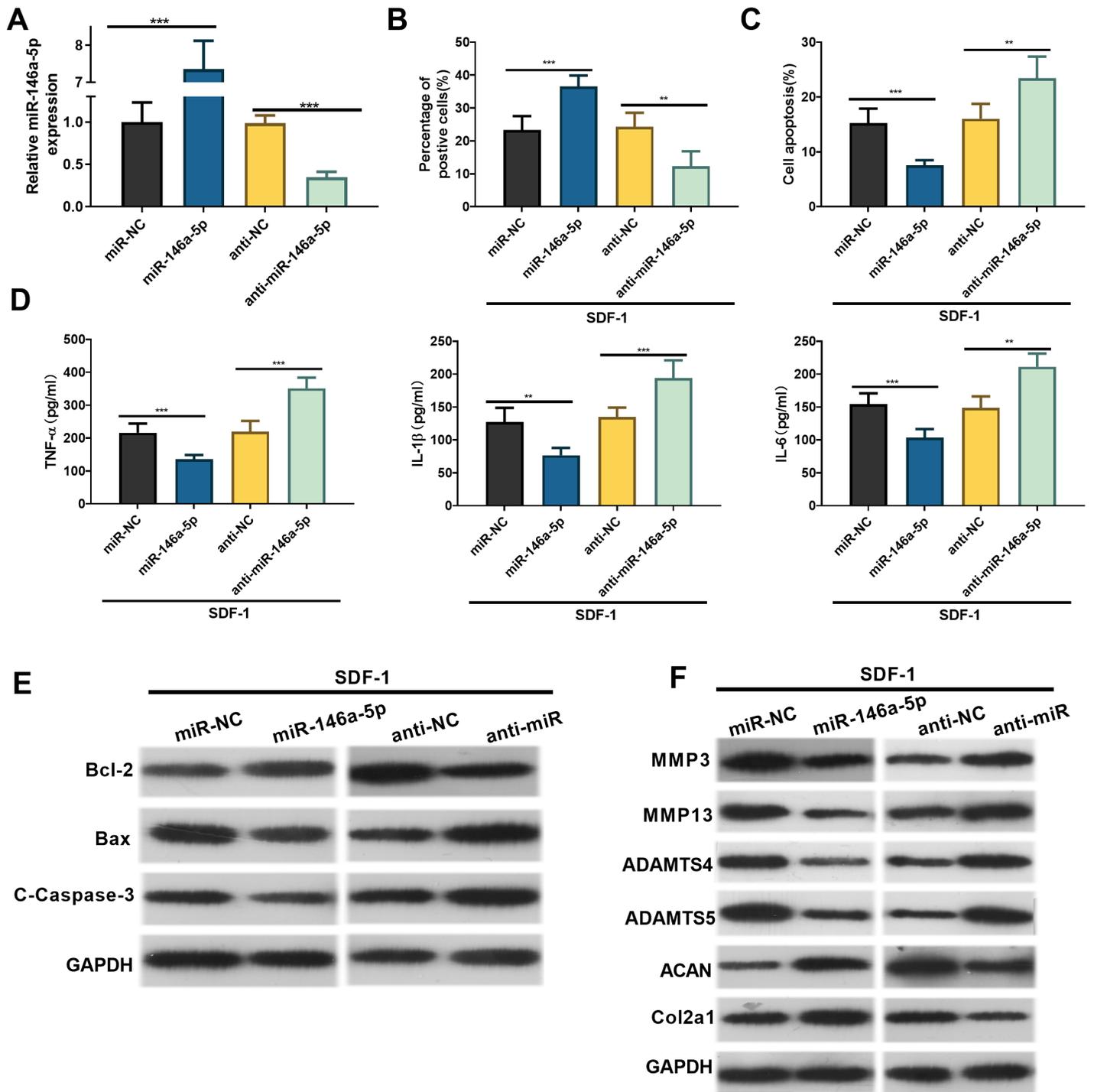


Figure 2

miR-146a-5p inhibited SDF-1 induced chondrocyte apoptosis and ECM degradation. A: RT-PCR confirmed the establishment of miR-146a-5p high and low expression cell models. B: BrdU test was carried out to detect the effect of miR-146a-5p on chondrocyte viability. C: Flow cytometry measurement of cell apoptosis. D: ELISA was performed to evaluate the expressions of inflammatory factors TNF- α , IL-1 β , IL-6. E-F: The expressions of apoptosis-related proteins cleaved-Caspase 3, Bax, Bcl-2 and ECM-related

