miR-335-5P contributes to human osteoarthritis by targeting HBP1

XIAOKUN LU*, YU LI*, HUIMIN CHEN, YUANCHENG PAN, RAN LIN and SHUNYOU CHEN

Department of Pediatric Orthopaedics, Fuzhou Second Hospital Affiliated to Xiamen University, Fuzhou, Fujian 350007, P.R. China

Received October 15, 2019; Accepted April 9, 2020

DOI: 10.3892/etm.2020.9541

Abstract. MicroRNA (miR)-335-5P has the ability to regulate chondrogenic differentiation and promote chondrogenesis in mouse mesenchymal stem cells. It is also abnormally elevated in human osteoarthritic chondrocytes. However, the biological function of miR-335-5P in osteoarthritis (OA) is not well understood. The present study investigated the mechanism of miR-335-5P in the pathogenesis of OA. To investigate the effect of miR-335-5P on the pathogenesis of OA *in vitro*, a miR-335-5P mimic and inhibitor were transfected into chondrocytes. Cell Counting kit-8 assay and flow cytometry were used to observe the effects of miR-335-5P on chondrocyte apoptosis and the expression of cartilage-specific genes, such as aggrecan, collagen II, matrix metalloproteinase 13 and collagen X, were detected by reverse transcription-quantitative PCR and western blot analysis. Moreover, the current study assessed whether HMG-box transcription factor 1 (HBP1) is a novel target of miR-335-5P with dual luciferase reporter assays. Finally, a rescue experiment was used to prove the regulation between miR-335-5P and HBP1. The results revealed that HBP1 was a novel target of miR-335-5P, and that miR-335-5P mediated the apoptosis of chondrocytes and changes in cartilage-specific genes via targeting HBP1. Overall, the present study revealed that miR-335-5P mediated the development of OA by targeting the HBP1 gene and promoting chondrocyte apoptosis. These data suggested that miR-335-5P may be used to develop novel early-stage diagnostic and therapeutic strategies for OA.

Correspondence to: Dr Shunyou Chen, Department of Pediatric Orthopaedics, Fuzhou Second Hospital Affiliated to Xiamen University, 47 Shangteng Road, Cangshan, Fuzhou, Fujian 350007, P.R. China
E-mail: lxk_362@163.com

*Contributed equally

Key words: osteoarthritis, microRNA, apoptosis, miR-335-5P, HMG-box transcription factor 1

Introduction

Osteoarthritis (OA) is a type of disease in which articular cartilage becomes fibrotic and ulcerated and can be lost (1). The common clinical manifestations of OA are joint pain and tenderness, joint stiffness, joint swelling, bone friction and joint weakness (2). In general, OA can be pathologically summarized as cartilage degeneration and synovitis, and its main features are the degradation of articular cartilage tissue and the apoptosis of chondrocytes (3). Although the cause of OA is still unclear, studies have shown that microRNAs (miRNAs/miRs) are closely related to OA (4-7). Therefore, discovering and exploring the functions of miRNAs may provide new strategies for OA treatment in the near future.

In recent years, 990 known miRNAs and 1,621 potential miRNAs have been found in human OA chondrocytes (5). It has been demonstrated that miRNAs are not only involved in cartilage development, including growth plate development, osteogenic and chondrogenic differentiation, and osteoclast formation, but also participate in the development of OA related to chondrocyte apoptosis and abnormal cartilage metabolism (6,8). Moreover, miRNAs and cytokines accelerate the course of disease or delay the course of disease through mutual synergy and antagonism (9).

miRNAs are a group of endogenous small noncoding RNAs (22-25 nucleotides) that regulate gene expression at the post-transcriptional level by directly targeting the 3'-untranslated region (3'-UTR) of a gene. They are important regulators of different biological processes, including cell proliferation, differentiation, migration, apoptosis and tumorigenesis (10). Under normal conditions, miRNA expression has strict tissue and temporal specificity, and is highly conserved in evolution, while abnormally expressed miRNAs may cause disease. miR-335-5P was originally discovered as a tumour metastasis suppressor targeting the transcription factors Sry- box transcription factor 4 and tenascin C and is encoded by the second intron of the mesoderm-specific transcript gene (11). miR-335-5P can inhibit the proliferation and migration of primary bone marrow-derived human mesenchymal stem cells (MSCs) (12). In addition, it has been reported that the expression of four miRNAs, namely miR-138-5P, miR-146a-5P, miR-335-5P and miR-9-5P, was significantly upregulated in human knee joint OA according to gene chip and *in situ*

hybridization techniques, suggesting that miR-335-5P may play an important role in the development of OA (13). However, the biological function of miR-335-5P in OA is not well understood. Thus, the present study investigated the effect of miR-335-5P on chondrocytes and whether miR-335-5P may be a potential target for OA treatment.

Materials and methods

Ethics statement. Human articular cartilage was harvested from a patient following a traumatic amputation. The patient provided their written informed consent. All protocols were approved by the Ethics Committee of Fuzhou Second Hospital Affiliated to Xiamen University (Fuzhou China).

Isolation, culture and transfection of human primary articular chondrocytes. Under sterile conditions, cartilage slices were dissected from a sample of a male patient with traumatic amputation (age, 32 years) on May 10, 2018 with no history of OA. The excess fibrous connective tissue in the specimen was removed, and the cartilage tissue was cut to a size of ~1 mm^3 and washed with PBS containing 1% penicillin and gentamicin double antibiotic solution. Then, 5 μl 0.25% trypsin were added, the tissue was digested in a 37˚C incubator for 30 min, and the supernatant was discarded. Next, 6ml 0.2% type II collagenase (Sigma-Aldrich; Merck KGaA) was added, the tissue was digested in a 37˚C incubator for 16 h, and cells were collected every 4 h. The cell suspension was filtered through a 75 μm mesh filter and centrifuged at 4˚C, 500 x g for 5 min, and the supernatant was discarded. The suspension was washed three times with DMEM Complete Medium (Beijing Dingguo Changsheng Biotechnology Co., Ltd). containing 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Finally, the cells were seeded into a culture flask at a density of $1x10^5$ cells/ml and cultured at 37˚C in a 5% CO_2 incubator as described previously (5). For the primary cell culture, only chondrocytes from the normal articular cartilage harvested from the femoral condyles and tibial plateaus of the tissue donor was used. The cells were identified by toluidine blue (Sigma-Aldrich; Merck KGaA) staining and type II collagen (cat. no. ab34712; Abcam) immunohistochemistry staining (Fig. S1). The immunohistochemistry staining procedure was as follows: The slides were rewarmed at 37˚C for 45 min after incubating with 50 μl of type II collagen antibody (1:500) at 4˚C overnight. After washing with PBS three times, the slides were incubated with 40-50 μl goat anti-rabbit IgG antibody (1:500; cat. no. ab 205718; Abcam) at room temperature for 1 h. Slides were then observed under a light microscope after incubation with freshly prepared 1 mg/ml DAB solution (Sigma-Aldrich; Merck KGaA) for 5-10 min in the dark at 37˚C. After rinsing with tap water for 10 min, the slides were counterstained with hematoxylin (Sigma-Aldrich; Merck KGaA) at 37˚C for 1 min. After thoroughly rinsing each sample in water, the cells were immersed in 1% hydrochloric acid alcohol and then 1% aqueous ammonia, followed by thorough washing in water. The cells from each sample were then dehydrated in 70% ethanol for 2 min, 80% ethanol for 2 min, 90% ethanol twice for 2 min, 95% ethanol twice for 2 min and 100% ethanol twice for 2 min. The cells were then immersed in xylene solution twice for 2 min and mounted on a glass slide in neutral resin, sealed with a neutral gum seal and finally examined using a Nikon TE2000 microscope.

