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# Knockdown of FOXM1 attenuates inflammatory response in human osteoarthritis chondrocytes

Run-ming Zeng[a,*], Xiao-hui Lu[a], Jing Lin[b], Jun Hu[a], Zhi-Jie Rong[a], Wei-Cai Xu[a], Ze-Wa Liu[b], Wan-ting Zeng[c]

[a] Department of Orthopedic Surgery, The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, China
[b] Department of Oncology, The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, China
[c] Division of Medicine, University College London, London WC1E 6BT, United Kingdom

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ABSTRACT

Osteoarthritis (OA) is the most common inflammatory joint disease that is mainly characterized by articular cartilage destruction. Forkhead box M1 (FOXM1) is a transcription factor that acts as a critical mediator of inflammatory response. However, the role of FOXM1 in OA has not been investigated. Interleukin (IL)-1β is a major proinflammatory cytokine, which is associated with cartilage destruction in the pathophysiology of OA. In the present study, we used IL-1β to stimulate chondrocytes for the establishment of OA in vitro model. We found that FOXM1 was up-regulated in IL-1β-induced chondrocytes. Knockdown of FOXM1 attenuated IL-1β-caused decrease in cell viability. Knockdown of FOXM1 suppressed the IL-1β-induced production of inflammatory cytokines including tumor necrosis factor (TNF)-α, and IL-6. Besides, several inflammatory mediators, such as nitric oxide (NO), prostaglandin E2 (PGE2), inducible nitric oxide synthases (iNOS), and cyclooxygenase-2 (COX-2) were also repressed by knockdown of FOXM1. FOXM1 silencing also inhibited the production of matrix metalloproteinases (MMPs) including MMP-3 and MMP-13. Furthermore, we found that knockdown of FOXM1 blocked the IL-1β-induced NF-κB activation in chondrocytes. These findings indicated that FOXM1 might play an important role in the pathogenesis of OA, suggesting that FOXM1 might be a potential therapeutic target for the treatment of OA.

## 1. Introduction

Osteoarthritis (OA) is the most common degenerative and progressive joint disease, which is characterized by articular cartilage destruction, synovial membrane inflammation, and subchondral bone remodeling [1]. OA affects around 250 million people in the world and remains a major cause of disability, resulting in limitations on quality of life and a great healthcare burden [2]. It has been demonstrated that OA is associated with a variable degree of inflammation [3,4]. A variety of cell types such as chondrocytes, osteoblasts, osteoclasts, synovial fibroblasts, adipocytes, and immune cells are involved in inflammatory and catabolic processes [5]. The inflammatory response is regulated by a complex network of proteolytic enzymes, chemokines and cytokines [5].

Among the various pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor (TNF) seem to be the main players involved in the pathophysiology of OA [1,5]. They are produced by chondrocytes, mononuclear cells, osteoblasts and synovial tissues, and then induce the production of a number of inflammatory and catabolic factors, thus initiate and propagate inflammation [1]. It has been found that IL-1β is associated with cartilage destruction, and TNF contributes to drive the inflammatory cascade [1]. Therefore, inhibition of IL-1β-induced inflammation may contribute to alleviate the cartilage tissue degradation.

Forkhead box M1 (FOXM1), a transcription factor of the Forkhead box family, is a critical mediator of inflammatory response [6,7]. Fatty Acid Binding Protein 4 (FABP4) results in airway inflammation and epithelial barrier dysfunction via ROS-activated FoxM1 [6]. Inhibition of FoxM1 suppresses the effect of FABP4 on inflammatory response and improves epithelial barrier dysfunction [6]. Besides, the lncRNA

*Abbreviations:* OA, osteoarthritis; FOXM1, Forkhead box M1; IL, interleukin; NO, nitric oxide; PGE2, prostaglandin E2; iNOS, inducible nitric oxide synthases; COX-2, cyclooxygenase-2; FABP4, Fatty Acid Binding Protein 4

* Corresponding author at: Department of Orthopedic Surgery, The First Affiliated Hospital of Shantou University Medical College, No. 57 of Changping Road, Shantou 515041, China.
*E-mail address:* runming_zeng@163.com (R.-m. Zeng).

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colorectal neoplasia differentially expressed (CRNDE) overexpression accelerates LPS-induced apoptosis and inflammation via up-regulation of FOXM1 in embryonic lung-derived diploid fibroblasts [7]. These previous studies suggest that FOXM1 plays important roles in inflammation. However, the effect of FOXM1 on inflammatory response in human OA chondrocytes remains unclear. Thus, the objective of this study was to evaluate the effect of FOXM1 on inflammatory response in chondrocytes exposed to IL-1β.

