BJR

RESEARCH

Silencing of microRNA-138-5p promotes IL-1β-induced cartilage degradation in human chondrocytes by targeting FOXC1

MIR-138 PROMOTES CARTILAGE DEGRADATION

Y. Yuan,
G. Q. Zhang,
W. Chai,
M. Ni,
C. Xu,
J. Y. Chen

Chinese PLA General Hospital, Beijing, China

■ Y. Yuan, MM, Department of Orthopaedics, Chinese PLA General Hospital, No.28 Fuxing Road,Haidian District,Beijing 100853,China and, Jinan Military General Hospital, No.25, Shifan Road, Tianqiao District, Jinan 250031, Shandong, China.
■ G. Q. Zhang, MD, Department of Orthopaedics, Chinese PLA General Hospital,
■ W. Chai, MD, Department of Orthopaedics, Chinese PLA General Hospital,
■ M. Ni, MD, Department of Orthopaedics, Chinese PLA General Hospital,
■ C. Xu, MM, Department of Orthopaedics, Chinese PLA General Hospital,
■ J. Y. Chen, MD, Department of Orthopaedics, Chinese PLA General Hospital, No.28 Fuxing Road, Haidian District, Beijing 100853, China.

Correspondence should be sent to J. Y. Chen at chenjiying5693@126.com

doi: 10.1302/2046-3758.510.BJR-2016-0074.R2

Bone Joint Res 2016;5:523–530.

Received: 8 March 2016;
Accepted: 8 August 2016

Objectives

Osteoarthritis (OA) is characterised by articular cartilage degradation. MicroRNAs (miRNAs) have been identified in the development of OA. The purpose of our study was to explore the functional role and underlying mechanism of miR-138-5p in interleukin-1 beta (IL-1β)-induced extracellular matrix (ECM) degradation of OA cartilage.

Materials and Methods

Human articular cartilage was obtained from patients with and without OA, and chondrocytes were isolated and stimulated by IL-1β. The expression levels of miR-138-5p in cartilage and chondrocytes were both determined. After transfection with miR-138-5p mimics, allele-specific oligonucleotide (ASO)-miR-138-5p, or their negative controls, the messenger RNA (mRNA) levels of aggrecan (ACAN), collagen type II and alpha 1 (COL2A1), the protein levels of glycosaminoglycans (GAGs), and both the mRNA and protein levels of matrix metalloproteinase (MMP)-13 were evaluated. Luciferase reporter assay, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blot were performed to explore whether Forkhead Box C1 (FOCX1) was a target of miR-138-5p. Further, we co-transfected OA chondrocytes with miR-138-5p mimics and pcDNA3.1 (+)-FOXC1 and then stimulated with IL-1β to determine whether miR-138-5p-mediated IL-1β-induced cartilage matrix degradation resulted from targeting FOXC1.

Results

MiR-138-5p was significantly increased in OA cartilage and in chondrocytes in response to IL-1β-stimulation. Overexpression of miR-138-5p significantly increased the IL-1β-induced downregulation of COL2A1, ACAN, and GAGs, and increased the IL-1β-induced over expression of MMP-13.We found that FOXC1 is directly regulated by miR-138-5p. Additionally, co-transfection with miR-138-5p mimics and pcDNA3.1 (+)-FOXC1 resulted in higher levels of COL2A1, ACAN, and GAGs, but lower levels of MMP-13.

Conclusion

miR-138-5p promotes IL-1β-induced cartilage degradation in human chondrocytes, possibly by targeting FOXC1.

Cite this article: *Bone Joint Res* **2016;5:523–530.**

Keywords: MicroRNA-138; IL-1β; osteoarthritis; cartilage degradation; FOXC1

Article focus

■ Does miR-138-5p play a pivotal role in interleukin-1 beta (IL-1β)-induced extracellular matrix (ECM) degradation of osteoarthritic (OA) cartilage and, if so, what is the underlying mechanism?

Key messages

■ In the present study, we found that miR-138-5p directly contributed to IL-1β-induced chondrocyte ECM degradation by targeting Forkhead Box C1, providing new insights into the pathological mechanism of OA.

Strengths and limitations

- MiR-138 may provide new insights into the pathological mechanism and therapeutic strategy for OA.
- The detailed molecular mechanism is still unknown and need to be explored in the future.

Introduction

Osteoarthritis (OA) is the most prevalent chronic articular disease and particularly affects elderly individuals.[1] The prevalence of OA escalates with age, imposing a huge economic burden and impairing health-related quality of life.[2] Although multiple factors contribute to the development of OA, the progressive loss of cartilage matrix and destruction of articular cartilage are major hallmarks of OA.[3] The imbalance between extracellular matrix (ECM) synthesis and degradation is associated with cartilage destruction.[4] Chondrocytes are important cell sources that are responsible for anabolic and catabolic metabolism during the pathogenesis of OA. For example, the synthesis of aggrecan (ACAN) and collagen type II alpha 1 (COL2A1) is reduced, while matrix metalloproteinase (MMP)-13 is increased in chondrocytes.[5]

Recently, the catabolic and anabolic effects of microRNAs (miRNAs) on OA cartilage have received focused attention.[6] A class of non-coding small RNAs, miRNAs are involved in various human diseases by regulating multiple biological processes, such as cell proliferation, survival, apoptosis, differentiation, migration, metabolism, and tumorigenesis.[7] MiRNAs regulate gene expression by binding to complementary sites within the 3'untranslated regions (3'-UTRs) of their target protein-coding messenger RNAs (mRNAs), repressing mRNA translation and promoting mRNA degradation.[8] Accumulating evidence suggests that miRNAs have been identified in the development of OA.[9-11] Among miRNAs, miR-138 has been reported to be involved in osteogenesis[12] and regulation of the chondrocyte phenotype.[13] Additionally, miR-138 is involved in the processes of OA cartilage breakdown in response to interleukin-1 beta (IL-1β).[14] However, the exact mechanism by which miR-138-5p exerts its ECM degradation of OA chondrocytes is unknown.