A total of $5x10^4$ chondrocytes per well were seeded into six-well plates, and the cells grown to ~80% confluence. Transfection was performed according to the instructions for Lipofectamine® 3000 transfection reagent (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, mimics or inhibitors (75 nM of each) were mixed with the transfection agent in Opti-MEM for 20 min, and then added to serum-free medium. After 6 h of transfection, the solution was changed. Cells were cultured for another 48 h, subjected to TRIzol® (Thermo Fisher Scientific, Inc.) RNA isolation and cell images were captured using a Nikon TE2000 light microscope (magnification, x100) or cells were cultured for 72 h to be used for western blot analysis. The hsa-miR-335-5P mimic and inhibitor were biosynthesized by Shanghai GenePharma Co., Ltd. The hsa-miR-335-5P mimic sequence was 5'-UAC AGUACUGUGAUAACUGAA-3' and the mimic negative control (NC) sequence was 5'-GTTCTCCGAACGTGTCAC GT-3', the hsa-miR-335-5P inhibitor sequence was 5'-ACA UUUUUCGUUAUUGCUCUUGA-3' and the inhibitor NC sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. The overexpression vector pCMV3-C-Myc-HMG-box transcription factor 1 (HBP1) and empty vector were purchased from Sino Biological, Inc.

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. To detect miR-335-5P expression, the TaqMan MicroRNA RT system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to generate cDNA, and the expression of miR-335-5P was measured using the miR-335-5P TaqMan microRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). Data were analysed using the comparative $2^{-\Delta\Delta Cq}$ method (6), and values were normalised to U6 expression. For the detection of gene expression, RT was performed using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara Bio, Inc.), followed by qPCR according to the instructions provided with the SYBR Premix Ex Taq kit (Takara Bio, Inc.). The expression levels of genes were normalized to the expression of GAPDH. The primers for the genes and miR-335-5P are shown in Table I. For RT-qPCR, the following thermal and thermocycling conditions were used: 50˚C for 3 min and 95˚C for 3 min for RT; and 40 cycles of 94˚C for 1 min and 56˚C for 1 min for the q-PCR. For miRNA, the following thermal and thermocycling conditions were used: 95˚C for 10 min for RT; followed by 40 cycles of 94˚C for 1 min, 56˚C for 1 min and 72˚C for 1 min for qPCR. Relative expression of the genes was evaluated with the $2^{-\Delta\Delta Cq}$ method (6).

Western blot analysis. A total of 48 h post-transfection, chondrocyte proteins were extracted using cell lysis buffer (cat. no. 9803S; Cell Signaling Technologies, Inc). The protein concentration was determined by the BCA method. A total of 30 μg protein was loaded per lane. The proteins were subjected to SDS-PAGE on 10% gels and then transferred to a PVDF membrane. After blocking with 5% skim milk powder at room temperature for 1 h, anti-aggrecan (ACAN; cat. no. ab3778; Abcam), anti-collagen II (cat. no. ab34712;

Table I. Primer sequences for aggrecan, collagen II, MMP13, collagen X and GAPDH.

Gene	Primer sequence (5'to 3')
Aggrecan	F: CTTCCGCTGGTCAGATGGAC
	R: CGTTTGTAGGTGGTGGCTGT
Collagen II	F: CCTACAATAATAATATATACCCCACCA
	R: ATGTGTTTTCAGTGATCATGTTTTC
MMP13	F: GCACTTCCCACAGTGCCTAT
	R: AGTTCTTCCCTTGATGGCCG
Collagen X	F: AAAGGCCCACTACCCAACAC
	R: GTGGACCAGGAGTACCTTGC
IL-1α	F: AGCTATGGCCCACTCCATGAAG
	R: ACATTAGGCGCAATCCAGGTGG
IL-6	F: AGACAGCCACTCACCTCTTCA
	R: CACCAGGCAAGTCTCCTCATT
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA
	R: CTGGAAGATGGTGATGGGATTTC

MMP13, matrix metalloproteinase 13; F, forward; R, reverse.

Abcam), anti-collagen X (cat. no. ab58632; Abcam), anti-matrix metalloproteinase (MMP)13 (cat. no. ab39012; Abcam), anti-Bax (cat. no. ab32503; Abcam), anti-Bcl2 (cat. no. ab32124; Abcam), anti- NF-κB (cat. no. ab16502; Abcam), anti-IκB (cat. no. ab32518; Abcam), anti-IL-1α (cat. no. ab7632; Abcam), anti-IL-6 (cat. no. ab6672; Abcam) and anti-β-actin (cat. no. MA5-15739; Sigma-Aldrich; Merck KGaA) primary antibodies (1:1,000) were incubated with the membrane overnight at 4˚C. After rinsing three times with TBS-Tween (TBST; 0.1% Tween-20; 10 min/wash), the horseradish peroxidase-conjugated AffiniPure Goat anti-rabbit IgG (cat. no. 31460) or goat anti-mouse IgG (cat. no. A24512) secondary antibodies (1:5,000) (Pierce; Thermo Fisher Scientific, Inc.) were incubated with the membrane for 1 h at 4˚C. The blots were washed with TBST three times (10 min/wash) the protein of interest was developed by electrochemiluminescence (Thermo Fisher Scientific, Inc.). The intensities of the proteins in western blots were semi-quantified using ImageJ version 1.52 (National Institute of Health).

Cell Counting kit-8 (CCK-8) assay. Cell viability was assessed using a CCK-8 kit (Dojindo Molecular Technologies, Inc.), according to the manufacturer's instructions. Chondrocytes were seeded at a density of $1x10^4$ cells/well into 96-well culture plates and cultured in DMEM/F12 containing 10% FBS at 37˚C in a 5% CO_2 incubator, with five duplicate wells in each group. At 24, 48 and 72 h after transfection, the cells were incubated with 100 μl WST-8 at 37˚C for 4 h. The absorbance of cells was measured by a spectrophotometer at 570 nm.

