

miR-10a-5p Promotes Chondrocyte Apoptosis in Osteoarthritis by Targeting HOXA1

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Osteoarthritis (OA) is a common joint disease characterized by degradation of the articular cartilage and joint inflammation. Studies have revealed the importance of microRNAs in the regulation of chondrocyte apoptosis. MicroRNA deep sequencing of control and osteoarthritic cartilage has revealed that miR-10a-5p is significantly upregulated in osteoarthritic tissues. However, its role in these tissues remains unknown. The present study was conducted to investigate the effect of miR-10a-5p in promoting OA. miR-10a-5p expression was increased in chondrocytes after interleukin-1 β treatment *in vitro*. Transfection with a miR-10a-5p inhibitor abrogated interleukin-1 β -induced apoptosis. A luciferase activity assay showed that miR-10a-5p targeted the 3' UTR of the homeobox gene *HOXA1*, inhibiting its expression. Treatment with *HOXA1* siRNA reversed the rescuing effect of the miR-10a-5p inhibitor on chondrocyte apoptosis. Additionally, an OA model was established in mice by anterior cruciate ligament transection. AntagomiR-10a-5p improved the cartilage surfaces of osteoarthritic mice, whereas agomiR-10a-5p worsened them. A terminal deoxynucleotidyl transferase dUTP nick-end labeling assay indicated reduced apoptosis and increased *HOXA1* expression in osteoarthritic mice after miR-10a-5p knockdown. These findings reveal a novel mechanism regulating OA progression and demonstrate the potential of miR-10a-5p and homeobox protein *HOXA1* as therapeutic targets.

INTRODUCTION

Osteoarthritis (OA) is a debilitating pathological condition that causes significant pain and stiffness in the joints. Millions of people worldwide are affected by OA each year.¹ Its symptoms typically include the degradation of articular cartilage, synovitis, chondrocyte apoptosis, inflammation, and the remodeling of subchondral bone.² Obesity, mechanical stress, genetic predisposition, gender, and aging are key factors in the development of OA.³ Currently, pain management and symptom control are the two primary treatment goals. However, the underlying molecular mechanisms of OA pathogenesis and new therapeutic approaches have been widely investigated in recent years, including numerous studies evaluating the roles of microRNAs (miRNAs) in the development and progression of OA.⁴⁻⁷

miRNAs are a subclass of small non-coding RNAs (approximately 17–24 nt in length) that are important regulators of gene expression at the posttranscriptional level.⁸ They have been a focal point of recent research, owing to their distinctive functions in various basic cell activities that are achieved via imperfect base pairing with the 3' UTRs of their target mRNAs, leading to reduced translation and/or degradation of target gene expression.⁹ Additionally, increasing evidence indicates that miRNAs play significant roles in chondrogenesis and cartilage remodeling⁴ and serve as important regulators of the inflammatory response, which is crucial to OA progression.⁵ Many miRNAs with aberrant expression profiles have been identified and associated with OA. For example, the upregulation of miR-34a levels in OA inhibits cell proliferation by affecting the SIRT1/p53 signaling pathway and triggering apoptosis in chondrocytes.⁶ Additionally, miR-210 has been shown to promote OA chondrocyte proliferation by targeting hypoxia-inducible factor (HIF)-3 α *in vitro*.⁷ These studies demonstrate that miRNAs represent promising targets for OA treatment. Therefore, exploring the expression of other miRNAs to identify additional targets that impact different pathways could be useful.

In this study, we examined the role of miR-10a-5p in interleukin (IL)-1-induced apoptosis of human chondrocytes. Analyses were also performed to investigate the target gene by which miR-10a-5p

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regulates functional proteins and mediates chondrogenic cell apoptosis and OA progression.

RESULTS

Identification of Differentially Expressed miRNAs in OA Cartilage Tissues

Primary chondrocytes were isolated from human osteoarthritic and control articular cartilage tissues (Figure 1A). To identify miRNAs that may play a role in OA, we examined the miRNA expression profiles of OA and control chondrocytes by high-throughput sequencing (Figures 1B and 1C). The top six most upregulated miRNAs in OA chondrocytes were confirmed by qRT-PCR in 10 patients with OA and 10 control subjects. miR-10a-5p showed the greatest degree of upregulation (Figures 1D and S1A) and an inhibitory effect on chondrocyte proliferation (Figure S1B), and thus it was further investigated.

Upregulation of miR-10a-5p in IL-1 β -Induced Chondrocytes and Anterior Cruciate Ligament Transection-Induced OA Mice

As miR-10a-5p was upregulated in chondrocytes extracted from human OA cartilage, miR-10a-5p expression in human OA and control cartilage tissues was analyzed by *in situ* hybridization. The percentage of miR-10a-5p-positive cells was higher in OA than in control cartilage (Figure 2A). Next, primary mouse chondrocytes were extracted from sham- and anterior cruciate ligament transection (ACLT)-induced OA mice, and miR-10a-5p expression was found to be upregulated in ACLT-induced OA cartilage (Figure 2F). Additionally, miR-10a-5p expression was increased both in IL-1 β -induced human and mouse chondrocytes (Figures 2B and 2G). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was used to evaluate the role of apoptosis in OA pathogenesis, and the percentage of TUNEL-positive cells was upregulated in both human OA cartilage and ACLT-induced OA mouse cartilage compared to that in controls (Figures 2C and 2E).

Effect of miR-10a-5p on Chondrocyte Proliferation and Apoptosis

To further explore the role of miR-10a-5p in OA pathogenesis, a miR-10a-5p mimic and inhibitor were used to modulate its endogenous expression in chondrocytes. The efficiency of miR-10a-5p overexpression and knockdown in human chondrocytes and mouse chondrocytes is shown in Figure 3A. Chondrocyte viability was significantly lower after treatment with IL-1 β , while miR-10a-5p knockdown clearly promoted chondrocyte proliferation (Figure 3B). Similarly, miR-10a-5p overexpression facilitated IL-1 β -induced inhibition of chondrocyte viability (Figure 3B). Additionally, the miR-10a-5p mimic significantly promoted chondrocyte apoptosis, while miR-10a-5p inhibition decreased IL-1 β -induced apoptosis (Figures 3C and 3D). Moreover, we detected the expression of apoptosis-related proteins, including cleaved caspase-3, cleaved PARP, and BCL-2. As shown in Figures 3E and 2A, the miR-10a-5p mimic augmented cleaved caspase-3 and cleaved PARP expression levels, both of which are induced by IL-1 β , and decreased BCL-2 expression. In contrast, the miR-10a-5p inhibitor rescued

the changes in cleaved caspase-3, cleaved PARP, and BCL-2 induced by IL-1 β .

Direct Targeting of Homeobox Gene *HOXA1* by miR-10a-5p in Chondrocytes

To investigate the molecular mechanism of miR-10a-5p, its potential targets were predicted using TargetScan (<http://www.targetscan.org/>), mirTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>), and PicTar (https://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi). Four target genes with high binding scores were selected from the overlapping gene set. Luciferase and qRT-PCR assays were further performed to determine the most likely target gene of miR-10a-5p (Figure S3). Based on these analyses, *HOXA1* was selected as the most likely target of miR-10a-5p during apoptosis. The putative binding sites of miR-10a-5p and *HOXA1* were analyzed using TargetScan. Moreover, luciferase activity in chondrocytes was significantly suppressed by the miR-10a-5p mimic and increased by the inhibitor (Figures 4A and 4B). Furthermore, we conducted western blotting and qRT-PCR experiments to detect the effects of miR-10a-5p overexpression and knockdown on *HOXA1* expression levels. Overexpression of miR-10a-5p significantly reduced *HOXA1* mRNA and protein levels, while miR-10a-5p knockdown had the opposite effect (Figures 4C and 4D).