Therefore, we investigated the functional role as well as the underlying mechanism of miR-138-5p in OA. In the present study, we found that the increased expression of miR-138-5p in response to IL-1β was responsible for the impaired ECM in OA chondrocytes, probably in part by targeting Forkhead Box C1 (FOCX1). Inhibition of miR-138-5p may have potential therapeutic applications.

Materials and Methods

Specimen selection. Between April 2014 and May 2015, human OA articular cartilage was obtained from 16 patients with OA (nine males and seven females, at a mean age of 66.5 years, 57 to 76) who underwent total knee arthroplasty. OA was diagnosed according to the criteria of clinical and radiological evaluations published by the American College of Rheumatology (ACR).[15] Normal articular knee cartilage was harvested from six patients (aged 43 years, 24 to 67) who underwent amputation as a result of trauma. These patients had no previous history of OA or other joint disorders. This study was approved by our hospital medical ethics committees, and informed consent was obtained from all patients.

Human chondrocytes isolation and culture. After collection of the cartilage specimens, the specimens were cut into small pieces. The small pieces were then predigested in 0.1% trypsin (Invitrogen, Grand Island, New York) for 30 minutes and digested in 1.5 mg/ml collagenase type II (Sigma Chemical Co., St Louis, Missouri) for 16 hours at 37°C. The suspension was passed through a 100 μm nylon cell strainer (Falcon, Becton Dickinson Labware GmbH, Heidelberg, Germany), and then washed with Dulbecco's modified Eagle's medium (DMEM, Invitrogen). The isolated cells were seeded in T-75 culture flasks in DMEM containing 10% foetal bovine serum (FBS, Invitrogen), 100 units/mL penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen), in a humidified incubator containing 5% CO_2 at 37°C. The culture medium was replaced every three to four days.

Transient transfection. OA chondrocytes at 70% to 80% confluence were used in the experiment. Briefly, the chondrocytes were seeded in 48-well plates prior to the transfection. The miR-138-5p mimics (50 nM), allele-specific oligonucleotide (ASO) -miR-138-5p (150 nM), or pcDNA3.1 (+)-FOXC1 (50 nM), and their negative controls, were transfected into OA chondrocytes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The miR-138-5p mimics, ASO-miR-138-5p, or pcDNA3.1 (+)-FOXC1, and their negative controls, were designed and produced by GenePharma (Shanghai, China). Twenty-four hours later, OA chondrocytes were starved for 12 hours and then were treated with IL-1β (R&D Systems, Minneapolis, Minnesota) at a concentration of 5 ng/mL for 24 hours. The cells were then harvested and the cell lysates were prepared.

Luciferase reporter assay. The 3'-UTRs sequence of wild-type (WT) or mutated (Mut) FOXC1 were amplified by polymerase chain reaction (PCR)- and linked into the psiCHECK vector (GenePharma) at 70% to 80% cell confluence. HEK293 cells were seeded in 48-well plates and were co-transfected with the described luciferase reporter constructs (500 ng), miR-138-5p mimics and ASO-miR-138-5p using Lipofectamine 2000. After 48 hours of transfection, firefly and renilla luciferase activities were measured consecutively by using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Tests were carried out in duplicate.

Target prediction. We screened the potential target gene of miR-138-5p using TargetScan 6.2[16] (http://www.targetscan.org) and/or microRNA.org.[17] (http://www.microrna.org).

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from both cartilage tissues and chondrocytes using TRIzol (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. MiRNAs were isolated using the mirVana miRNA isolation kit (Ambion Ltd, Cambridgeshire, United Kingdom). For analysis of miRNAs expression, reaction was carried out at 37°C for 60 minutes and 70°C for ten minutes. For analysis of mRNA expression, reaction was performed at 42°C for 60 minutes and 70°C for ten minutes. qRT-PCR was performed on the Prism 7500 real-time PCR system (Applied Biosystems, Foster City, California) by using standard SYBR green assay protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal loading controls for mRNA and miRNA, respectively. Data were analysed using the $2^{-\Delta\Delta CT}$ method.

Western blot. Protein was extracted from chondrocytes after transfection. The protein concentration was assessed by using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calfornia). The samples were subjected to 10% to 12% sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE), and then transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, United Kingdom). Membranes were blocked with 5% non-fat dried milk in Tris buffered saline with Tween (TBST) for two hours and probed with anti-FOXC1 antibody (ab55178, Abcam, Cambridge, Massachusetts) at 4°C overnight. GAPDH was used as an internal control. The blots were then washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody (Sigma) at room temperature for two hours. The blots were visualised using enhanced chemiluminescence (Amersham Pharmacia Biotech Inc, Piscataway, New Jersey). The gray value of the bands was analysed by Image-pro plus 6.0 software (Media Cybernetics, Inc., Rockville, Maryland).

Proteoglycan analysis. The chondrocytes were digested in 0.5% (v/v) papain solution (Sigma) at 65°C for three hours. The concentrations of sulfated glycosaminoglycans (GAGs) were determined using the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor Ltd, Carrickfergus, United Kingdom) according to the manufacturer's instructions. GAGs content was normalised to the DNA content. Absorbance at 525 nm was measured by SpectraMax M2 microplate spectrofluorometer (Molecular Devices, LLC, Sunnyvale, California).