Flow cytometry. An Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to detect apoptotic activity according to the manufacturer's instructions. Chondrocytes were seeded into six-well plates at a density of $1x10^6$ cells/well in DMEM containing 10% FBS. When the cells reached 60% confluence, they were transfected. After 48 h, the cells were digested with 0.5% trypsin and resuspended in 300 μl binding buffer containing 5 μl Annexin V-FITC and 1 μl propidium iodide solution (100 μg/ml). After incubation for 20 min in the dark at room temperature, the stained cells were analysed by BD FACS Calibur with CellQuest Pro Software 5.1 (BD Biosciences).

Luciferase reporter assay. The miR-335-5P response element in the 3'-UTR of HBP1 (both wild-type and mutant HBP1) was cloned into the pMIR-REPORT miRNA luciferase reporter vector (Ambion, Inc.; Thermo Fisher Scientific, Inc.) containing firefly luciferase confirmed by sequencing. The region of HBP1 was predicted using TargetScan 7.2 (http://www.targetscan.org/). 293T cells (The Cell Bank of Type Culture Collection of the Chinese Academy of Science) were co-transfected with the miR-335-5P mimic and wild-type or mutant HBP1 3'-UTR luciferase reporters together with the *Renilla* plasmid for 48 h using the miR-335-5P mimic NC as the negative control. Then, firefly and *Renilla* luciferase activities were measured according to the manufacturer's instructions using a Dual-Luciferase Reporter Assay System (Promega Corporation), and the firefly luciferase activity was normalized to the value of the *Renilla* luciferase activity. Each experiment was repeated in triplicate. The statistical data are expressed as the mean ± standard error of the mean (SEM).

Statistical analysis. Data were analysed using GraphPad Prism 6 software (GraphPad Software, Inc.). The statistical data are expressed as the mean ± SEM. A two-tailed Student's t-test was used to compare two groups. One-way ANOVA with Bonferroni post hoc test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-335-5P mimic promotes the apoptosis of chondrocytes. To investigate the effect of miR-335-5P on chondrocyte apoptosis, the miR-335-5P mimic and the miR-335-5P inhibitor were transfected into chondrocytes. After 48 h, the expression levels of miR-335-5P were detected by RT-qPCR (Fig. 1B). The results showed that the level of miR-335-5P was significantly higher in the mimic group than in the mimic NC group, and the level in the inhibitor group was significantly lower than that in the inhibitor NC group (Fig. 1B), indicating that the miR-335-5P mimic and inhibitor were successfully transfected into chondrocytes. Images of cell morphology were captured (Fig. 1A), cell activity was detected by CCK-8 assay (Fig. 1C, $P<0.05$, vs. control group), apoptosis was detected using flow cytometry (Fig. 1D and E), and Bax and Bcl2 expression were detected by western blotting (Fig. 1F and G, $P<0.01$, vs. control group). For miR-335-5p mimic, miR-335-5p mimic NC was used as the control, for miR-335-5p inhibitor, miR-335-5p inhibitor NC was used as the control. The results showed that compared with the mimic control group, miR-335-5P mimic transfection inhibited the viability of chondrocytes and promoted chondrocyte apoptosis.

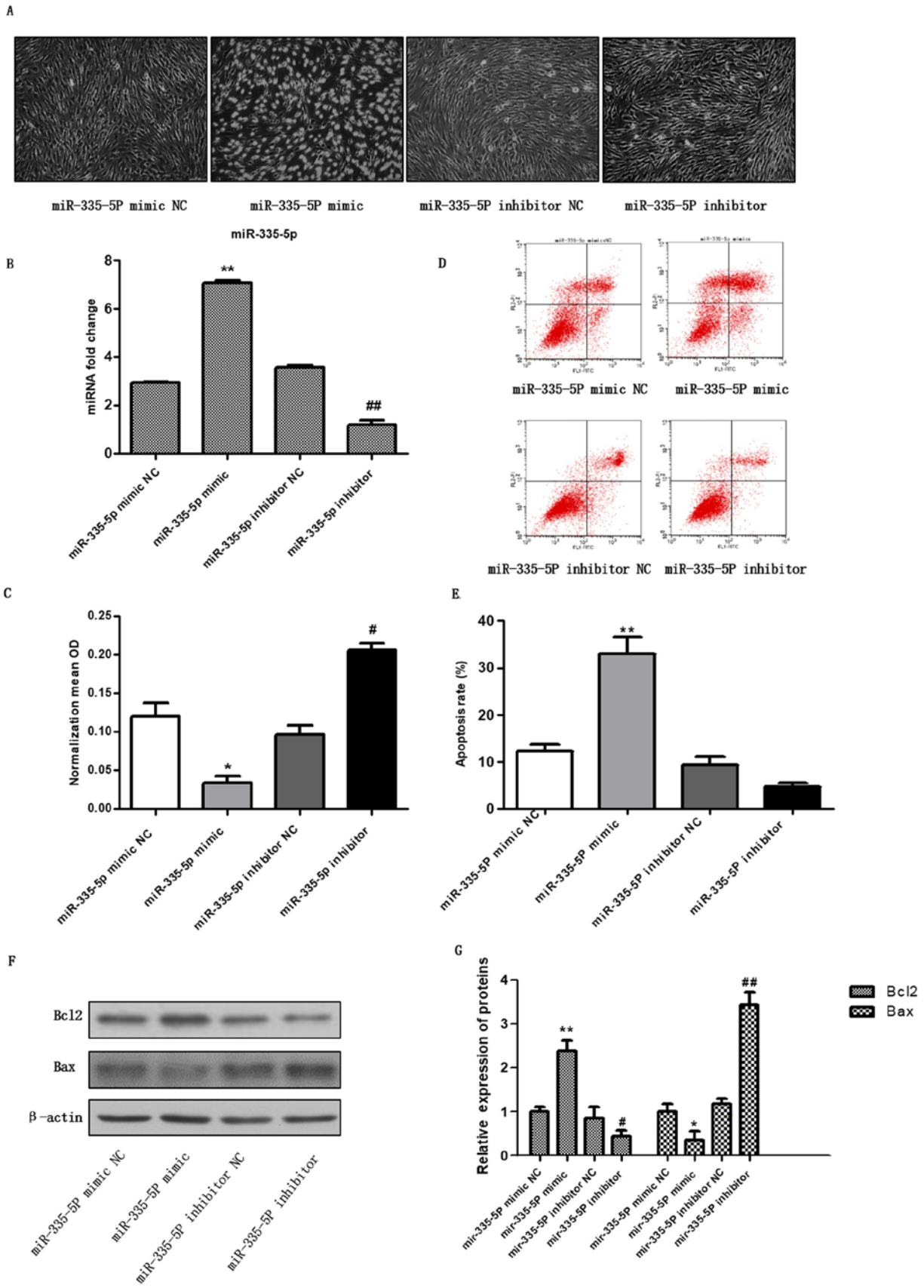

Figure 1. miR-335-5P promotes chondrocyte apoptosis *in vitro*. (A) Chondrocytes were transfected with the miR-335-5P mimic NC, miR-335-5P mimic, miR-335-5P inhibitor NC or miR-335-5P inhibitor. Cell images were taken with a Nikon TE2000 microscope after 48 h (x100). (B) Relative expression levels of miR-335-5P were detected in the different treatment groups. (C) Chondrocytes were transfected and a Cell Counting Kit-8 assay was used to determine cell viability 48 h post-transfection. (D and E) Chondrocyte apoptosis (including early and late apoptosis) was analysed by flow cytometry. Cell apoptosis data are shown as the mean ± SEM of three independent experiments. (F and G) Western blotting was used to study the expression of Bax and Bcl2. *P<0.05, **P<0.01 compared with the miR-335-5P mimic NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the miR-335-5P inhibitor NC group. miR/miRNA, microRNA; NC, negative control; OD, optical density.