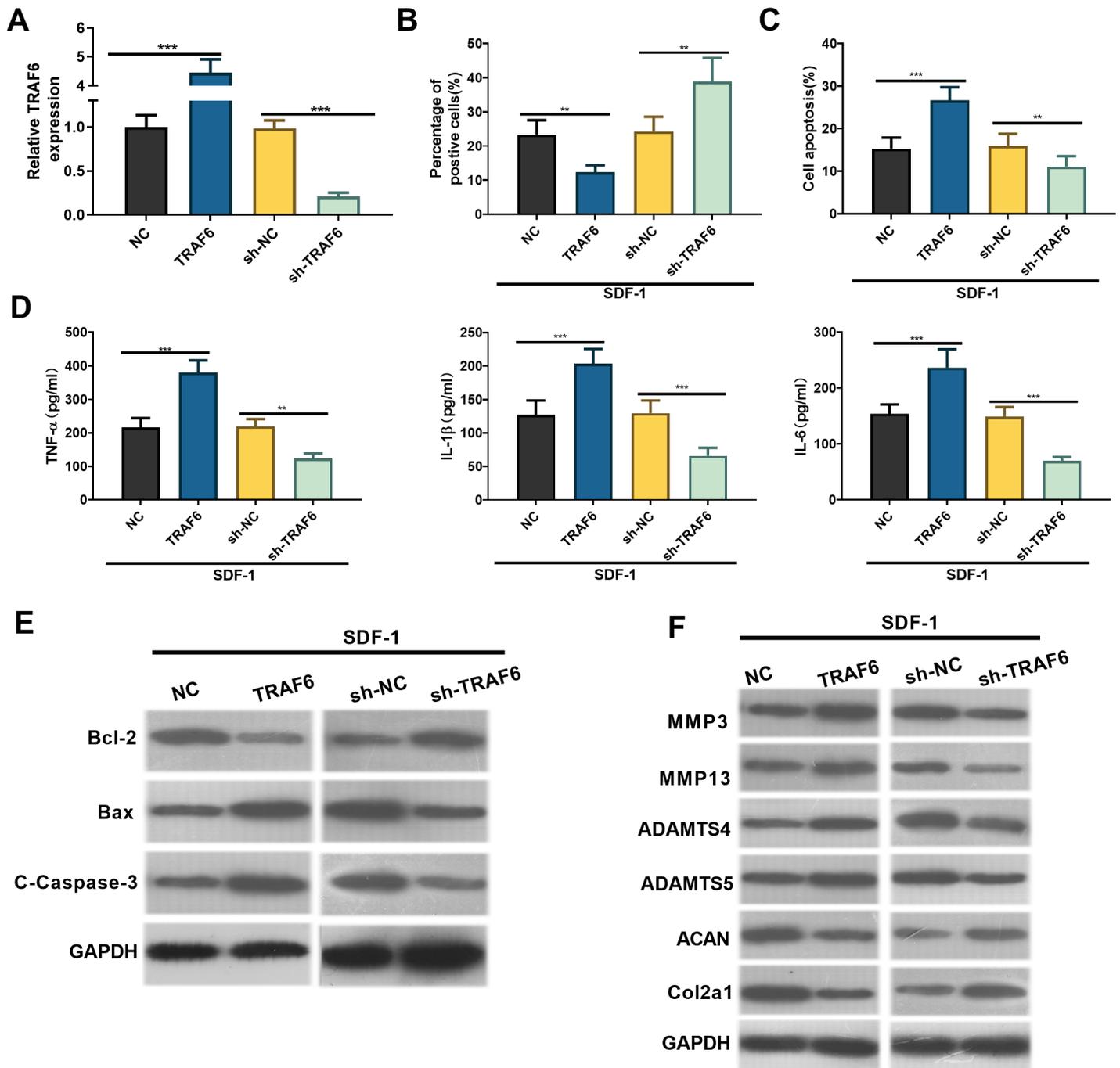


Figure 4

TRAF6 promoted SDF-1 induced chondrocyte apoptosis and ECM degradation. A: RT-PCR confirmed the establishment of TRAF6 high and low expression cell models. B: BrdU test to detect the effect of TRAF6 on chondrocyte viability. C: Flow cytometry detection of cell apoptosis. D: ELISA was employed to measure the expressions of inflammatory factors TNF- α , IL-1 β , IL-6. E-F: The expressions of apoptosis-related proteins cleaved-Caspase 3, Bax, Bcl-2 and ECM-related proteins ACAN, Col2a1, MMP3, MMP13, ADAMTS5, ADAMTS4 were measured via WB. * P < 0.05, ** P < 0.01, *** P < 0.001.