Enzyme-linked immunosorbent assay (ELISA). The levels of MMP-13 secreted by chondrocytes in the culture supernatants were evaluated by using commercially available ELISA kits (R&D Systems) following the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (VersaMax Tunable Microplate Reader; Molecular Devices).

Statistical analysis. Data are expressed as the mean and standard deviation (SD). Statistical analyses were conducted using Statistic Package for Social Science (SPSS, version 18.0, SPSS Inc., Chicago, Illinois) statistical software. Student's *t*-test was performed to determine statistical comparisons. A value of $p < 0.05$ was considered significant unless otherwise described.

Results

miR-138-5p is increased in OA cartilage and in chondrocytes in response to IL-1β. To explore the potential functional role of miR-138-5p in the development of OA, we examined its expression levels in normal and OA articular cartilage, as well as in chondrocytes. As shown in (Fig. 1), the results showed that compared with the normal groups without OA, the expression levels of miR-138-5p were significantly upregulated in both OA articular cartilages tissues and in chondrocytes (both $p < 0.01$). Next, the expression level of miR-138-5p in IL-1β-stimulated human chondrocytes was determined. As expected, the expression levels of miR-138-5p were significantly elevated in normal ($p < 0.05$) and OA chondrocytes ($p < 0.01$) treated by IL-1β (Fig. 1c).

Alteration of miR-138-5p affects IL-1β-induced inhibition of ACAN and COL2A1 synthesis in OA chondrocytes. To investigate whether miR-138-5p influences the IL-1β-induced matrix degradation, we transfected either miR-138-5p mimics or ASO-miR-138-5p into the OA chondrocytes and then stimulated the cells with IL-1β. As indicated in Figures 2a and 2b, the expression levels of ACAN and COL2A1 were significantly downregulated by IL-1β stimulation (both $p < 0.01$). Overexpression of miR-138-5p further significantly aggravated the IL-1β-induced decrease of ACAN and COL2A1 mRNA expression (both $p < 0.05$). However, suppression of miR-138-5p statistically inhibited IL-1β-induced downregulation of ACAN and COL2A1 expression.

The effect of miR-138-5p on IL-1β-induced cartilage matrix degradation. We further analysed the effect of miR-138-5p on proteoglycan content by using a dimethylmethylene blue (DMMB) spectrophotometric analysis. As shown in Figure 2c, IL-1β stimulation led to a significant downregulation of GAGs content ($p < 0.01$). Overexpression of miR-138-5p resulted in significantly lower levels of GAGs content in OA chondrocytes stimulated with IL-1β ($p < 0.01$), while suppression of miR-138-5p statistically inhibited the IL-1β-induced decrease of GAGs content ($p < 0.01$). MMP-13, one of the mammalian collagenases, plays a key role in the catabolic processes of OA by cleaving key ECM proteins such as COL2A1 and ACAN. Hence, both the mRNA and protein expression levels of MMP-13 in OA chondrocytes were examined by qRT-PCR and ELISA, respectively, after transfection with either miR-138-5p mimics or ASO-miR-138-5p followed by stimulation with IL-1β. We observed that IL-1β stimulation resulted in a significant upregulation of protein and mRNA levels of MMP-13 (both $p < 0.01$). Both the protein

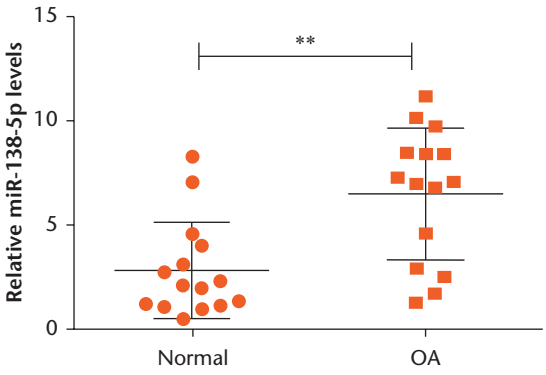

Fig. 1a

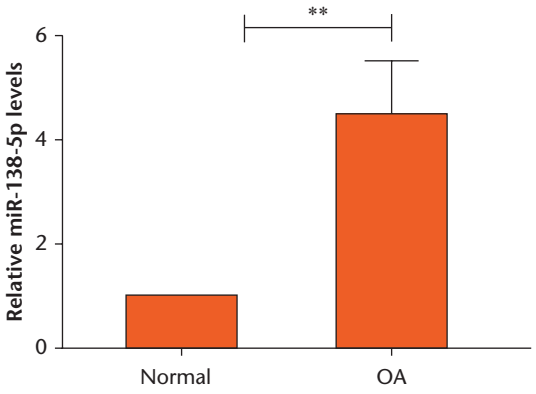

Fig. 1b

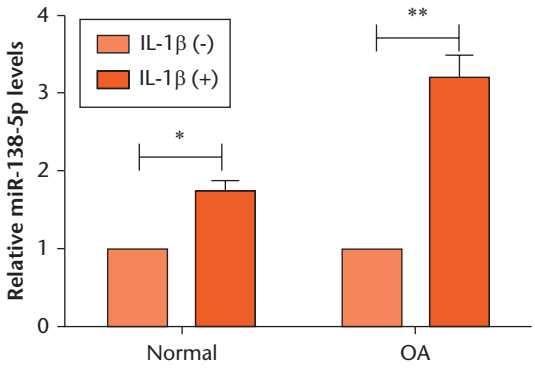

Fig. 1c

miR-138-5p is upregulated in OA cartilage and in chondrocytes in response to IL-1β; a) relative expression of miR-138-5p in normal and OA cartilage; b) relative expression of miR-138-5p in normal and OA chondrocytes; c) relative expression of miR-138-5p stimulated by IL-1β. (miR, microRNA; OA, osteoarthritis; IL-1β, interleukin-1 beta.) * $p < 0.05$; ** $p < 0.01$.

and mRNA levels of MMP-13 were further significantly increased by overexpression of miR-138-5p ($p < 0.05$ or $p < 0.01$). Conversely, inhibition of miR-138-5p reversed the effects ($p < 0.05$) (Figs 2d and 2e).