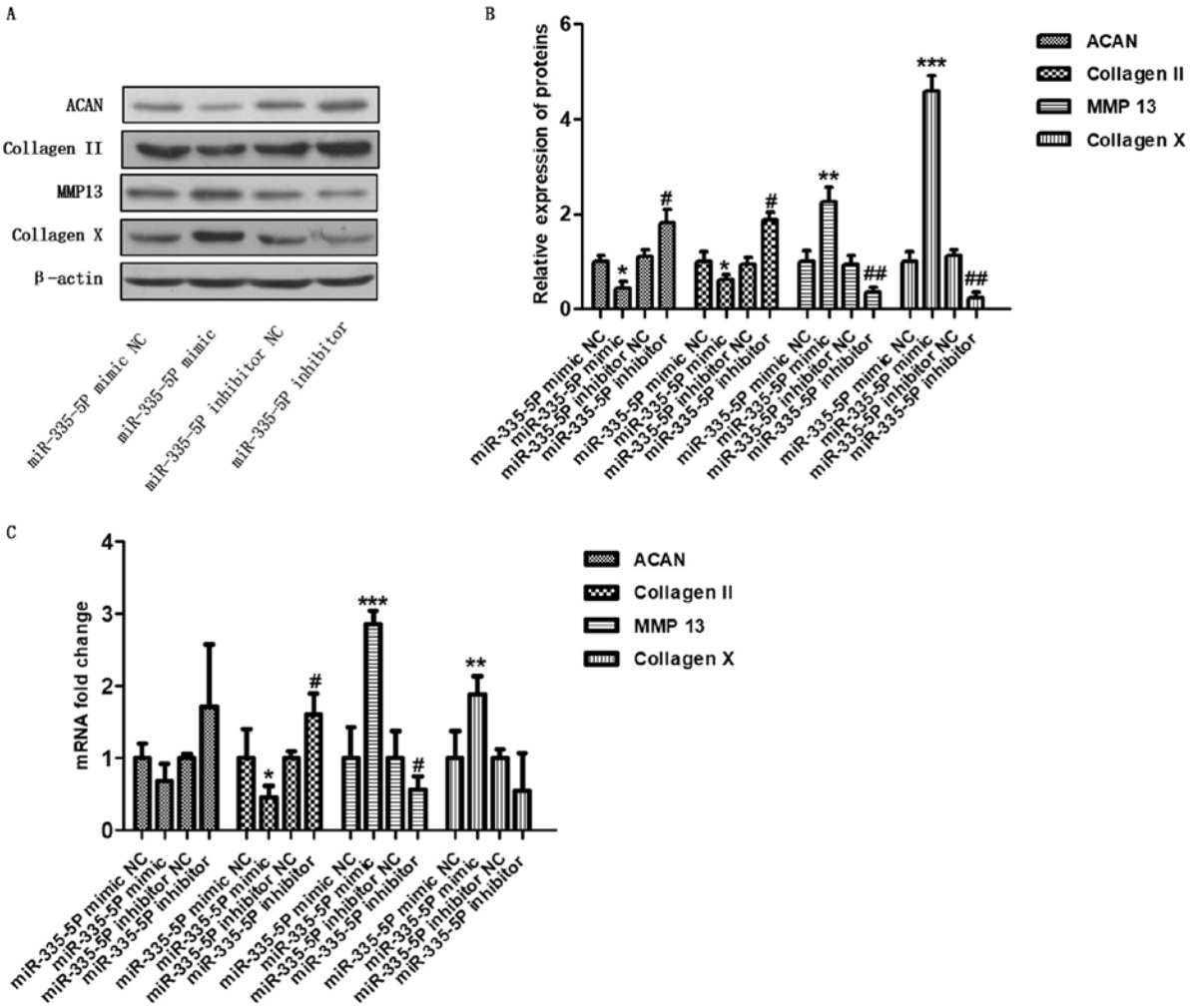

Figure 2. miR-335-5P regulates cartilage-specific genes in human chondrocytes *in vitro*. Chondrocytes were transfected with the miR-335-5P mimic NC, miR-335-5P mimic, miR-335-5P inhibitor NC or miR-335-5P inhibitor. After 48 h, the levels of ACAN, collagen II, MMP13, and collagen X were analysed by (A and B) western blotting and (C) reverse transcription-quantitative PCR. *P<0.05, **P<0.01, ***P<0.001 compared with the miR-335-5P mimic NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the miR-335-5P inhibitor NC group. ACAN, aggrecan; MMP13, matrix metalloproteinase 13; miR, microRNA; NC, negative control.

miR-335-5P regulates cartilage-specific genes in human chondrocytes in vitro. To determine the regulation of cartilage-specific genes by miR-335-5P, cells were harvested 48 h after transfection, and the expression of cartilage-specific genes was detected by western blotting and RT-qPCR. As shown in Fig. 2A and B, the miR-335-5P mimic significantly downregulated the protein expression of ACAN (P<0.05) and collagen II (P<0.05), and significantly upregulated the expression levels of MMP13 (P<0.01) and collagen X (P<0.001) compared with the mimic control. Conversely, the miR-335-5P inhibitor significantly upregulated the expression levels of ACAN (P<0.05) and collagen II (P<0.05) and downregulated the protein expression of MMP13 (P<0.01) and collagen X (P<0.01) compared with the inhibitor control. In addition, compared with the mimic NC group, the overexpression of miR-335-5P significantly downregulated the mRNA expression levels of collagen II (P<0.05), and upregulated the mRNA expression levels of MMP13 (P<0.001) and collagen X (P<0.01). Conversely, inhibition of miR-335-5P significantly upregulated the mRNA expression levels of collagen II (P<0.05) and downregulated the mRNA expression levels of MMP13 (Fig. 2C, P<0.05) compared with the inhibitor NC. These results suggested that miR-335-5P may inhibit the expression of the anabolic genes ACAN and collagen II, and promote the expression of the OA-related genes MMP13 and collagen X. Because inflammation usually accompanies OA, the NF-κB signalling pathway and inflammatory factors were measured in cells. The results showed that the NF-κB signalling pathway was activated, and the levels of IL-1α (P<0.01) and IL-6 (P<0.01) in cells transfected with miR-335-5p mimics were significantly increased compared with the NC group. Conversely, the NF-κB signalling pathway was suppressed and the expression of IL-1α (P<0.05) and IL-6 (P<0.05) in cells transfected with miR-335-5p inhibitors was significantly decreased compared with the inhibitor control (Fig. S2).