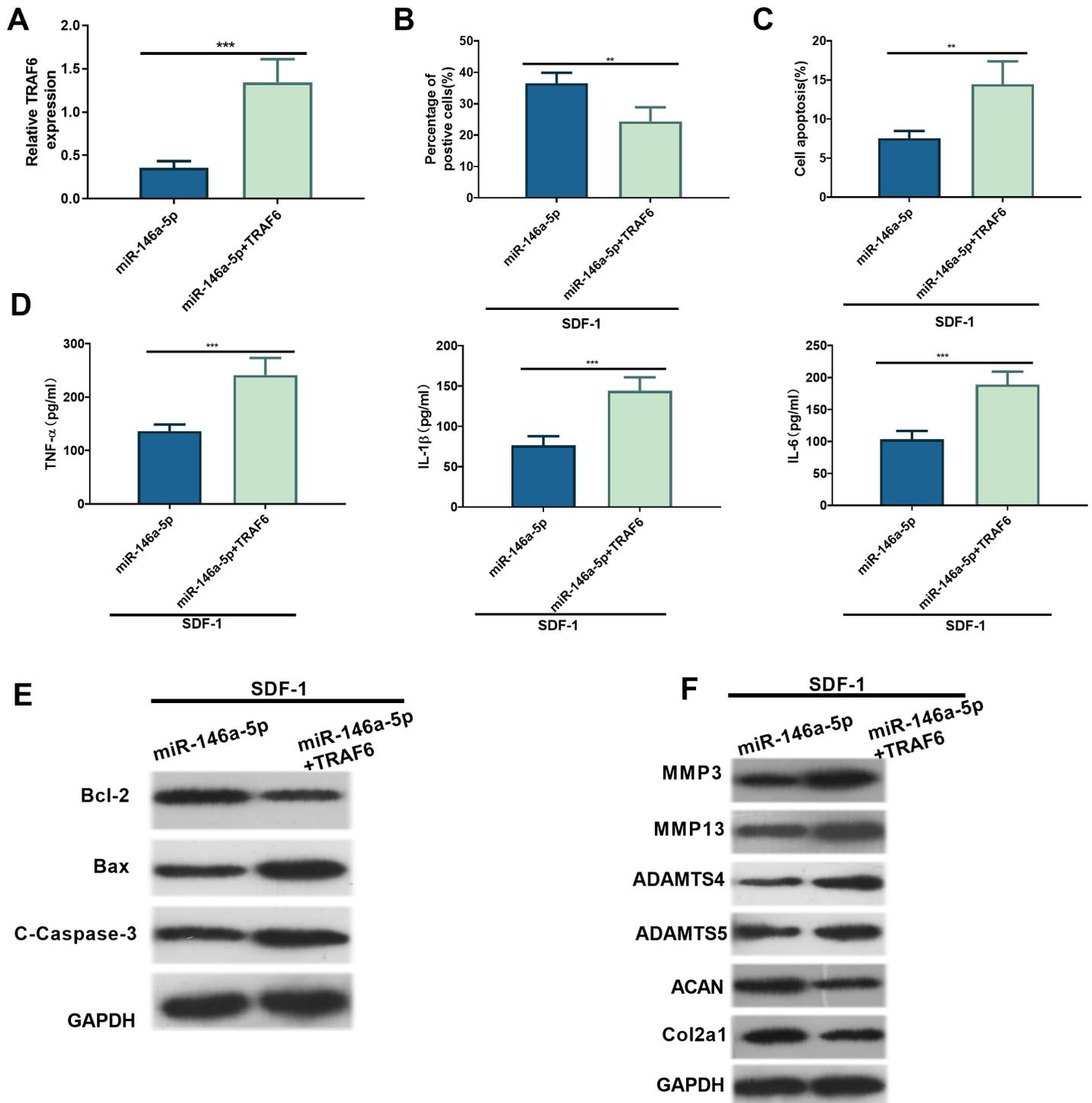


Figure 5

miR-146a-5p regulated SDF-1 induced chondrocyte apoptosis via TRAF6. A: Altered TRAF6 expression after miR-146a-5p mimic supplemented with over-expressed TRAF6 plasmid was examined via RT-PCR. B: BrdU experiment was carried out to detect altered chondrocyte viability when miR-146a-5p mimic was supplemented with over-expressed TRAF6 plasmid. C: Flow cytometry detection of cell apoptosis. D: ELISA was taken to detect the expressions of inflammatory factors TNF- α , IL-1 β , IL-6. E-F: The