Downregulation of *HOXA1* in OA Cartilage and IL-1 β -Induced Chondrocytes

We further investigated the function of *HOXA1* in OA progression. Immunohistochemistry (IHC) showed that *HOXA1* expression was significantly downregulated in human OA cartilage and the ACLT-induced mouse OA model (Figures 5A and 5B). Further, immunofluorescence experiments confirmed that *HOXA1* expression was reduced by IL-1 β treatment in chondrocytes (Figure 5C). Moreover, as shown in Figures 5D and 5E, IL-1 β reduced both mRNA and protein levels of *HOXA1* in chondrocytes.

Protection against IL-1 β -Induced Chondrocyte Proliferation Inhibition and Apoptosis by Targeting of *HOXA1* with miR-10a-5p Inhibitor

We then transfected *HOXA1* small interfering RNA (siRNA) into human and mouse chondrocytes. Figures 6A and 6B show that the *HOXA1* mRNA and protein levels were efficiently knocked down by siHOXA1. A cell-counting kit (CCK)-8 assay indicated that the downregulation of *HOXA1* decreased chondrocyte viability. IL-1 β inhibits chondrocyte proliferation, and this was significantly rescued by treatment with miR-10a-5p inhibitor, while *HOXA1* knockdown inhibited OA chondrocyte proliferation (Figure 6C). An apoptosis assay demonstrated that siHOXA1 augmented the apoptotic rate and reversed the rescuing effect of the miR-10a-5p inhibitor on IL-1 β -induced chondrocyte apoptosis (Figures 6D and 6E). Moreover, when administered in combination with the miR-10a-5p inhibitor, siHOXA1 enhanced the protein expression of cleaved caspase-3 and cleaved PARP and reduced the protein levels of BCL-2 (Figures 6F and S2B). These results suggest that siHOXA1 neutralized the effects of the miR-10a-5p inhibitor, indicating that *HOXA1* is the target gene of miR-10a-5p.

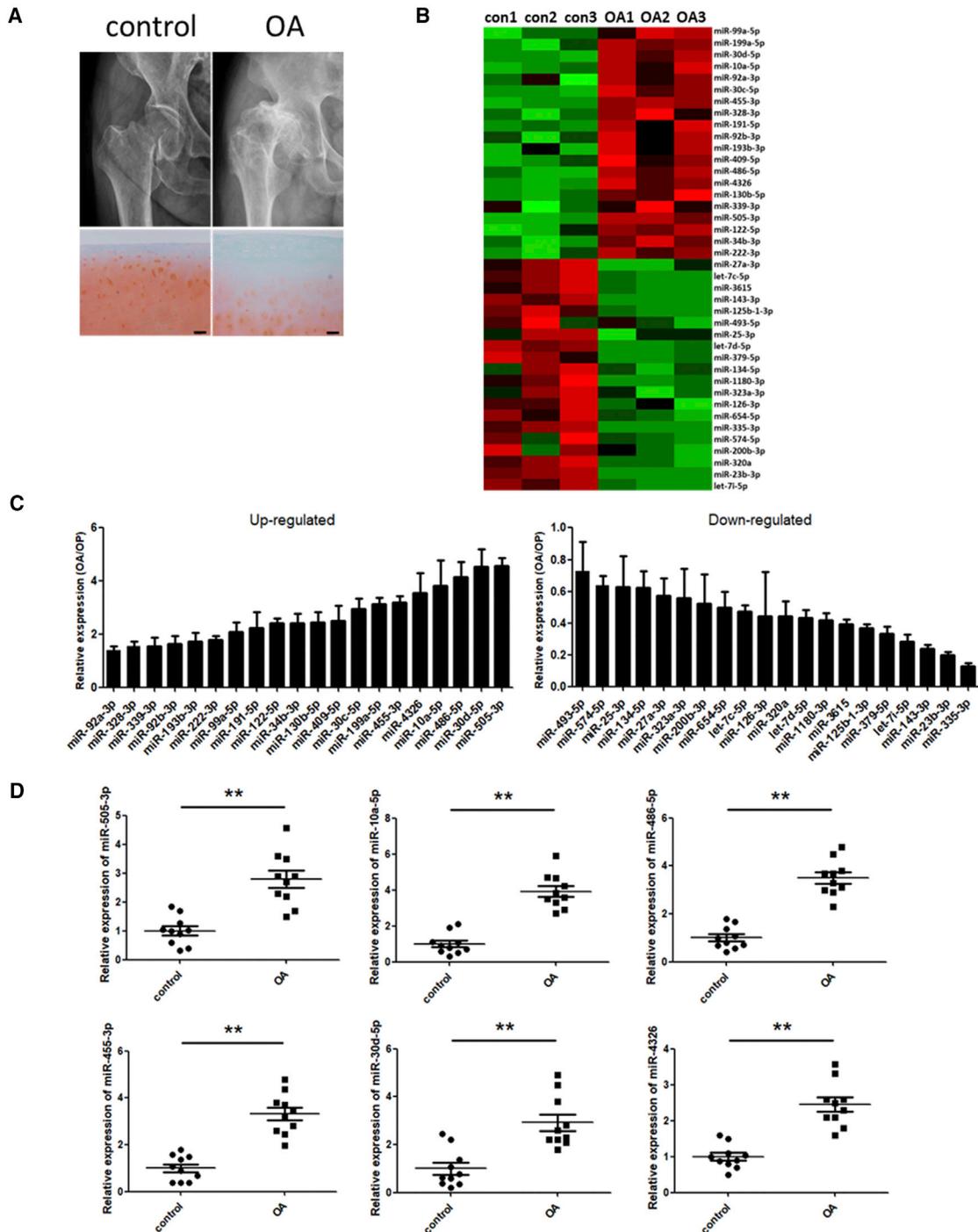


Figure 1. Identification of Differentially Expressed miRNAs in OA Cartilage Tissues

(A) X-ray of hip and safranin O-Fast Green staining of cartilage tissues from OA patients and controls. (B) Heatmap of miRNAs differentially expressed in OA and control cartilage. Upregulation is indicated in red, while downregulation is shown in blue (n = 3). (C) Expression levels of miRNAs in the heatmap. (D) qRT-PCR validation of the top six most upregulated miRNAs in human chondrocytes from OA and control cartilage (n = 10). Data represent the mean ± SEM. *p < 0.05, **p < 0.01.