Prediction of HBP1 as a target of miR-335-5P. To further investigate the molecular mechanism of miR-335-5P in OA, the miR-335-5P mimic and miR-335-5P inhibitor were transfected into chondrocytes. After 48 h, the expression of HBP1 was detected by RT-qPCR and western blot analysis. As expected, the overexpression of miR-335-5P non-significantly restricted

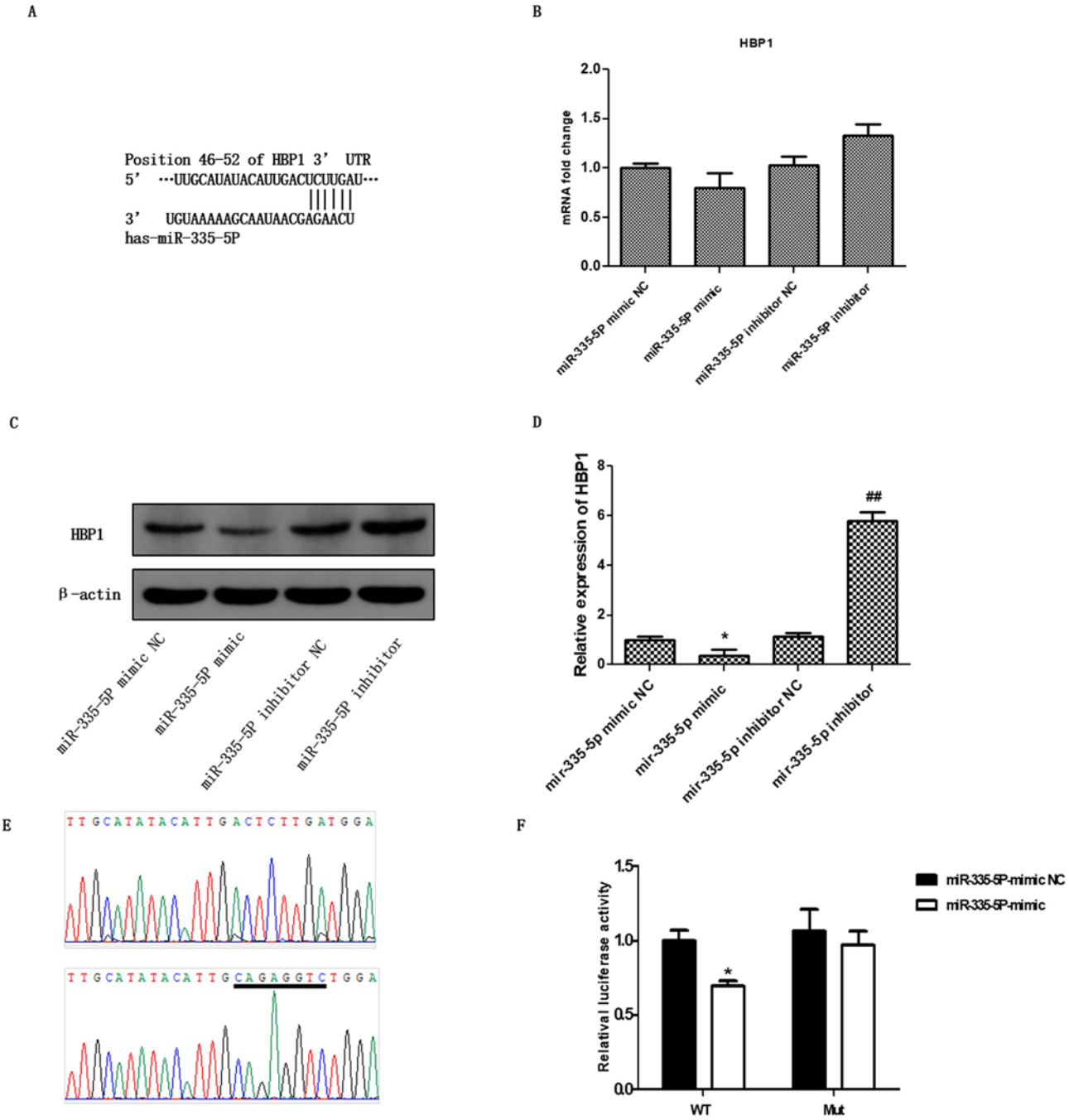

Figure 3. HBP1 expression is regulated by miR-335-5P and represents a novel direct target of miR-335-5P. (A) Target sequence in the 3'-UTR of human HBP1 predicted to bind hsa-miRNA-335-5P. HBP1 expression was analysed by (B) reverse transcription-quantitative PCR and (C) western blotting after chondrocytes were transfected with the miR-335-5P mimic NC, miR-335-5P mimic, miR-335-5P inhibitor NC or miR-335-5P inhibitor. (D-E) The 3'-UTR+1-+1,000 sequence of HBP1 and the fragment of the binding site mutation were cloned into the vector pMIR-REPORT and confirmed by sequencing (underlined sequence represents mutant sites). (F) In 293T cells, the luciferase reporter assay was used to test the activity of the HBP1 3'-UTR reporter. The miR-335-5P mimic was cotransfected into cells with the wild-type HBP1 3'-UTR or mutant-HBP1-3'-UTR. The miR-335-5P mimic NC served as a NC. *P<0.05 compared with the miR-335-5P mimic NC group; $^{\#\#}$P<0.01 compared with the miR-335-5P inhibitor NC group. HBP1, HMG-box transcription factor 1; miR, microRNA; mut, mutant; NC, negative control; WT, wild type.

the mRNA expression of HBP1, whereas the downregulation of miR-335-5P non-significantly enhanced the mRNA expression of HBP1 (Fig. 3B; P>0.05;). In addition, the western blotting results showed that the miR-335-5P mimic significantly downregulated the protein expression of HBP1 (P<0.05) compared with the mimic NC. By contrast, the miR-335-5P inhibitor significantly upregulated the levels of HBP1 (Fig. 3C, P<0.01) compared with the inhibitor NC.