## 2. Materials and methods

### 2.1. Clinical samples

Clinical articular cartilage samples were obtained from articular joints of 12 OA patients and 12 patients with other joint diseases undergoing total knee replacement surgery at the First Affiliated Hospital of Shantou University Medical College (Shantou, China). All the procedures have been approved by the Clinical Studies Ethical Committee of the First Affiliated Hospital of Shantou University Medical College Hospital. Informed consent was obtained from each patient.

### 2.2. Primary human OA chondrocyte culture

Primary chondrocytes were isolated from human OA articular cartilage samples as described previously [8]. Briefly, cartilage samples were dissected, sequentially digested with pronase and collagenase P, and subjected to filtration. The obtained cells were centrifuged, washed with DMEM/F12, and cultured in 6-well tissue culture plates. To obtain a sufficient number of cells, cells were serially passaged. The first-passage was plated in 12-well tissue culture plate and cultured in DMEM/F12 containing 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C. For the IL-1β induction group, chondrocytes were treated with IL-1β (40 ng/ml) for 48 h.

### 2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from chondrocytes using TRIzol (Invitrogen) in accordance with the manufacturer's protocol. Then the cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The PCR was conducted using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX Connect Real-Time PCR Detection System (Bio-Rad). The results were calculated using the $2^{-\Delta\Delta CT}$ method, and the gene expression levels of FOXM1, inducible nitric oxide synthases (iNOS), cyclooxygenase-2 (COX-2) were respectively normalized to the β-actin.

### 2.4. SiRNA knockdown experiment

Chemically synthesized siRNAs targeting FOXM1 (si1-FOXM1 and si2-FOXM1) or a scramble siRNA (si-scramble) (Invitrogen) were transfected into chondrocytes with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 48 h of transfection, the cells were collected for further investigations.

### 2.5. Western blot

To extract the total cellular proteins, the cells were collected and then lysed using the RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). The nuclear and cytoplasmic proteins were isolated using the extraction kit (Beyotime), as recommended by the manufacturer. Protein concentration was measured using a protein assay kit (Bio-Rad). Then the same amounts of protein (50 μg) were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA). After blocking with 5% bovine serum albumin (BSA) for 1.5 h at room temperature, the membranes were subsequently incubated with the 1: 800 diluted primary antibodies against iNOS (1:500 dilution; Abcam, Cambridge, MA, USA), COX-2 (1:500 dilution; Abcam), p-p65 (1:500 dilution; Invitrogen), p65 (1:500 dilution; Invitrogen), IκBα (1:500 dilution; Abcam), or β-actin (1:500 dilution; Invitrogen) overnight with gentle agitation at 4 °C. The membranes were incubated with respective horseradish peroxidase (HRP)-linked secondary antibodies (1:3000 dilution; Invitrogen) for 1.5 h at room temperature. The immunoreactive bands were visualized by an ECL reagent (Thermo). The bands on the membranes were analyzed with Image J program (National Institutes of Health, NIH, Bethesda, MD, USA).

### 2.6. Cell viability assay

Cell viability was determined by MTT assay. In brief, chondrocytes were seeded into 96-well plates at a density of $2 \times 10^4$ cells per well to adhere overnight and treated with IL-1β (40 ng/ml) for 48 h. Then, each well was added with 10 μl of 5 mg/ml MTT solution and subjected to an additional 4 h incubation. Then 150 μl DMSO was added to dissolve the formazan crystals. Finally, absorbance was measured at 490 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

### 2.7. Nitric oxide (NO) measurement

The levels of NO in the supernatant of the medium were assessed by Griess method using the Nitric Oxide Assay Kit (Beyotime) as described by the manufacturer's instructions. Briefly, 50 μl of cultured medium was mixed with 100 μl of Griess reagent. After incubation for indicated time, the absorbance was measured at 540 nm using a microplate reader (Bio-Tek).

### 2.8. ELISA

The levels of prostaglandin E2 (PGE2), TNF-α, IL-6, matrix metalloproteinase (MMP)-3 and MMP-13 in the supernatant of the medium were assessed using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.9. Statistical analysis

Results of the present study are expressed as the mean ± standard deviation (SD). The statistical analysis was conducted by using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). All data were analyzed by one-way analysis of variance (ANOVA), followed by Turkey-Kramer's test for post hoc analysis. Differences were considered significant when $p < 0.05$.