FOXC1 is a direct target of miR-138-5p. To determine how miR-138-5p exerts its ECM degradation induced by IL-1β, two algorithm programmes (TargetScan 6.2[16] and microRNA.org[17]) were performed to predict the potential target genes. We found that FOXC1, an important transcription factor (TF) playing a key role in the development of OA,[18] was a putative target gene of miR-138-5p. FOXC1 3′-UTR contains several potential binding sites of miR-138-5p (Fig. 3a). To validate whether FOXC1 is indeed regulated by miR-138-5p in chondrocytes, we generated reporter plasmids (WT-FOXC1 3′-UTR or its mutant form MUT-FOXC1 3′-UTR). The reporter assay showed that co-transfection of miR-138-5p mimics with WT-FOXC1 3′-UTR resulted in a significant suppression of luciferase activity compared with the control group ($p < 0.01$), while no significant difference could be found with co-transfection of miR-138-5p mimics with MUT-FOXC1 3′-UTR (Fig. 3b). In addition, the reporter assay showed that the luciferase activity was significantly increased by co-transfection of ASO-miR-138-5p with WT-FOXC1 3′-UTR compared with the control group ($p < 0.01$), however, there was no significant difference in the luciferase activity with co-transfection of ASO-miR-138-5p with WT-FOXC1 3′-UTR (Fig. 3c). We further explored the regulatory relationship between miR-138-5p and FOXC1. An inverse correlation between miR-138-5p and FOXC1 expression was observed (Figs 3d to 3f). Both the mRNA and protein levels of FOXC1 were significantly downregulated by overexpression of miR-138-5p ($p < 0.05$). Conversely, both the mRNA and protein levels of FOXC1 were statistically upregulated by suppression of miR-138-5p ($p < 0.01$). These results strongly suggested that FOXC1 is a direct target of miR-138-5p and that FOXC1 is negatively regulated by miR-138-5p.

Overexpression of FOXC1 reverses the effects of overexpression of miR-138-5p-mediated IL-1β-induced cartilage matrix degradation in OA chondrocytes. Although the above results suggested that miR-138-5p could directly inhibit the expression of FOXC1, it has not been confirmed that miR-138-5p-mediated IL-1β-induced cartilage matrix degradation was achieved by targeting FOXC1. Therefore, we co-transfected OA chondrocytes