Subsequent bioinformatics analysis was used to assess the targeted regulatory relationship between miR-335-5P and HBP1. It was found that the 3'-UTR of HBP1 has a potential binding site for miR-335-5P (Fig. 3A). This result suggested that HBP1 may be a potential target gene of miR-335-5P. To verify whether miR-335-5P directly binds to the 3'-UTR (46-52 bp) of HBP1, the 3'-UTR (+1 to +1,000 bp) of HBP1 and the fragment containing the binding site mutation were inserted

into the pMIR-REPORT miRNA luciferase reporter vector. The pMIR-Report-HBP1 wild-type and pMIR-Report-HBP1 mutant were constructed and confirmed by sequencing (Fig. 3D). Then, the luciferase reporter assay was used to confirm whether miR-335-5P regulates HBP1 transcriptional activity. The miR-335-5P mimic NC and the miR-335-5P mimic were transfected into pMIR-Report-HBP1 wild-type and pMIR-Report-HBP1-mutant 293T cells. After 48 h, the firefly and *Renilla* luciferase activities were assessed. The results showed that the luciferase activity of the miR-335-5P mimic group was significantly lower than that of the mimic control group in the transfected pMIR-Report-HBP1 wild-type cells but not in the pMIR-Report-HBP1-mutant cells (Fig. 3E, $P<0.05$). These results indicated that miR-335-5P may specifically bind to the 3'-UTR of HBP1 and that HBP1 could be a novel specific target gene of miR-335-5P.

Regulation of chondrocyte apoptosis and cartilage-specific genes by miR-335-5P via targeting HBP1. To further confirm whether miR-335-5P regulated HBP1 expression, promoted chondrocyte apoptosis and regulated the expression of cartilage-specific genes, miR-335-5 and pcDNA3.1-Myc-HBP1 plasmids were cotransfected into chondrocytes. After 48 h, RT-qPCR was performed to detect the miR-335-5P expression level ($P<0.05$; Fig. 4A), and western blotting was performed to detect HBP1 overexpression (Fig. 4A). The expression levels of ACAN ($P<0.05$, vs. control group) and collagen II ($P<0.05$) were found to be significantly upregulated, and the expression levels of MMP13 ($P<0.001$) and collagen X ($P<0.01$) were significantly downregulated after cotransfection with the miR-335-5P and pcDNA3.1-Myc-HBP1 plasmids compared with cotransfection with the miR-335-5P and vector (Fig. 4B and C). For pcDNA3.1-Myc-HBP1, empty vector was used as the control, for miR-335-5P mimic + Myc-HBP1, miR-335-5P mimic + empty vector was used as the control. Furthermore, cell viability was detected by CCK-8 assays (Fig. 4D, $P<0.05$, vs. control group), and apoptosis was detected using flow cytometry (Fig. 4G and H, $P<0.05$, vs. control group). Bax and Bcl2 expression was detected by western blotting to test chondrocyte apoptosis (Fig. 4E and F). These results suggested that HBP1 may have a role in the downstream effects of miR-335-5P on chondrocyte apoptosis and cartilage-specific gene expression to some degree.

Discussion

In previous studies, the expression of miR-335-5P was significantly increased in osteoarthritic chondrocytes compared with normal cartilage (7,13). However, to the best of our knowledge, the biological function of miR-335-5P in OA has not been reported, and the molecular mechanism is not yet clear. This study aimed to investigate the effects of miR-335-5P on chondrocyte apoptosis and its underlying molecular mechanisms in OA.

In the present study, a miR-335-5P mimic and inhibitor were transfected into chondrocytes. Notably, chondrocyte apoptosis was observed after transfection of miR-335-5P mimic. Although no relationship between chondrocyte apoptosis and the occurrence and development of OA has previously been demonstrated (3), the evidence provided here indicated that chondrocyte apoptosis may occur during the whole process of OA. Thus, inhibiting chondrocyte apoptosis may serve as a target for the treatment of OA. Moreover, miR-335-5P could inhibit the expression of extracellular matrix (ECM) synthesis-related genes, such as ACAN and collagen II, and promote the mRNA and protein levels of ECM degradation-related enzymes, such as MMP13 and collagen X. As inflammation usually accompanies OA, the NF-κB signalling pathway and inflammatory factors in cells were also measured. These results showed that the NF-κB signalling pathway was activated, and that the levels of IL-1α and IL-6 were significantly increased in cells that were transfected with miR-335-5p mimics compared with the control group. It is well-known that inflammation of chondrocytes regulates the expression of cartilage-specific genes, such as IL-1, which may suppress expression of cartilage-specific types II and X collagens and increase types I and III collagens in human chondrocytes. Hence, it was hypothesized that the molecular mechanisms underlying the effect of miR-335-5p on aberrant expression of cartilage-specific genes may be related to inflammation. In summary, these results indicated that miR-335-5P may have a negative regulatory role in the pathogenesis of OA. In a study of osteogenic differentiation induced by OA and normal human bone marrow-derived MSCs, it was demonstrated that miR-335-5P was associated with the OA process and had potential to aid in the development of OA therapies (7).

In addition, mechanistic studies have revealed that miR-335-5P may target a group of negative regulators, including dishevelled-associated activator of morphogenesis 1 and Rho kinase 1 of the SRY-box-transcription factor 9 (Sox9), and knocking out Sox9 caused chondrocyte defects (14). The level of miR-335-5P can affect the expression of its target dickkopf-related protein 1 (DKK1) and DKK1 can further act on the Wnt signalling pathway (15). In addition, it has been reported that the Wnt signalling pathway may play an important role in chondrocyte proliferation and differentiation, and the Wnt pathway is closely related to the occurrence and development of OA (16). It is believed that the expression of miR-335-5P may have a potential influence on the biology of chondrocytes and thus miR-335-5P may play a role in the development of OA. This study revealed HBP1 as a newly predicted target gene of miRNA-335-5P. Through online bioinformatics analysis and the dual luciferase assay, the 3'-UTR of HBP1 was verified to bind to miR-335-5P by Specific binding site. These results suggested that HBP1 is a potential new target of miR-335-5P *in vitro*.

The HBP1 gene encodes HMG-box transcription factor 1, which is involved in Wnt signalling inhibition and cell senescence (17,18). Most reports on this gene have focused on the activities of transcription factors in various types of human cancer but less so in OA (19,20). However, it is known that the Wnt pathway and cellular senescence play a role in the aetiology of OA (15,16). It was previously reported that the decreased expression of this gene was associated with OA susceptibility (21). Thus, the function of HBP1 in the activation of the Wnt pathway and the attenuation of senescence may be risk factors for the development of OA.