## 3. Results

### 3.1. FOXM1 levels were significantly up-regulated in OA articular tissues and IL-1β-induced chondrocytes

Firstly, the mRNA levels of FOXM1 in articular tissues of 12 OA patients and 12 patients with other joint diseases were measured using qRT-PCR analysis. The results in Fig. 1A showed that FOXM1 expression was markedly increased in OA articular tissues. Then we evaluated the FOXM1 expression in IL-1β-induced primary chondrocytes. As shown in Fig. 1B and C, the expressions of FOXM1 at both mRNA and protein levels were significantly up-regulated after IL-1β stimulation in chondrocytes.

### 3.2. Knockdown of FOXM1 inhibited IL-1β-induced reduction in cell viability

To further investigate the role of FOXM1 in vitro, the chondrocytes were transfected with si-FOXM1. The results of qRT-PCR and Western

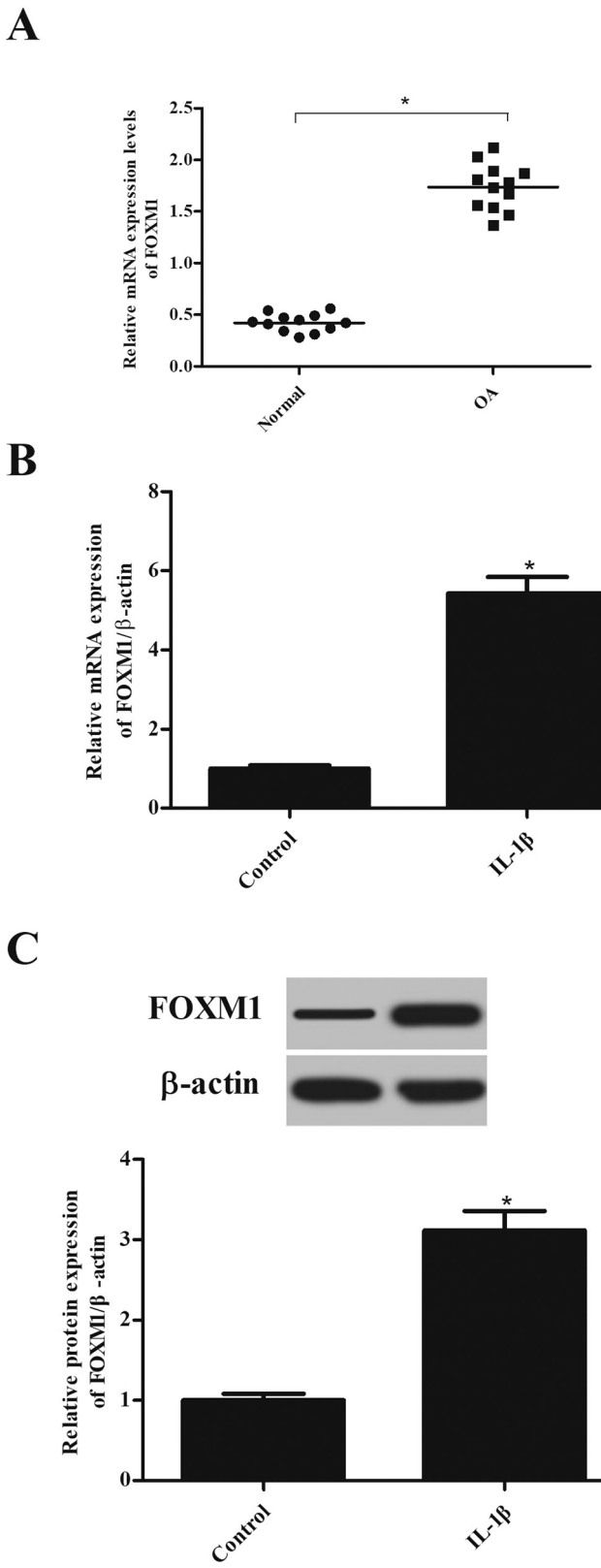


**Fig. 1.** Expressions of FOXM1 were significantly increased in clinical OA articular tissues and IL-1β-induced chondrocytes. (A) The mRNA levels of FOXM1 in articular tissues of 12 OA patients and 12 patients with other joint diseases were detected using qRT-PCR. (B and C) FOXM1 levels in primary chondrocytes with or without IL-1β (40 ng/l) stimulation for 48 h. $^{*}p < 0.05$ vs. control group.