Attenuation of OA Progression by miR-10a-5p Inhibitor

The previous experiments demonstrated the effects of miR-10a-5p on chondrocytes *in vitro*. To determine whether miR-10a-5p plays a role

in OA progression *in vivo*, agomiR-10a-5p and antagomiR-10a-5p were administered to ACLT-induced OA mice intra-articularly. Eight weeks after surgery, safranin O and Fast Green staining were

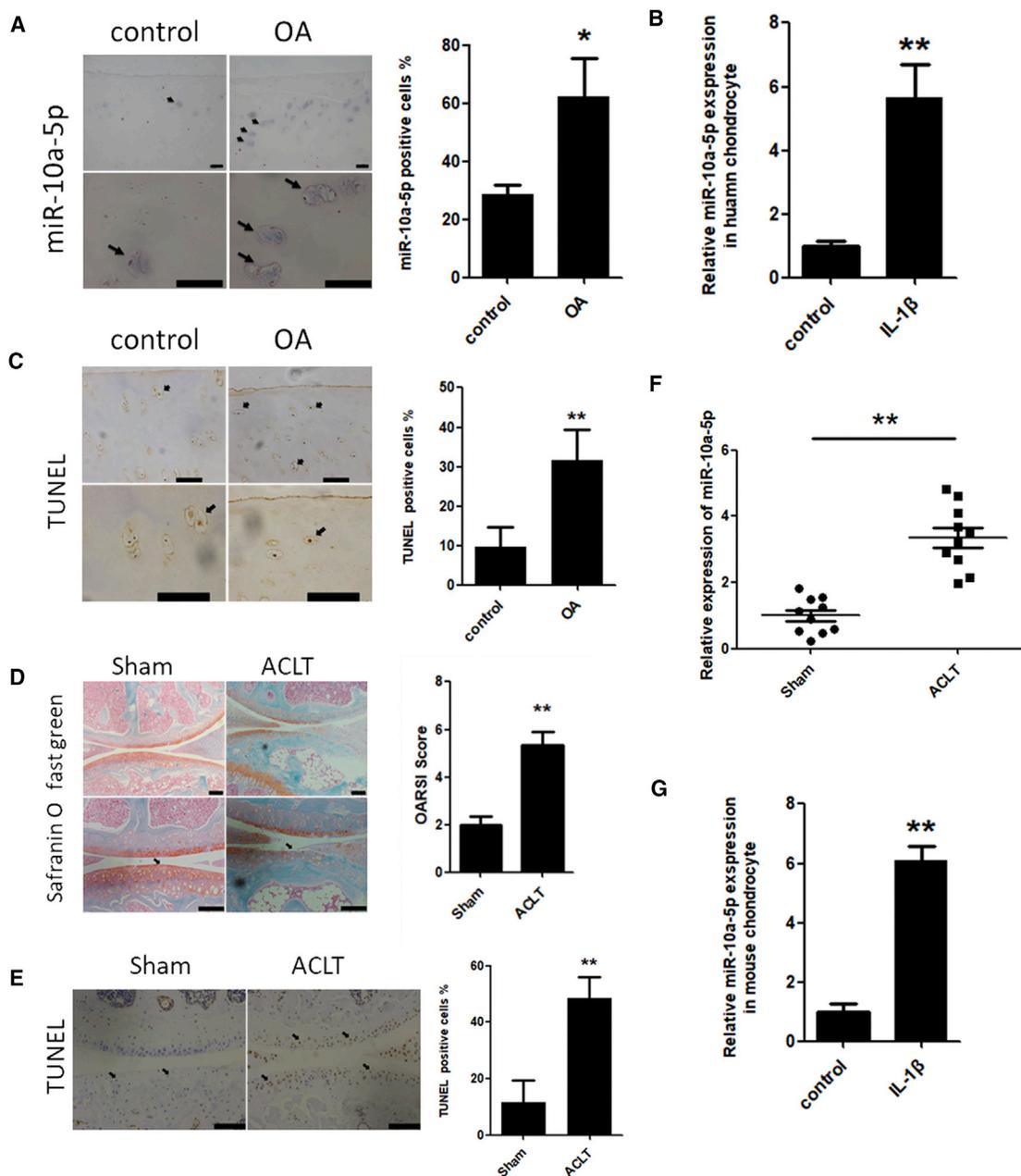
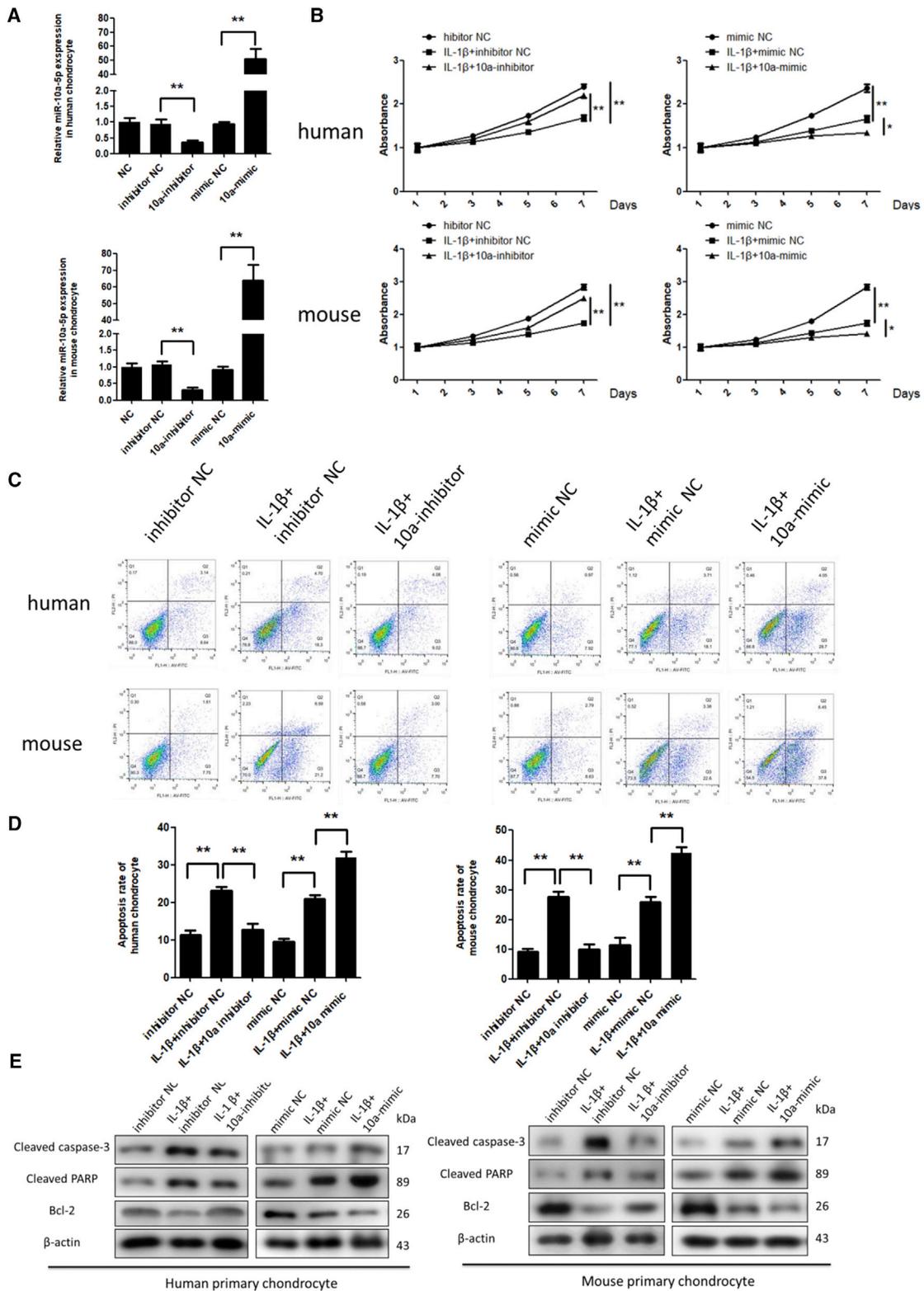