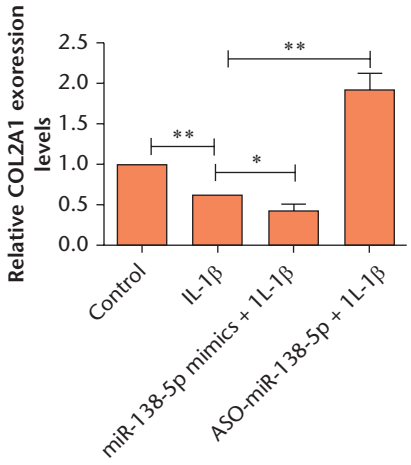

Fig. 2a

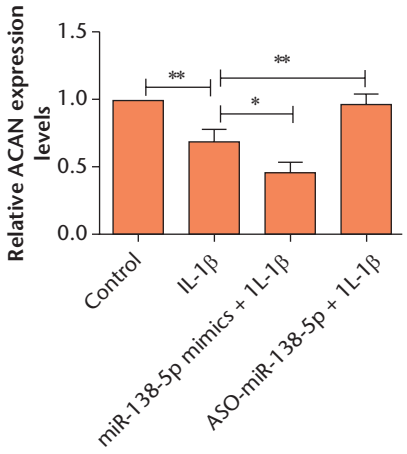

Fig. 2b

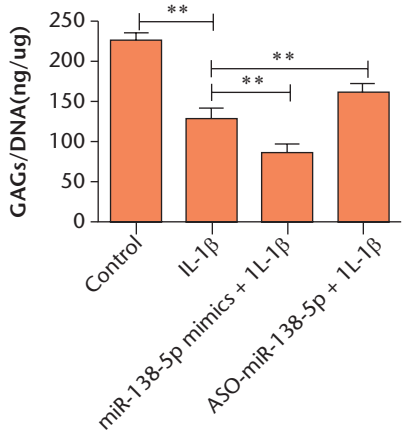

Fig. 2c

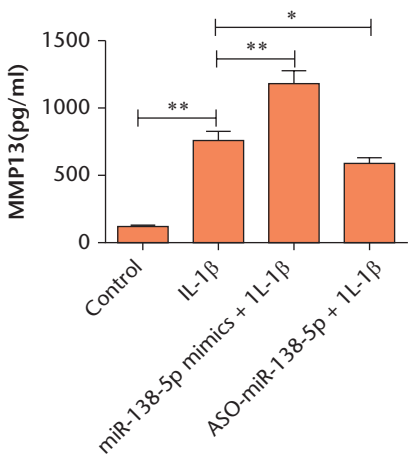

Fig. 2d

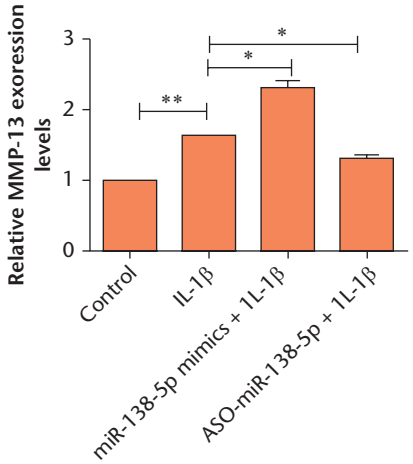

Fig. 2e

Alteration of miR-138-5p affects IL-1β-induced anabolic and catabolic metabolism in OA chondrocytes. The cells in the control group were maintained in culture media only, and the cells in other groups were treated with IL-1β alone or transfected with miR-138-5p mimics or ASO-miR-138-5p: a) relative expression of COL2A1; b) relative expression of ACAN; c) relative expression of GAGs; d) protein expression of MMP-13; e) relative mRNA expression levels of MMP-13. (miR-138, microRNA-138; OA, osteoarthritis; IL-1β, interleukin-1 beta; COL2A1, collagen, type II, alpha 1; ACAN, aggrecan; GAGs, glycosaminoglycans; MMP, matrix metalloproteinase; ASO, allele-specific oligonucleotide.) *p < 0.05; **p < 0.01.

with miR-138-5p mimics and pcDNA3.1 (+)-FOXC1, and stimulated them with IL-1β. The levels of COL2A1, ACAN, GAGs, and MMP-13 were determined. As seen in Figure 4, the results showed that co-transfection of miR-138-5p mimics and pcDNA3.1 (+)-FOXC1 resulted in higher levels of COL2A1 ($p < 0.01$), ACAN ($p < 0.01$), and GAGs ($p < 0.05$), and led to both lower protein and mRNA levels of MMP-13 ($p < 0.01$) compared with the miR-138-5p mimics group. The results indicated that overexpression of FOXC1 reversed the effects of overexpression of miR-138-5p-mediated IL-1β-induced cartilage matrix degradation.

Discussion

In the present study, our results suggest that the expression levels of miR-138-5p are significantly increased in OA cartilage and chondrocytes, as well as in response to IL-1β stimulation. The results indicate that miR-138-5p might be involved in the pathogenesis of OA. Modulation of miR-138-5p efficiently affects IL-1β-induced ECM degradation in OA chondrocytes. Inhibition of miR-138-5p could reverse IL-1β-induced lower levels of COL2A1, ACAN, and GAGs, and reverse IL-1β-induced higher levels of MMP-13. Moreover, we find that FOXC1 is directly regulated by miR-138-5p. Overexpression of FOXC1 is able to reverse the effects of overexpression of miR-138-5p-mediated IL-1β-induced cartilage matrix degradation in OA chondrocytes.

MiRNAs are small, non-coding RNAs that regulate the expression of target mRNAs. It has been well demonstrated that miRNAs are involved in various physiological and pathological conditions, including OA and inflammation.[19,20] An increasing number of studies have been performed to examine the functional role of

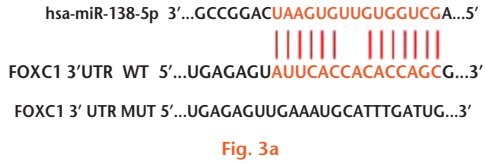

Fig. 3a

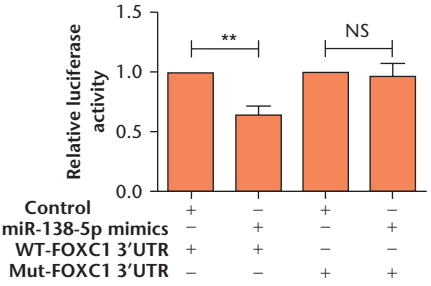

Fig. 3b

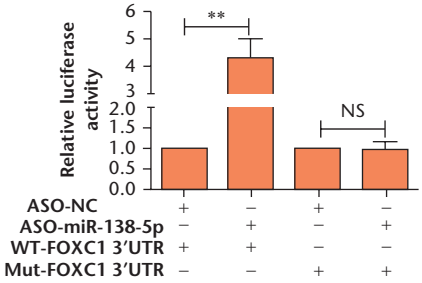

Fig. 3c

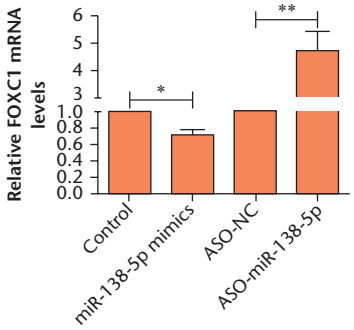

Fig. 3d

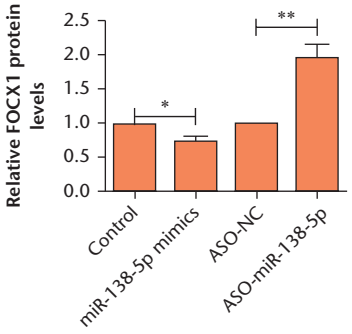

Fig. 3e

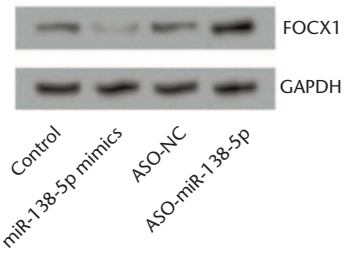

Fig. 3f

FOXC1 is a direct target of miR-138-5p and is negatively regulated by miR-138-5p. The cells in the control group were transfected with miR-138-5p mimics or ASO-miR-138-5p negative controls, and the cells in other groups were transfected with miR-138-5p mimics or ASO-miR-138-5p: a) software prediction of miR-138-5p potential binding sites on FOXC1 3′-UTR; b) relative luciferase activity in co-transfection of miR-138-5p mimics with WT or MUT-FOXC1 3′-UTR vectors; c) relative luciferase activity in co-transfection of ASO-miR-138-5p with WT or MUT-FOXC1 3′-UTR vectors; d) relative mRNA levels of FOXC1 after alteration of the expression of miR-138-5p; e) relative protein levels of FOXC1 after alteration of the expression of miR-138-5p; f) representative pictures of Western blot. (miR-138, microRNA-138; OA, osteoarthritis; FOXC1, Forkhead Box C1; UTR, untranslated regions; WT, wild type; MUT, mutant; ASO, allele-specific oligonucleotide; NC, negative control; NS, no significance.) $^{*}p < 0.05$;$^{**}p < 0.01$.