Another possible reason for the association between OA and HBP1 is that HBP1 plays a role in the regulation of superoxide production, which is a cause of OA aetiology (22). Grishko *et al* (23) demonstrated that oxidative stress in OA resulted in decreased cell viability, decreased mitochondrial

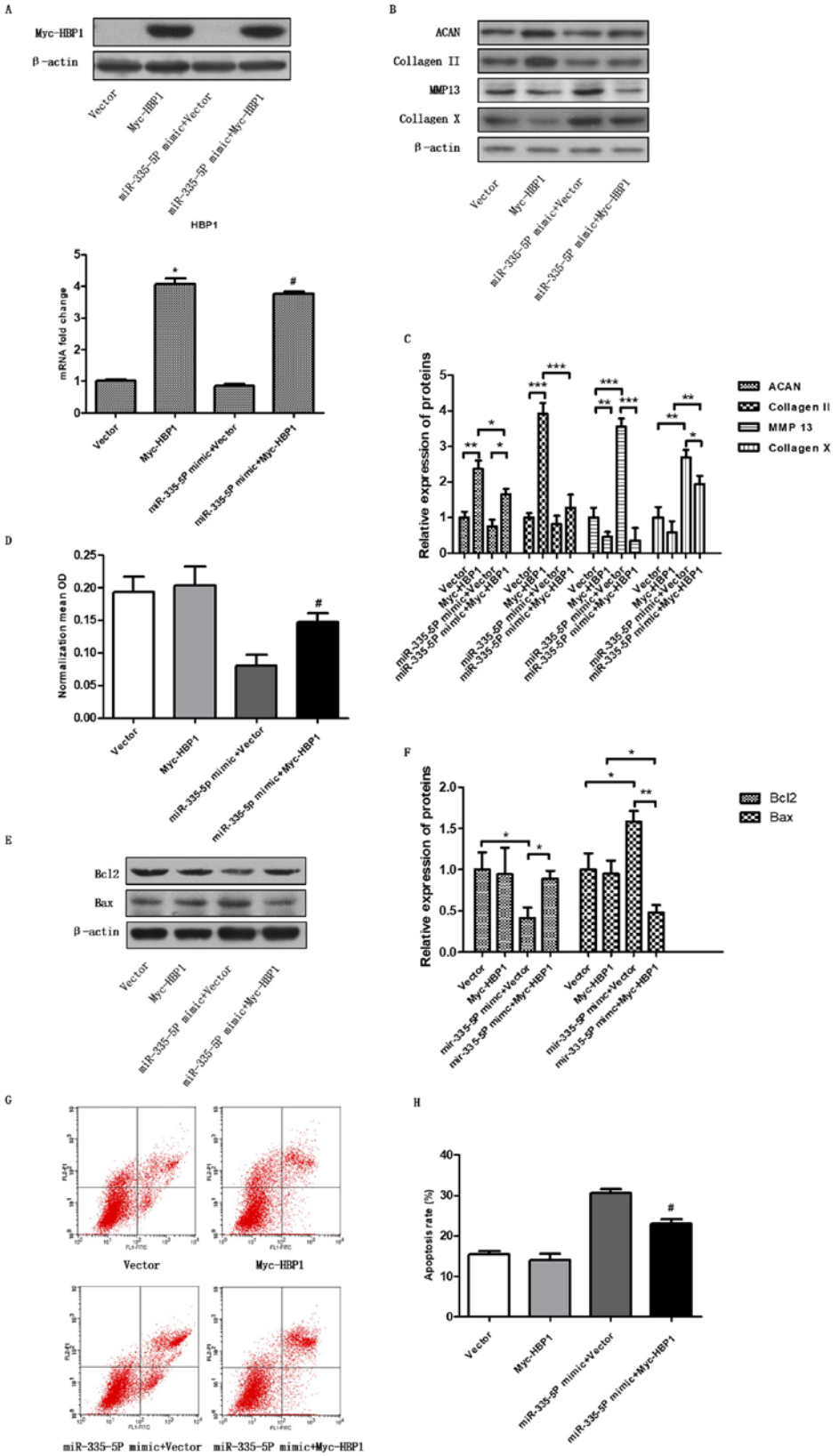

Figure 4. Cartilage-specific genes and chondrocyte apoptosis are regulated by miR-335-5P via targeting HBP1. (A) Overexpression of HBP1 was analysed by western blotting and reverse transcription-quantitative PCR after the chondrocytes were transfected with the vector, pcDNA3.1-Myc-HBP1, miR-335-5P mimic alone or miR-335-5P mimic + pcDNA3.1-Myc-HBP1. *P<0.05 compared with the vector group; $^{\#}$P<0.05 compared with the miR-335-5P mimic + vector group. (B and C) Chondrocytes were transfected and the expression levels of ACAN, collagen II, MMP13 and collagen X were assessed by western blotting. *P<0.05, **P<0.01, ***P<0.001 as indicated. (D) Cell Counting Kit-8 was used to determine cell viability, and the data are shown as the mean ± SEM of three independent experiments. (E and F) Western blot analysis was used to study the apoptosis-associated protein expression of Bax and Bcl2. *P<0.05, **P<0.01as indicated. (G and H) Chondrocyte apoptosis was analysed by flow cytometry. Cell apoptosis data are shown as the mean ± SEM of three independent experiments. *P<0.05 compared with the vector group; $^{\#}$P<0.05 compared with the miR-335-5P mimic + vector group. ACAN, aggrecan; miR, microRNA; MMP13, matrix metalloproteinase 13; HBP1, HMG-box transcription factor 1; OD, optical density.

DNA repair ability after reactive oxygen species (ROS) stimulation and an increased phenotype of apoptosis in OA pathogenesis, indicating that the decreased involvement of mitochondrial DNA damage and repair ability play an important role in the development of OA. Neutrophils are often found in the joints and synovial fluids when their surface is attacked by immune complexes and complement components, and large quantities of free radicals can be released (24). Under normal conditions, there is a balance between the oxidation system and the antioxidant system, and the presence of ROS can prevent the invasion of pathogens (25). However, under pathological conditions, the increased production of ROS inhibits the proliferation of chondrocytes, which results in their death and inhibits the synthesis of cartilage matrix proteoglycans and collagen (26-28). Additionally, the oxidation of cartilage collagen causes collagen cleavage, which changes the performance of collagen fibres, making them prone to fatigue damage, accelerating degradation of the cartilage matrix, reducing the elasticity and strength of cartilage, damaging chondrocytes and leading to the occurrence of OA (28). Although this study has unveiled the influence of miR-335-5P on chondrocytes *in vitro*, a lack of the effects of miR-335-3p on an animal model of OA *in vivo* is one limitation of the study. Hence, the effects of miR-335-3p on the animal model of OA remain to be explored.

In conclusion, the present study has revealed that miR-335-5P may act as a regulator of OA by inducing chondrocyte apoptosis, and HBP1 was identified as a novel target of miR-335-5P. Furthermore, HBP1 was involved in the occurrence of OA via miR-335-5P. Therefore, miR-335-5P may be a promising therapeutic target, providing a new repair pathway for the clinical treatment of OA.