Figure 2. miR-10a-5p Was Upregulated in IL-1 β -Induced Chondrocytes and ACLT-Induced OA mice

(A) *In situ* hybridization analysis of miR-10a-5p expression in OA and control cartilage tissues. The percentage of miR-10a-5p-positive cells was calculated. (B) Relative miR-10a-5p expression in IL-1 β -induced human chondrocytes. (C) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of OA and control cartilage tissues. The percentage of TUNEL-positive cells was calculated. (D) Safranin O-Fast Green staining of cartilage from sham-operated and ACLT-induced OA mice. Osteoarthritis Research Society International (OARSI) scores were calculated. (E) TUNEL staining of cartilage from sham-operated and ACLT-induced OA mice. The percentage of TUNEL-positive cells was calculated. (F) Relative expression of miR-10a-5p in mouse chondrocytes of sham-operated and ACLT-induced OA cartilage. (G) Relative miR-10a-5p expression in IL-1 β -induced mouse chondrocytes. Data are represented as the mean \pm SEM (n = 10). *p < 0.05; **p < 0.01.

conducted to evaluate the degradation of the cartilage matrix. Cartilage surfaces in ACLT-induced OA mice were improved and worsened upon treatment with antagomiR-10a-5p and agomiR-10a-5p, respectively (Figure 7A). Quantitative analysis employing

the Osteoarthritis Research Society International (OARSI) scoring system indicated that antagomiR-10a-5p treatment resulted in significantly lower OARSI scores, whereas agomiR-10a-5p treatment resulted in higher scores (Figure 7B). IHC results showed that



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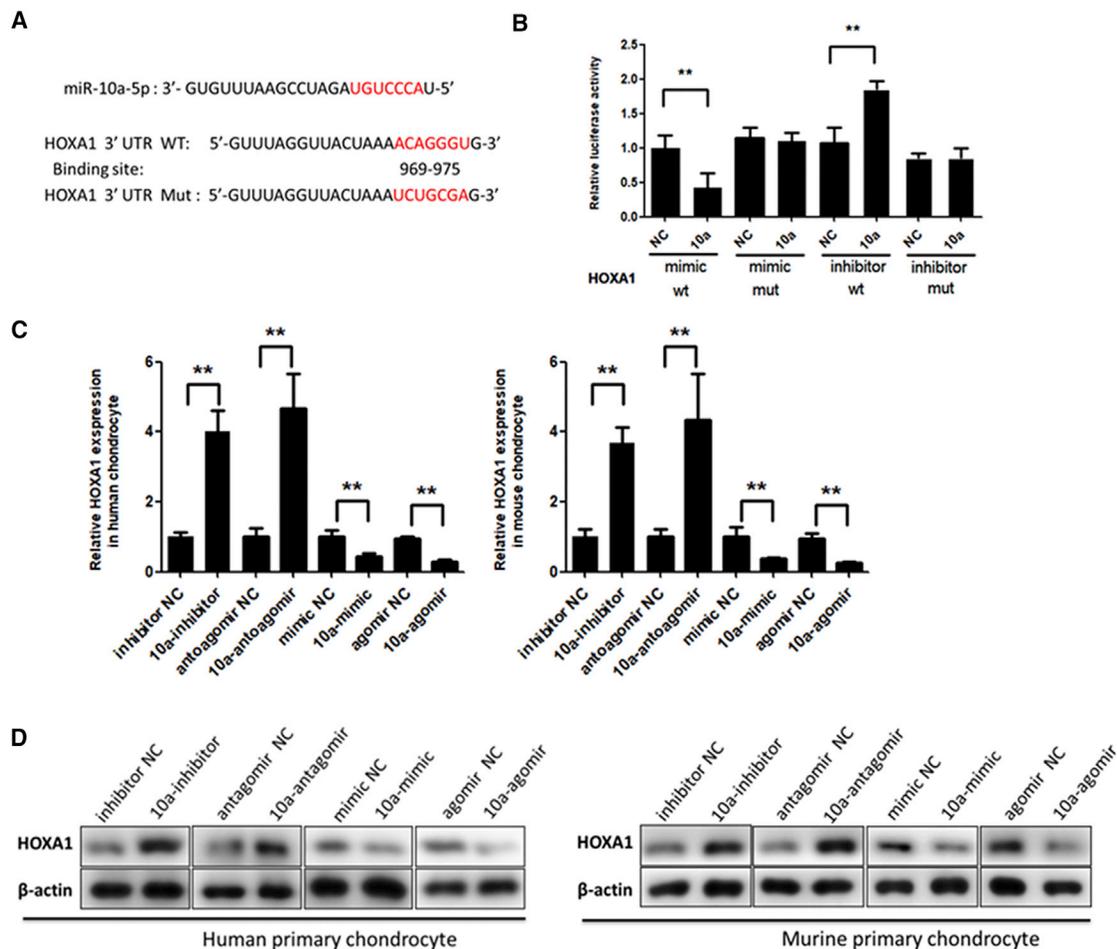


Figure 4. miR-10a-5p Directly Targets HOXA1 in Chondrocytes

(A) miR-10a-5p aligned with the 3' UTR of *HOXA1* mRNA. (B) HEK293T cells co-transfected with a reporter carrying a mutant or wild-type (wt) *HOXA1* 3'UTR, along with miR-10a-5p mimic or inhibitor, were analyzed by luciferase assay. (C) Human and mouse chondrocytes were transfected with a negative control (NC) inhibitor, miR-10a-5p inhibitor, NC mimic, miR-10a-5p mimic, NC antagonist, antagonist-miR-10a-5p, NC agomiR, or agomiR-miR-10a-5p, and *HOXA1* expression was then measured by qRT-PCR. (D) Human and mouse chondrocytes were transfected with NC inhibitor, miR-10a-5p inhibitor, NC mimic, miR-10a-5p mimic, NC antagonist, antagonist-miR-10a-5p, NC agomiR, or agomiR-miR-10a-5p, and *HOXA1* expression was then measured by western blotting. Data are represented as the mean \pm SEM (n = 3). *p < 0.05; **p < 0.01.

antagomiR-10a-5p-treated OA mice exhibited lower percentages of TUNEL-positive cells and higher percentages of HOXA1-positive cells than agomiR-10a-5p-treated OA mice (Figures 7C–7F).