miRNAs in cartilage homeostasis modulated by IL-1β in OA chondrocytes.[21,22] IL-1β, a common inflammatory cytokine and a major catabolic inducer, has been reported to be involved in OA.[4,23-25] However, the available results are inconsistent and controversial. For example, Sandy et al[26] confirmed that the activation of matrix genes, but not IL-1β-responsive genes, played a critical role in the pathology of human OA. However, many results suggested that IL-1β was a significant modulator in cartilage degradation and IL-1β-induced cartilage degradation was involved in the regulation of miRNAs. Yang et al[6] suggested that miR-145 plays a significant role in IL-1β-induced cartilage degradation probably by targeting mothers against decapentaplegic homolog 3 (Smad3). Zhang et al[14] suggested that miR-502-5p might protect IL-1β-induced chondrocyte injury by targeting tumour necrosis factor receptor-associated factor 2 (TRAF2) and inhibiting the nuclear factor-κB (NF-κB) signalling pathway. Silencing of miR-101 exhibits protective effects on IL-1β-induced ECM degradation in chondrocytes by directly targeting the SRY (Sex Determining Region Y)-Box 9 (Sox9).[27] miR-558 regulates IL-1β-induced catabolic effects in human articular chondrocytes by directly targeting cyclooxygenase-2 (COX-2).[28] However, very little information is available regarding miR-138-5p on IL-1β-induced catabolic effects in human articular chondrocytes.

In this study we evaluated the expression pattern of miR-138-5p in normal and OA cartilage. The data indicated that miR-138-5p was upregulated in OA cartilage compared with normal samples. We then cultured chondrocytes and verified that miR-138-5p is increased in OA chondrocytes, and in response to IL-1β. Our results suggest that the abnormal overexpression of miR-138-5p in OA chondrocytes in response to IL-1β may be, at least in part, responsible for the imbalance of anabolic-catabolic effects in OA cartilage. MiR-138-5p functioned as a positive effector in response to IL-1β, promoting ECM degradation of OA cartilage. Overexpression of miR-138-5p