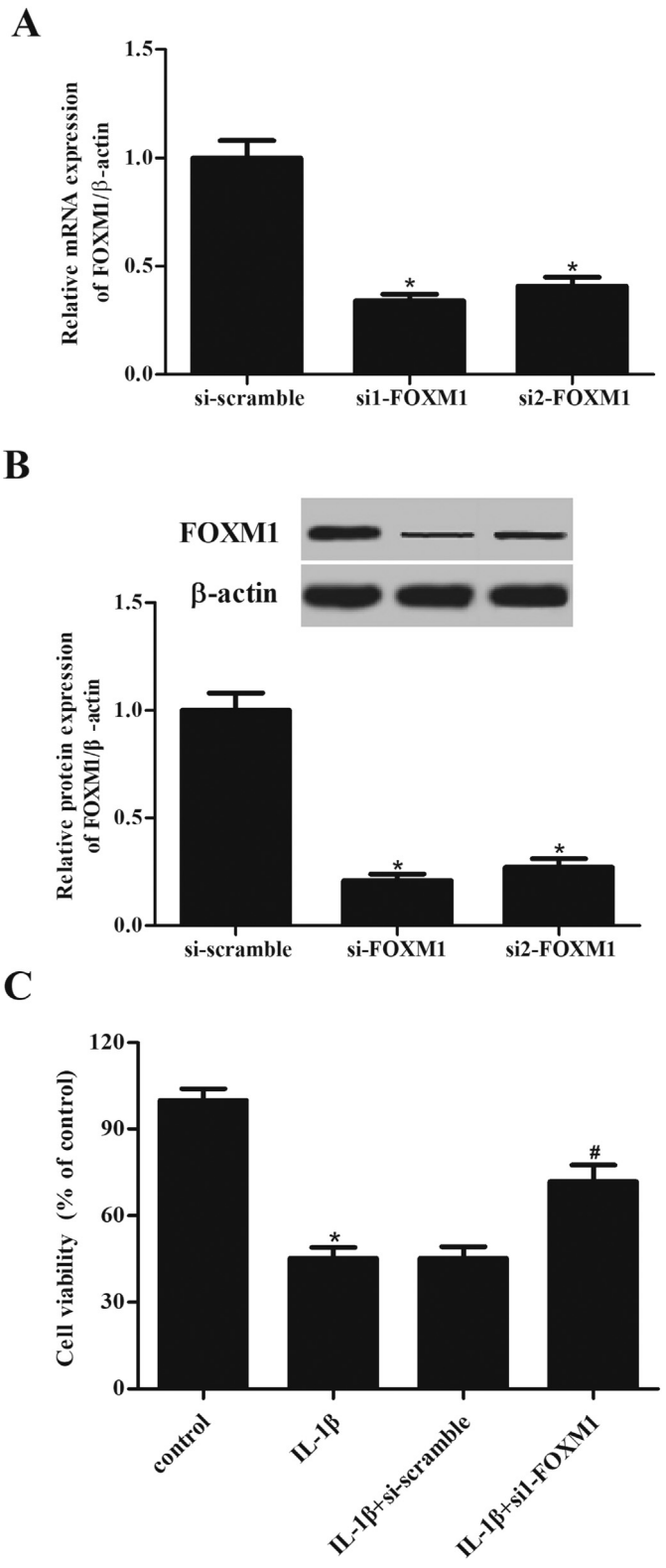


**Fig. 2.** Cell viability of IL-1β-induced chondrocytes was improved by knockdown of FOXM1. To knockdown FOXM1 in chondrocytes, cells were transfected with siRNAs-FOXM1. (A) The qRT-PCR was performed to evaluate mRNA levels of FOXM1. (B) Western blot was conducted to detect the protein levels of FOXM1. $^{*}p < 0.05$ vs. si-scramble group. (C) MTT assay was carried out to determine cell viability. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. IL-1β-induced chondrocytes.