DISCUSSION

OA is a complex degenerative joint disease affected by many factors. It is currently thought that a combination of mechanical and biological factors results in the degradation of chondrocytes, extracellular ma-

trix, and the subchondral bone, as well as an imbalance in the normal synthetic coupling of these elements. As the only cell type in articular cartilage, chondrocytes regulate the balance between the synthesis and decomposition of the cartilage matrix. When joints are stimulated by excessive mechanical stress, oxy-radicals, or inflammation, chondrocytes undergo degeneration and apoptosis, leading to an imbalance in the synthesis and decomposition of the cartilage matrix.¹⁰ Increasing evidence indicates that chondrocyte apoptosis is

Figure 3. Effect of miR-10a-5p on Chondrocyte Proliferation and Apoptosis

(A) Human and mouse chondrocytes were transfected with negative control (NC) inhibitor, miR-10a-5p inhibitor, NC mimic, or miR-10a-5p mimic for 72 h, and miR-10a-5p expression was measured by qRT-PCR. (B) The miR-10a-5p mimic suppressed chondrocyte proliferation, whereas miR-10a-5p inhibitor promoted proliferation, as determined using the Cell Counting Kit (CCK)-8 assay. (C) Analysis of chondrocyte apoptosis in miR-10a-5p mimic- or inhibitor-transfected primary human and mouse chondrocytes. (D) The rate of apoptosis in human and mouse chondrocytes was calculated. (E) Human and mouse chondrocytes were transfected with NC inhibitor, miR-10a-5p inhibitor, NC mimic, or miR-10a-5p mimic for 24 h, then induced by IL-1 β for 48 h. Cleaved caspase-3, cleaved PARP, and BCL-2 expression levels were measured by western blotting. Data are represented as the mean \pm SEM (n = 3). *p < 0.05; **p < 0.01.

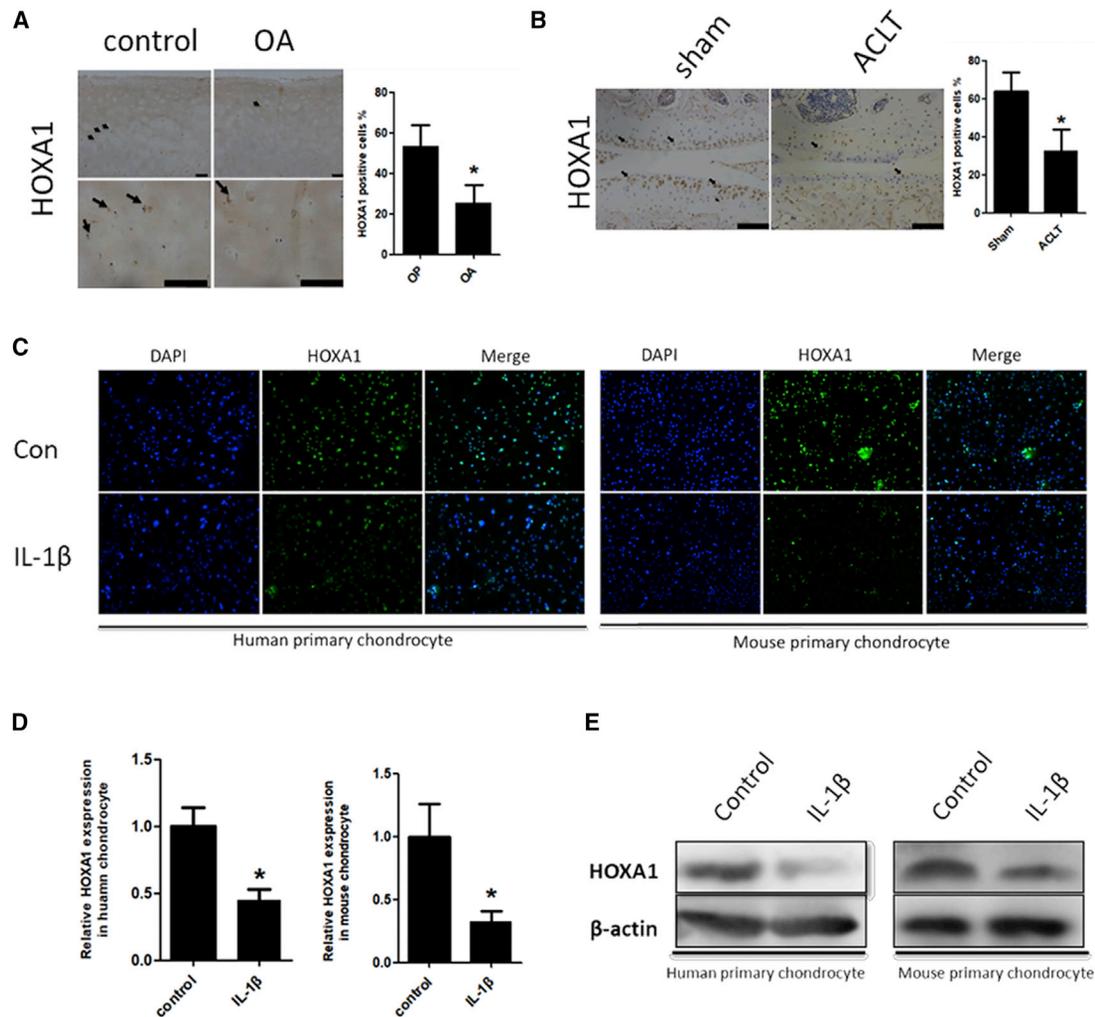


Figure 5. HOXA1 Was Downregulated in OA Cartilage and IL-1 β -Induced Chondrocytes

(A) Immunohistochemistry (IHC) of HOXA1 expression in human OA and control cartilage tissues. The percentage of HOXA1-positive cells was calculated. (B) IHC analysis of HOXA1 expression in sham-operated and ACLT-induced OA mouse cartilage tissues. The percentage of HOXA1-positive cells was calculated. (C) Immunofluorescence analysis of HOXA1 expression in control and IL-1 β -induced human and mouse chondrocytes. (D) Human and mouse chondrocytes were stimulated by IL-1 β , and HOXA1 expression was measured by qRT-PCR. (E) Human and mouse chondrocytes were stimulated by IL-1 β , and HOXA1 expression was measured by western blotting. Data are represented as the mean \pm SEM (n = 3). *p < 0.05; **p < 0.01.

closely associated with OA development.^{11–13} miRNAs are thought to be widely involved in the apoptosis and proliferation of tumor cells and to play an important role in the development of related diseases. In this study, we focused on the effects of miRNAs on chondrocyte apoptosis and proliferation.

First, transcriptome high-throughput sequencing showed that levels of miR-10a-5p, miR-486-5p, miR-455-3p, miR-30d-5p, miR-505-3p, and miR-4326 were significantly upregulated in human OA chondrocytes, while qRT-PCR confirmed that the greatest difference was found in miR-10a-5p expression. Previous studies have reported a reduction in miR-10a-5p expression in rheumatoid arthritis and increase in OA,¹⁴ but the specific role of miR-10a-5p in chondrocytes

has yet to be determined. We thus focused on miR-10a-5p in this analysis.

Studies have shown that IL-1 β plays an important role in the progression of OA by promoting the degeneration and apoptosis of chondrocytes, and it has been widely used to establish OA cell models.^{15,16} This is consistent with our results showing that IL-1 β stimulated miR-10a-5p expression in both human and murine primary chondrocytes. Additionally, silencing of miR-10a-5p significantly attenuated IL-1 β -induced apoptosis, whereas miR-10a-5p overexpression enhanced apoptosis. Cell proliferation experiments and apoptosis-related protein analysis also showed consistent results, suggesting that miR-10a-5p positively promotes chondrocyte apoptosis.