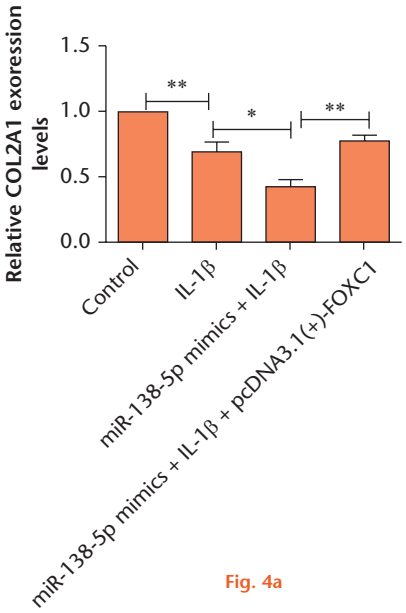

Fig. 4a

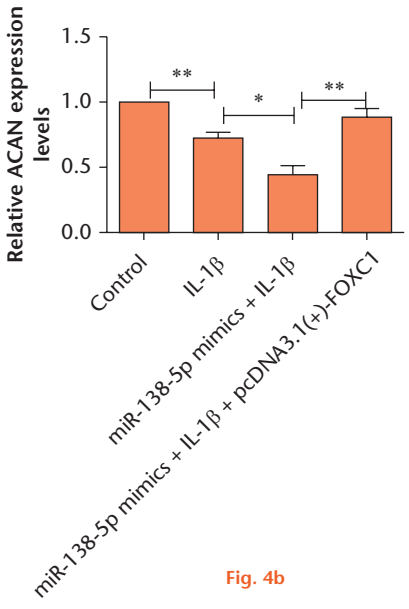

Fig. 4b

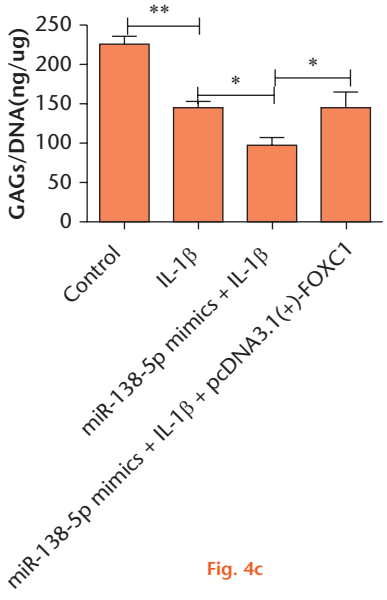

Fig. 4c

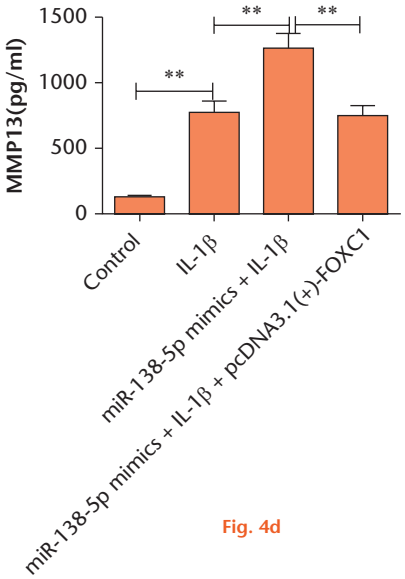

Fig. 4d

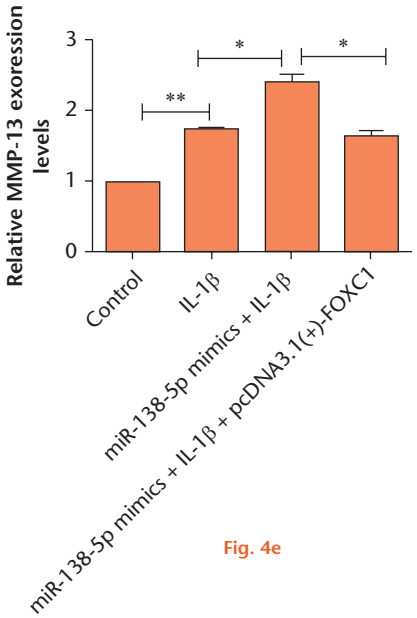

Fig. 4e

FOXC1 reverses the effects of miR-138-5p-mediated IL-1β-induced matrix degradation. The cells in the control group were maintained in culture media only, and the cells in other groups were treated with IL-1β alone or with miR-138-5p mimics or combined with pcDNA3.1 (+)-FOXC1. a) relative expression of COL2A1; b) relative expression of ACAN; c) relative expression of GAGs; d) protein expression of MMP-13; e) relative mRNA expression levels of MMP-13. (miR-138, microRNA-138; FOXC1, Forkhead Box C1; IL-1β, interleukin-1 beta; COL2A1, collagen, type II, alpha 1; ACAN, aggrecan; GAGs, glycosaminoglycans; MMP, matrix metalloproteinase; ASO, allele-specific oligonucleotide.) *p < 0.05; **p < 0.01.

increased IL-1β-induced downregulation of ACAN, COL2A1, and GAGs, and further increased IL-1β-induced upregulation of MMP-13. Reverse results were obtained by inhibition of miR-138-5p. Our results were similar to that of Seidl, Martinez-Sanchez and Murphy[13] who suggested that miR-138-5p is able to inhibit the expression of the major cartilage ECM component, COL2A1, by directly targeting specificity protein (Sp)-1 and hypoxia-inducible factor 2α (HIF-2α). Sp-1 and HIF-2α are two important TFs that are essential for COL2A1 transcription. To further explore the underlying mechanism regarding miR-138-5p on ECM degradation of OA cartilage, we used bioinformatic analyses to search for potential target genes of miR-138-5p. We found that miR-138-5p may directly target FOXC1 mRNA because FOXC1 contains potential binding sites for miR-138-5p in its 3′-UTR. FOXC1 is a member of the forkhead class of gene, which plays key roles in embryogenesis, tumorigenesis, and

regulation of tissue-specific gene expression.[29-32] In addition, it has been reported that FOXC1 plays an essential role in osteoblast differentiation.[33] Moreover, a recent study has shown that FOCX1 is involved in the development of OA.[18] In the current study, we found that FOXC1 was a direct target gene of miR-138-5p and is also negatively regulated by miR-138-5p. Our results are in line with the study conducted by Yu et al[34] in which FOXC1 was also confirmed to be one of the targeting genes of miR-138-5p in pancreatic cancer cells. The results of that study suggested that miR-138-5p might play a critical role in regulating pancreatic cancer cell growth, possibly by targeting FOXC1. However, it could not be confirmed that miR-138-5p-mediated IL-1β-induced cartilage ECM degradation was directly through targeting FOXC1. Therefore, we co-transfected miR-138-5p mimics and pcDNA3.1 (+)-FOXC1 into OA chondrocytes and then stimulated the cells with IL-1β. After transfection, the levels of COL2A1, ACAN, GAGs, and MMP-13 were re-assessed. The results showed that co-transfection of miR-138-5p mimics and pcDNA3.1 (+)-FOXC1 reversed the effects of overexpression of miR-138-5p-mediated IL-1β-induced cartilage ECM degradation by upregulating the levels of COL2A1, ACAN, and GAGs, and by downregulating the levels of MMP-13.

In conclusion, our results suggest that miR-138-5p directly modulates the expression of FOXC1, contributing to IL-1β-induced ECM degradation in chondrocytes.