expressions of apoptosis-related proteins cleaved-Caspase 3, Bax, Bcl-2 and ECM-related proteins ACAN, Col2a1, MMP3, MMP13, ADAMTS5, ADAMTS4 were detected by WB. * P <0.05, ** P <0.01, *** P <0.001.

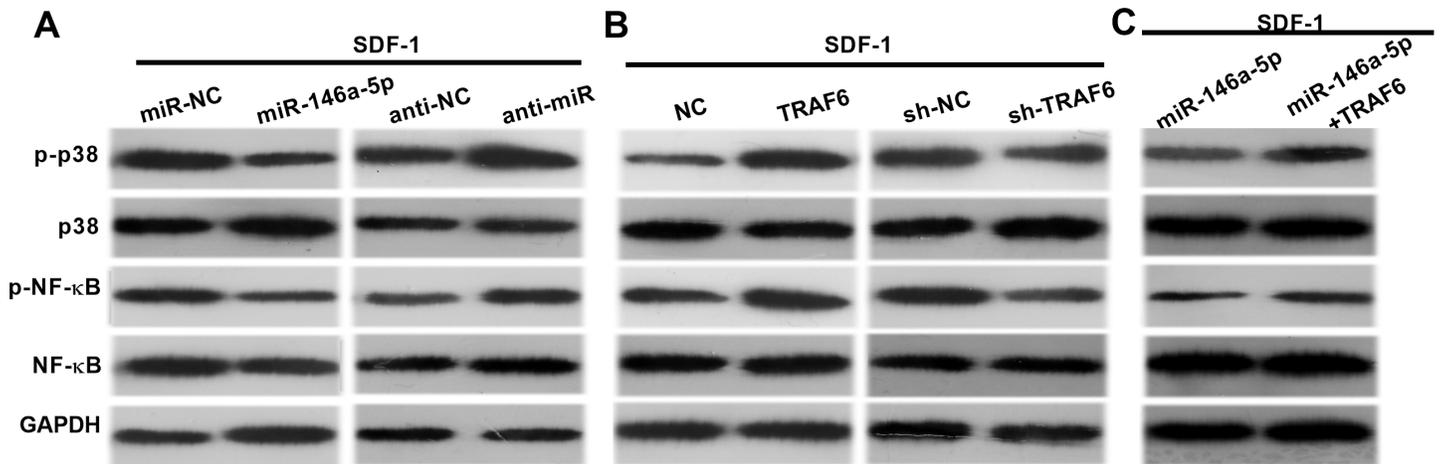


Figure 6

miR-146a-5p regulated p38 and NF-κB signaling pathway through TRAF6. A-B: WB detected the changes in p38 and NF-κB after regulating miR-146a-5p and TRAF6. C: After miR-146a-5p mimics supplemented with over-expressed TRAF6 plasmid, its regulatory effect on p38 and NF-κB.