Acknowledgements

The authors would like to thank Dr Yunfei Pu (School of Life Sciences, Xiamen University) for critically reading this manuscript.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

SC conceived the study. XL, YL and SC designed the study. XL and YL performed the experiments and wrote the manuscript. HC, YP and RL performed the statistical analysis. XL and YL drafted the manuscript. SC reviewed the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The sample was obtained with written informed consent from the donor. The study was approved by the Ethics Committee of Fuzhou Second Hospital Affiliated to Xiamen University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ko JY, Lee MS, Lian WS, Weng WT, Sun YC, Chen YS and Wang FS: MicroRNA-29a counteracts synovitis in knee osteoarthritis pathogenesis by targeting VEGF. Sci Rep 7: 3584, 2017.
2. Huskisson EC: Modern management of mild-to-moderate joint pain due to osteoarthritis: A holistic approach. J Int Med Res 38: 1175-1212, 2010.
3. Goggs R, Carter SD, Schulzetanzil G, Shakibaei M and Mobasheri A: Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet J 166: 140-158, 2003.
4. Papanagnou P, Stivarou T and Tsironi M: The role of miRNAs in common inflammatory arthropathies: Osteoarthritis and gouty arthritis. Biomolecules 6: 44, 2016.
5. Crowe N, Swingler TE, Le LT, Barter MJ, Wheeler G, Pais H, Donell ST, Young DA, Dalmay T and Clark IM: Detecting new microRNAs in human osteoarthritic chondrocytes identifies miR-3085 as a human, chondrocyte-selective, microRNA. Osteoarthritis Cartilage 24: 534-543, 2016.
6. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
7. Tornero-Esteban P, Rodríguez-Rodríguez L, Abásolo L, Tomé M, López-Romero P, Herranz E, González MA, Marco F, Moro E, Fernández-Gutiérrez B and Lamas JR: Signature of microRNA expression during osteogenic differentiation of bone marrow MSCs reveals a putative role of miR-335-5P in osteoarthritis. BMC Musculoskelet Disord 16: 182-190, 2015.
8. Mirzamohammadi F, Papaioannou G and Kobayashi T: microRNAs in cartilage development, homeostasis, and disease. Curr Osteoporos Rep 12: 410-419, 2014.
9. Steck E, Boeuf S, Gabler J, Werth N, Schnatzer P, Diederichs S and Richter W: Regulation of H19 and its encoded microRNA-675 in osteoarthritis and under anabolic and catabolic in vitro conditions. J Mol Med (Berl) 90: 1185-1195, 2012.
10. Ambros V: The functions of animal microRNAs. Nature 431: 350-355, 2004.
11. Negrini M and Calin GA: Breast cancer metastasis: A microRNA story. Breast Cancer Res 10: 203-206, 2008.
12. Tomé M, López-Romero P, Albo C, Sepúlveda JC, Fernández-Gutiérrez B, Dopazo A, Bernad A and González MA: miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. Cell Death Differ 18: 985-995, 2011.
13. Kopańska M, Szala D, Czech J, Gabło N, Gargasz K, Trzeciak M, Zawlik I and Snela S: miRNA expression in the cartilage of patients with osteoarthritis. J Orthop Surg Res 12: 51, 2017.
14. Lin X, Wu L, Zhang Z, Yang R, Guan Q, Hou X and Wu Q: miR-335-5P promotes chondrogenesis in mouse mesenchymal stem cells and is regulated through two positive feedback loops. J Bone Miner Res 29: 1575-1585, 2014.
15. Zhang J, Tu Q, Bonewald LF, He X, Stein G, Lian J and Chen J: Effects of miR-335-5P in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. J Bone Miner Res 26: 1953-1963, 2011.
16. Yates KE, Shortkroff S and Reish RG: Wnt influence on chondrocyte differentiation and cartilage function. DNA Cell Biol 24: 446-457, 2005.
17. Sampson EM, Haque ZK, Ku MC, Tevosian SG, Albanese C, Pestell RG, Paulson KE and Yee AS: Negative regulation of the Wnt-beta-catenin pathway by the transcriptional repressor HBP1. EMBO J 20: 4500-4511, 2014.
18. Paulson KE, Riegerchrist K, Mcdevitt MA, Kuperwasser C, Kim J, Unanue VE, Zhang X, Hu M, Ruthazer R, Berasi SP, *et al*: Alterations of the HBP1 transcriptional repressor are associated with invasive breast cancer. Cancer Res 67: 6136-6145, 2007.

19. Yee AS, Paulson EK, Mcdevitt MA, Rieger-Christ K, Summerhayes I, Berasi SP, Kim J, Huang CY and Zhang X: The HBP1 transcriptional repressor and the p38 MAP kinase: Unlikely partners in G1 regulation and tumor suppression. Gene 336: 1-13, 2004.
20. Chen YC, Zhang XW, Niu XH, Xin DQ, Zhao WP, Na YQ and Mao ZB: Macrophage migration inhibitory factor is a direct target of HBP1-mediated transcriptional repression that is overexpressed in prostate cancer. Oncogene 29: 3067-3078, 2010.
21. Raine EV, Wreglesworth N, Dodd AW, Reynard LN and Loughlin J: Gene expression analysis reveals HBP1 as a key target for the osteoarthritis susceptibility locus that maps to chromosome 7q22. Ann Rheum Dis 71: 2020-2027, 2012.
22. Berasi SP, Xiu M, Yee AS and Paulson KE: HBP1 repression of the p47phox gene: Cell cycle regulation via the NADPH oxidase. Mol Cell Biol 24: 3011-3024, 2004.
23. Grishko VI, Ho R, Wilson GL and Pearsall AW IV: Diminished mitochondrial DNA integrity and repair capacity in OA chondrocytes. Osteoarthritis Cartilage 17: 107-113, 2009.
24. Stanczyk J, Kowalski ML, Grzegorczyk J, Szkudlinska B, Jarzebska M, Marciniak M and Synder M: RANTES and chemotactic activity in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Mediators Inflamm 2005: 343-348, 2014.
25. Aikawa C, Nozawa T, Maruyama F, Tsumoto K, Hamada S and Nakagawa I: Reactive oxygen species induced by Streptococcus pyogenes invasion trigger apoptotic cell death in infected epithelial cells. Cell Microbiol 12: 814-830, 2010.
26. Huang Z, Li J, Du S, Chen G, Qi Y, Huang L, Xiao L and Tong P: Effects of UCP4 on the proliferation and apoptosis of chondrocytes: Its possible involvement and regulation in osteoarthritis. PLoS One 11: e0150684, 2016.
27. Yu SM and Kim SJ: Production of reactive oxygen species by withaferin A causes loss of type collagen expression and COX-2 expression through the PI3K/Akt, p38, and JNK pathways in rabbit articular chondrocytes. Exp Cell Res 319: 2822-2834, 2013.
28. Lepetsos P and Papavassiliou AG: ROS/oxidative stress signaling in osteoarthritis. Biochim Biophys Acta 1862: 576-591, 2016.