References

1. **Blalock D, Miller A, Tilley M, Wang J.** Joint instability and osteoarthritis. *Clin Med Insights Arthritis Musculoskelet Disord* 2015;8:15-23.
2. **Litwic A, Edwards MH, Dennison EM, Cooper C.** Epidemiology and burden of osteoarthritis. *Br Med Bull* 2013;105:185-199.
3. **Pereira D, Peleteiro B, Araújo J, et al.** The effect of osteoarthritis definition on prevalence and incidence estimates: a systematic review. *Osteoarthritis Cartilage* 2011;19:1270-1285.
4. **Hashimoto M, Nakasa T, Hikata T, Asahara H.** Molecular network of cartilage homeostasis and osteoarthritis. *Med Res Rev* 2008;28:464-481.
5. **Sandell LJ, Aigner T.** Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 2001;3:107-113.
6. **Yang B, Kang X, Xing Y, et al.** Effect of microRNA-145 on IL-1β-induced cartilage degradation in human chondrocytes. *FEBS Lett* 2014;588:2344-2352.
7. **Baranwal S, Alahari SK.** miRNA control of tumor cell invasion and metastasis. *Int J Cancer* 2010;126:1283-1290.
8. **Bartel DP.** MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
9. **Nugent M.** MicroRNAs: exploring new horizons in osteoarthritis. *Osteoarthritis Cartilage* 2016;24:573-580.
10. **Goldring MB, Marcu KB.** Epigenomic and microRNA-mediated regulation in cartilage development, homeostasis, and osteoarthritis. *Trends Mol Med* 2012;18:109-118.
11. **Le LT, Swingler TE, Clark IM.** Review: the role of microRNAs in osteoarthritis and chondrogenesis. *Arthritis Rheum* 2013;65:1963-1974.
12. **Eskildsen T, Taipaleenmäki H, Stenvang J, et al.** MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. *Proc Natl Acad Sci U S A* 2011;108:6139-6144.
13. **Seidl CI, Martinez-Sanchez A, Murphy CL.** Derepression of MicroRNA-138 contributes to loss of the human articular chondrocyte phenotype. *Arthritis Rheumatol* 2016;68:398-409.
14. **Zhang G, Sun Y, Wang Y, et al.** MiR-502-5p inhibits IL-1β-induced chondrocyte injury by targeting TRAF2. *Cell Immunol* 2016;302:50-57.
15. **Altman R, Asch E, Bloch D, et al.** Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. *Arthritis Rheum* 1986;29:1039-1049.
16. **No authors listed.** TargetScan 6.2. http://www.targetscan.org (date last accessed 07 October 2016).
17. **No authors listed.** microRNA.org-Targets and Expression. http://www.microrna.org/microrna/home.do (date last accessed 07 October 2016).[[bibmisc]]
18. **Fei Q, Lin J, Meng H, et al.** Identification of upstream regulators for synovial expression signature genes in osteoarthritis. *Joint Bone Spine* 2016;S1297-319X00281-X.
19. **Swingler TE, Wheeler G, Carmont V, et al.** The expression and function of microRNAs in chondrogenesis and osteoarthritis. *Arthritis Rheum* 2012;64:1909-1919.
20. **Jones SW, Watkins G, Le Good N, et al.** The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-alpha and MMP13. *Osteoarthritis Cartilage* 2009;17:464-472.
21. **Díaz-Prado S, Cicione C, Muiños-López E, et al.** Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes. *BMC Musculoskelet Disord* 2012;13:144.
22. **Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A.** Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One* 2008;3:e3740.
23. **Kobayashi M, Squires GR, Mousa A, et al.** Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum* 2005;52:128-135.
24. **Daheshia M, Yao JQ.** The interleukin 1beta pathway in the pathogenesis of osteoarthritis. *J Rheumatol* 2008;35:2306-2312.
25. **Westacott CI, Sharif M.** Cytokines in osteoarthritis: mediators or markers of joint destruction? *Semin Arthritis Rheum* 1996;25:254-272.
26. **Sandy JD, Chan DD, Trevino RL, Wimmer MA, Plaas A.** Human genome-wide expression analysis reorients the study of inflammatory mediators and biomechanics in osteoarthritis. *Osteoarthritis Cartilage* 2015;23:1939-1945.
27. **Dai L, Zhang X, Hu X, Zhou C, Ao Y.** Silencing of microRNA-101 prevents IL-1β-induced extracellular matrix degradation in chondrocytes. *Arthritis Res Ther* 2012;14:R268.
28. **Park SJ, Cheon EJ, Kim HA.** MicroRNA-558 regulates the expression of cyclooxygenase-2 and IL-1β-induced catabolic effects in human articular chondrocytes. *Osteoarthritis Cartilage* 2013;21:981-989.
29. **Wang WH, McNatt LG, Shepard AR, et al.** Optimal procedure for extracting RNA from human ocular tissues and expression profiling of the congenital glaucoma gene FOXC1 using quantitative RT-PCR. *Mol Vis* 2001;7:89-94.
30. **Chung TK, Lau TS, Cheung TH, et al.** Dysregulation of microRNA-204 mediates migration and invasion of endometrial cancer by regulating FOXC1. *Int J Cancer* 2012;130:1036-1045.
31. **Huang L, Chi J, Berry FB, et al.** Human p32 is a novel FOXC1-interacting protein that regulates FOXC1 transcriptional activity in ocular cells. *Invest Ophthalmol Vis Sci* 2008;49:5243-5249.
32. **Omatsu Y, Seike M, Sugiyama T, Kume T, Nagasawa T.** Foxc1 is a critical regulator of haematopoietic stem/progenitor cell niche formation. *Nature* 2014;508:536-540.
33. **Mirzayans F, Lavy R, Penner-Chea J, Berry FB.** Initiation of early osteoblast differentiation events through the direct transcriptional regulation of Msx2 by FOXC1. *PLoS One* 2012;7:e49095.
34. **Yu C, Wang M, Li Z, et al.** MicroRNA-138-5p regulates pancreatic cancer cell growth through targeting FOXC1. *Cell Oncol (Dordr)* 2015;38:173-181.

Funding Statement
- None declared

Author Contribution
- Y. Yuan: Designed the study, Conducted experiments, Analysed data, Prepared the manuscript.
- G. Q. Zhang: Conducted experiments, Analysed data, Helped to prepare the manuscript.
- W. Chai: Conducted experiments, Analysed data, Helped to prepare the manuscript.
- M. Ni: Collected literature, Analysed data, Helped to prepare the manuscript.
- C. Xu: Collected literature, Analysed data, Helped to prepare the manuscript.
- J. Y. Chen: Designed the study, Analysed data, Prepared the manuscript.

ICMJE conflict of interest
- None declared