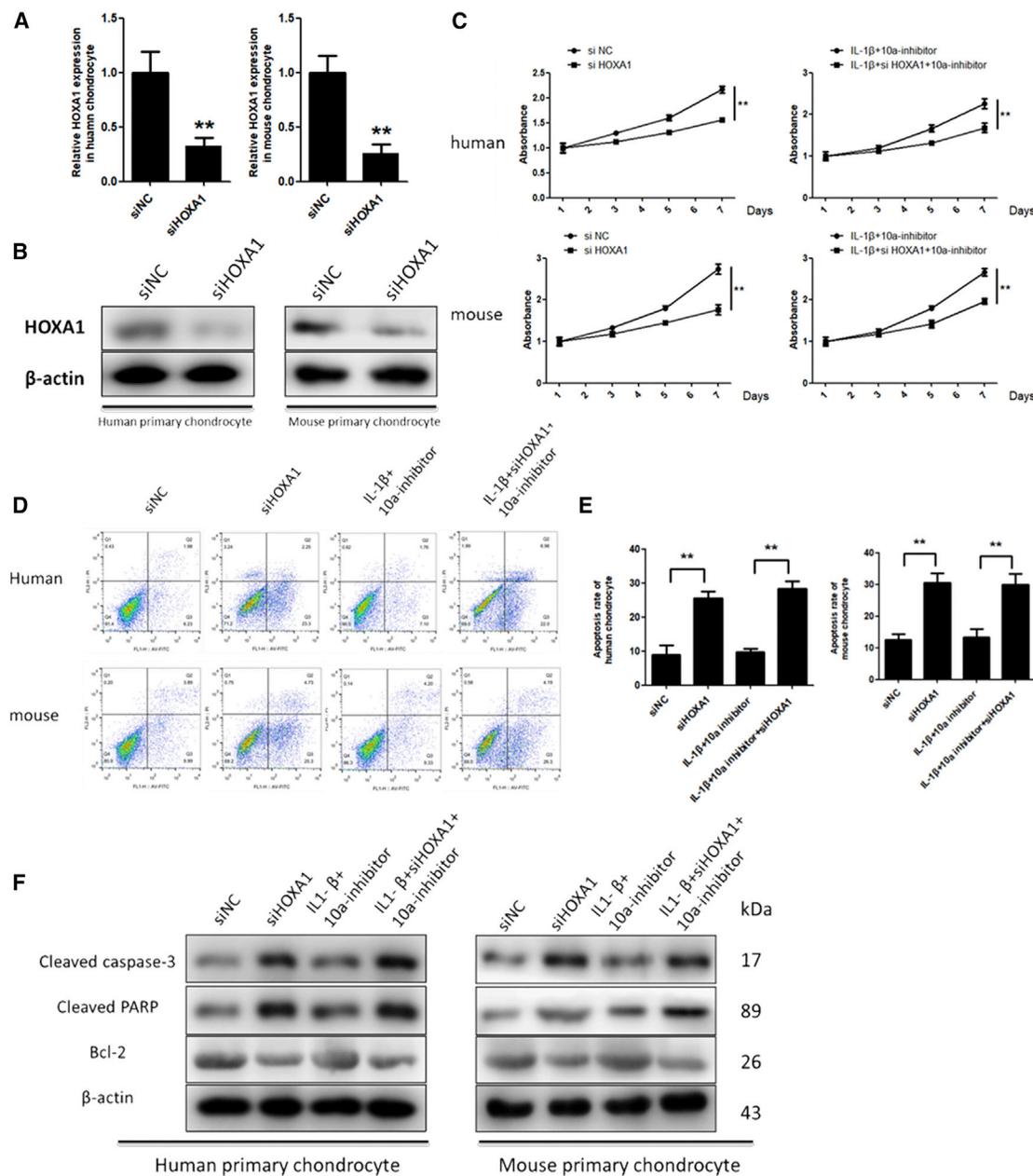


Figure 6. miR-10a-5p Inhibitor Protects against IL-1β-Induced Chondrocyte Apoptosis by Targeting HOXA1

(A) Human and mouse chondrocytes were transfected with small interfering negative control (siNC) or siHOXA1, and relative *HOXA1* expression was measured by qRT-PCR. (B) Human and mouse chondrocytes were transfected with siNC or siHOXA1, and relative *HOXA1* expression was measured by western blotting. (C) siHOXA1 suppressed chondrocyte proliferation and reversed the rescuing effect of miR-10a-5p inhibitor on IL-1β-induced chondrocyte proliferation, as determined by CCK-8 assay. (D) Analysis of chondrocyte apoptosis in siNC- or siHOXA1-transfected, as well as siNC- and miR-10a-5p inhibitor-co-transfected or siHOXA1- and miR-10a-5p inhibitor-co-transfected primary human and mouse chondrocytes. (E) The rate of apoptosis in human and mouse chondrocytes was calculated. (F) Cleaved caspase-3, cleaved PARP, and BCL-2 expression levels were measured by western blotting. Data are represented as the mean ± SEM (n = 3). *p < 0.05; **p < 0.01.

Moreover, miR-10a-5p induces apoptosis in a variety of tumor cells,^{17,18} supporting the results of this study to some extent.

Further bioinformatics analyses predicted the downstream target genes of miR-10a-5p. Preliminary screening revealed that

HOXA1 was highly regulated by miR-10a-5p and downregulated in OA articular cartilage and IL-1β-stimulated chondrocytes. This is consistent with a previous study in which IL-1β treatment reduced *HOXA1* expression in decidual cells.¹⁹ Other studies have observed that *HOXA1* inhibition induces apoptosis in a variety of