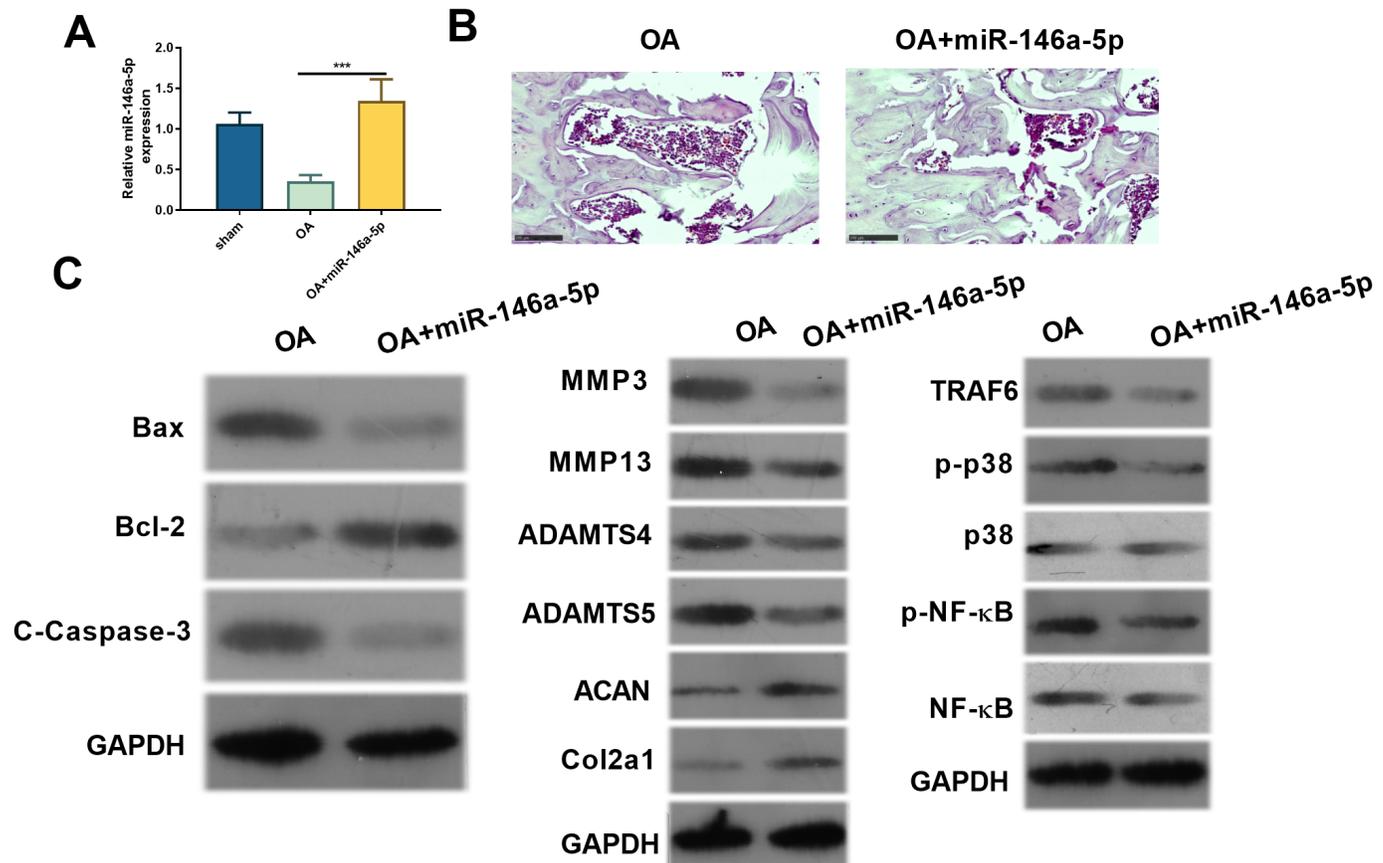


Figure 7

miR-146a-5p alleviates cartilage degradation by regulating TRAF6 in vivo. A: miR-146a-5p was examined via RT-PCR in OA model with miR-146a-5p mimic. B: HE staining was used to detect the change of cartilage degradation. C: WB detected the changes apoptosis-related proteins cleaved-Caspase 3, Bax, Bcl-2 and ECM-related proteins ACAN, Col2a1, MMP3, MMP13, ADAMTS5, ADAMTS4, and in TRAF6, p38 and NF- κ B after regulating miR-146a-5p.