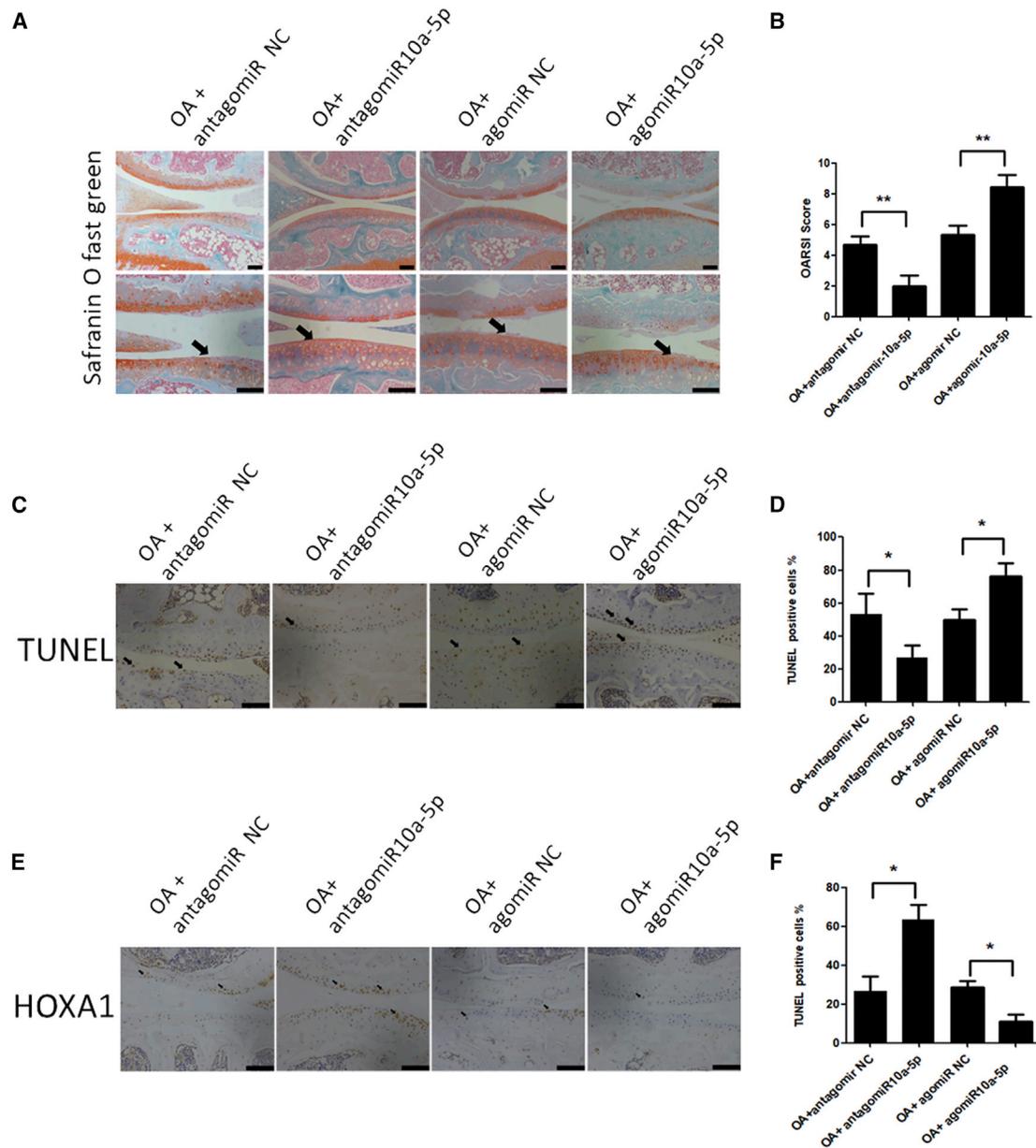


Figure 7. miR-10a-5p Inhibitor Attenuated OA Progression

(A) ACLT-induced OA mice were treated with negative control (NC) antagomiR, antagomiR-10a-5p, NC agomiR, or antagomiR-10a-5p, and then the degree of knee OA was evaluated by safranin O-Fast Green staining. (B) OARSI scores were determined according to the staining results. (C) TUNEL staining of ACLT-induced OA mice treated with NC antagomiR, antagomiR-10a-5p, NC agomiR, or antagomiR-10a-5p. (D) The percentage of TUNEL-positive cells was calculated according to staining. (E) IHC analysis of HOXA1 expression from ACLT-induced OA mice treated with NC antagomiR, antagomiR-10a-5p, NC agomiR, or antagomiR-10a-5p. (F) The percentage of HOXA1-positive cells was calculated according to IHC data. Data are represented as the mean \pm SEM ($n = 8$). * $p < 0.05$; ** $p < 0.01$.

tumor cells.^{20–22} The present study further confirmed that the silencing of HOXA1 promotes apoptosis in chondrocytes and rescues the effect of miR-10a-5p inhibition on IL-1 β -induced chondrocyte apoptosis. This suggests that the miR-10a-5p-mediated HOXA1 axis may play an important role in chondrocyte apoptosis.

We also demonstrated the role of miR-10a-5p in human and murine primary chondrocytes. Experiments involving the injection of antagomiR and agomiR into the knee joints of mice have been previously evaluated,^{23,24} we thus synthesized antagomiR-10a-5p and agomiR-10a-5p for further animal experiments. AntagomiR-10a-5p attenuated OA progression, whereas agomiR-10a-5p aggravated OA

progression. Additionally, IHC analysis of mouse specimens showed that the antagomiR-10a-5p group exhibited increased HOXA1 expression and a smaller percentage of apoptotic cells (TUNEL-positive), whereas those in the agomiR-10a-5p group exhibited decreased HOXA1 expression and a higher percentage of apoptotic cells. This suggests that miR-10a-5p promotes apoptosis in chondrocytes by inhibiting HOXA1 expression, thereby aggravating the progression of OA in mice.

The femoral cartilage tissue of patients is often used as a control for OA,²⁵ and such specimens are available from clinical trials in our lab. In this study, we first identified and determined the function of miR-10-5p in primary cells extracted from patient specimens and then evaluated mouse primary chondrocytes. The experiments showed similar results, indicating that miR-10-5p plays the same role in human and murine chondrocytes. Thus, we conducted *in vivo* mouse experiments, which showed that miR-10a-5p promotes chondrocyte apoptosis by inhibiting HOXA1 expression and aggravating OA progression in mice.

This study had some limitations. Although IL-1 β is routinely used to establish cellular OA models, it does not fully mimic the OA environment, as several other inflammatory factors are involved in the course of OA.²⁶ Whether other inflammatory factors, such as tumor necrosis factor- α and IL-6, trigger similar changes remains unclear. Additionally, OA is often accompanied by synovial membrane inflammation and sclerosis of the subchondral bone; however, this study focused only on the effects of miR-10-5p on chondrocytes. Therefore, investigation of the subchondral bone and the synovium is needed to fully elucidate the impact of miR-10-5p on OA.

To our knowledge, this is the first study to examine the role of the miR-10a-5p/HOXA1 axis in regulating apoptosis in chondrocytes. The role of this axis in OA development was verified in our mouse model. Our results provide a basis for targeting the miR-10a-5p/HOXA signaling axis for the prevention and treatment of OA.

MATERIALS AND METHODS

Human Cartilage and OA Mouse Model

Human cartilage samples were collected according to protocols approved by the Ethics Committee of Sir Run Run Shaw Hospital (Zhejiang, China). The methods were carried out in accordance with approved guidelines. Written informed consent was obtained from all subjects. Control cartilage was harvested from patients with femoral neck fractures without OA history at the time of total hip replacement surgery ($n = 40$), while pathological cartilage was acquired from patients with end-stage symptomatic hip or knee OA at the time of total hip replacement surgery ($n = 20$). All C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal (Shanghai, China), and animal handling and experimental procedures were performed with approval from the Institute of Health Sciences Institutional Animal Care and Use Committee. The OA mouse model was induced by ACLT, as previously described.²⁷

Histological Analysis, TUNEL Staining, and IHC

Cartilage specimens were fixed in 4% paraformaldehyde for paraffin embedding. Each paraffin-embedded cartilage sample was sectioned at 5 μ m, and every 10th section was stained with 0.1% safranin O solution and 0.001% Fast Green solution (Sigma-Aldrich, St. Louis, MO, USA). Cartilage destruction of mouse knee joints was scored by two observers blinded to group-identifying information using the OARSI grading system.²⁸ TUNEL staining was performed using the *In Situ* Cell Death Detection kit, POD (Sigma-Aldrich), according to the manufacturer's protocol. For IHC analysis, the sections were incubated at 4°C with antibodies for HOXA1 (Abcam, Cambridge, UK) overnight and for 2 h at room temperature with secondary antibodies (Beyotime Institute of Biotechnology, Shanghai, China). The number of positively stained cells on the entire articular surface (including the femoral condyle and tibial plateau area) per specimen was counted, and the percentage of positive cells was calculated.²⁹

Chondrocyte Culture

Chondrocytes were isolated from human and mouse articular cartilage tissues, as described previously.³⁰ Chondrocytes were maintained in DMEM containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h at 37°C. The cells were filtered through a 0.075-mm cell strainer and washed with sterile PBS before culturing or miRNA and mRNA isolation. Primary chondrocytes at 80% confluence were used for the experiments. During the culture period, the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

RNA Extraction and qRT-PCR Analysis

Total cellular RNA was extracted from the cultured chondrocytes, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was stored at -80°C. Reverse transcription was performed using 1.0 μ g total RNA and a miRNA cDNA kit and HiFiScript cDNA kit (CWBI, Beijing, China), which were used to investigate the expression of miRNA and HOXA1, respectively. Amplification reactions were conducted in 20- μ L reaction volumes containing amplification primers and UltraSYBR Mixture (with ROX) (CWBI) detected by an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). 1- μ L volume cDNA and 1- μ L volume primer (Sangon Biotech, Shanghai, China) were used in each amplification reaction. The following cycling conditions were used: 40 cycles of denaturation at 95°C for 5 s and amplification at 60°C for 24 s. All reactions were conducted in triplicate and normalized using the miRNA housekeeping gene U6 or mRNA housekeeping gene β -actin. Primer sequences were as follows: human HOXA1 (forward: 5'-CGGCTTCCTGTGCTAAGTCT-3' and reverse: 5'-TAGCCCAGCCAAATACACGG-3'), mouse Hoxa1 (forward: 5'-CCAGAGAGCTGGTTCGTAG-3' and reverse: 5'-GAACCATGGTGAATGTGCTG-3'), human BCL6 (forward: 5'-CCCATGTGTCTTCAGCTTTCT-3' and reverse: 5'-CTCCTCAGTGGCAGGTTGTT-3'), mouse Bcl6 (forward: 5'-TGAGGGGTTTTGCATCCTCC-3' and reverse: 5'-TCACGGGGAGGTTAAGTGC-3'), human USF2 (forward: 5'-AATGGAGGACAGACAGGAACAC-3' and reverse: 5'-TTTACTCGCTCCCGTCTTGC-3'), mouse Usf2

(forward: 5'-CCGGACACACCCCTATTCTC-3' and reverse: 5'-TGCTCCTGTCTTGTCTGTTGT-3'), human *NCOR2* (forward: 5'-GCATCATGGGTGGCGG-3' and reverse: 5'-ATCAGGGGGTTGTA GGGGAA-3'), mouse *Ncor2* (forward: 5'-GATCCCTCTGGAAGCA GCAG-3' and reverse: 5'-GCTGCGAGGTGATGTAGTCA-3'), human β -actin (forward: AGCGAGCATCCCCAAAGTT and reverse: GGGCACGAAGGCTCATCATT), mouse β -actin (forward: 5'-CCTCTATGCCAACACAGT-3' and reverse: 5'-AGCCACCAATCCACACAG-3'), U6 (forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTACGAATTTGCGT-3'), miR-10a-5p (forward: 5'-CGCTACCTGTAGATCCGAATTTGTG-3'), miR-486-5p (forward: 5'-TCCTGTACTGAGCTGCCCC-3'), miR-4326 (forward: 5'-CGTGTTCCTCTGTCTCCCAGAC-3'), miR-30d-5p (forward: 5'-CGTGTAACATCCCCGACTGGAAG-3'), miR-505-3p (forward: 5'-CCGTCAACACTTGCTGGTTTCCCT-3'), miR-455-3p (forward: 5'-CGGCAGTCCATGGGCATATACAC-3'), universal miRNA reverse (reverse: 5'-GTGCAGGGTCCGAGGT-3').

Transfection

Chondrocytes were cultured in 6- or 96-well plates with appropriate cells for 24 h. miR-10a-5p mimic and inhibitor, agomiR-10a-5p, antagomiR-10a-5p, siHOXA1, and their negative controls, all purchased from Ruibo (Guangzhou, China), were transfected into chondrocytes, using Lipofectamine 3000 (Invitrogen) as previously described.²⁷ Twenty-four hours after transfection, the cells were used for subsequent experiments.

Luciferase Assays

miR-10a-5p mimic and inhibitor, its miR-control, and wild-type or mutant *HOXA1* 3'UTR-Luc reporter plasmids were co-transfected into HEK293T cells, using Lipofectamine 3000 (Invitrogen). The Luciferase Reporter Gene system (Sigma-Aldrich) was used to detect activity, according to the manufacturer's instructions.

Flow Cytometry Assay

Chondrocytes subjected to different treatments were collected using trypsin (Thermo Fisher Scientific) and washed with PBS. Apoptosis was detected with an Annexin V-FITC Apoptosis Kit (BD Biosciences, Franklin Lakes, NJ, USA). The collected cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide for 15 min, according to the manufacturer's instructions. Apoptosis was detected with a FACS flow cytometer (BD Biosciences).

Western Blotting

Following stimulation with IL-1 β or transfection, culture supernatants were removed, and human or mouse chondrocytes were washed with cold PBS and lysed in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Proteins were resolved on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20, the membranes were incubated with primary antibodies against

HOXA1, cleaved caspase-3, cleaved PARP, BCL-2, and β -actin (Abcam). After the membranes were washed, primary antibodies were detected using horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (Beyotime Institute of Biotechnology) and visualized with an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Dallas, TX, USA).

CCK-8 Cell Growth Assay

Chondrocytes were seeded into 96-well plates at 2×10^3 cells/well and incubated for 1–7 days. At each time point, CCK-8 (Sigma-Aldrich) was added to each well. After 4 h of incubation, the absorbance of the solution at 450 nm was determined using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Immunofluorescence

Chondrocytes were grown in 20-mm glass-bottomed cell culture dishes (801001; Nest Biotechnology, Shanghai, China). The cells were fixed with 4% paraformaldehyde for 15 min, washed twice with PBS containing 0.05% Tween-20, permeabilized with 0.3% Triton X-100 for 5 min, and then blocked with 1% BSA for 30 min. The cells were then incubated with HOXA1 antibodies (1:200; Abcam) at 4°C overnight. After they were washed three times with PBS, the cells were incubated with goat anti-rabbit IgG conjugated to fluorescent Cy5 dye (1:100; Abcam) in PBS. DAPI (Life Technologies, Carlsbad, CA, USA) was used for nuclear staining. Immunofluorescence images were obtained using a Nikon Eclipse TI (Tokyo, Japan) and Zeiss LSM780 confocal microscope (Oberkochen, Germany) or Zeiss Colibri epifluorescence microscope and processed with ImageJ software (NIH, Bethesda, MD, USA).

In Situ Hybridization

Digoxigenin-labeled, locked nucleic acid miR-10a-5p probes were designed and synthesized by RiboBio (Guangzhou, China) using a Fluorescent *In situ* Hybridization Kit (RiboBio). Specimens were analyzed with a Nikon inverted fluorescence microscope.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, IL, USA). Data are represented as the mean \pm SEM. The significance of differences between two groups was determined by an independent-samples t test. The significance of differences between three or more groups was analyzed by one-way ANOVA and Tukey's *post hoc* test or Student's t test. A p value < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at <https://doi.org/10.1016/j.omtn.2018.12.012>.

AUTHOR CONTRIBUTIONS

Conceptualization: J.Z. and S.S.; Methodology: Y.M., Y.W., J.C., K.H., B.J., Q.W., and Z.C.; Investigation: J.Z., S.S., Y.M., Y.W., J.C., K.H., J.M., and B.J.; Writing: Y.M.; Supervision: J.Z. and S.S.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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