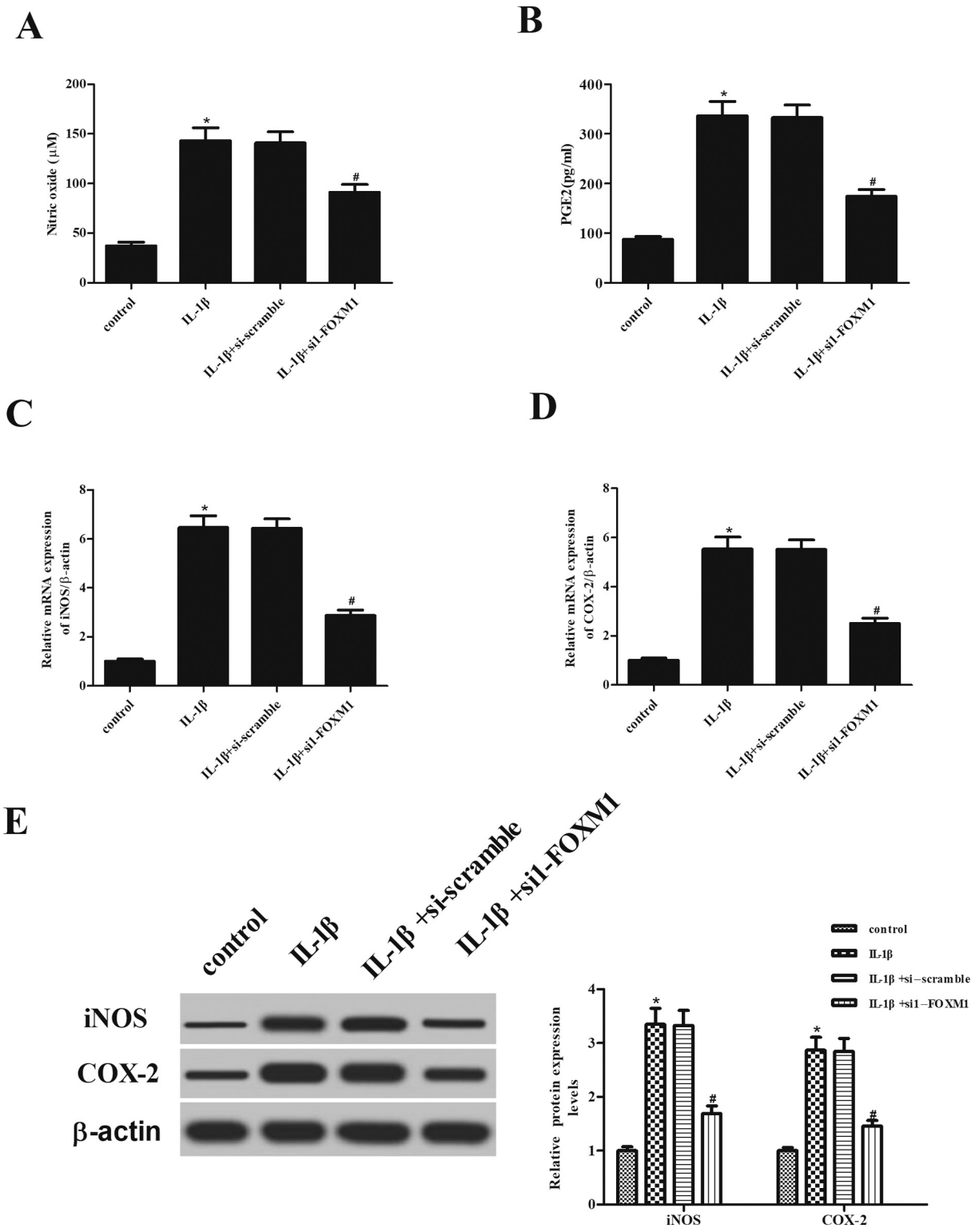


**Fig. 3.** NO and PGE2 production, and as well as iNOS and COX-2 expression in IL-1β-induced chondrocytes were suppressed by knockdown of FOXM1. Chondrocytes were transfected with si1-FOXM1 or si-scramble, and then stimulated with IL-1β (40 ng/ml) for 48 h. (A) Griess method was performed to examine NO level. (B) ELISA was carried out to determine the PGE2 level. (C and D) The qRT-PCR was performed to evaluate mRNA levels of iNOS and COX-2. (E) Western blot was conducted to detect the protein levels of iNOS and COX-2. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. IL-1β-induced chondrocytes.

blot indicated that the FOXM1 expression was markedly decreased after si-FOXM1 transfection. The FOXM1-silencing induced by siRNA1 was greater than that of siRNA2. We therefore concluded that siRNA1 transfection was able to silence FOXM1 expression (Fig. 2A and B). Then we evaluate the effect of si-FOXM1 on cell viability. The MTT assay showed that IL-1β caused significant decrease in cell viability of chondrocytes. The reason for this is that IL-1β could induce chondrocytes apoptosis [9,10]. However, the reduction was attenuated by si1-FOXM1 transfection (Fig. 2C).

### 3.3. Knockdown of FOXM1 suppressed the NO and PGE2 production, and as well as repressed iNOS and COX-2 expression in IL-1β-induced human OA chondrocytes

Subsequently, we evaluated the effect of si1-FOXM1 on the production of inflammation mediators including NO and PGE2. As illustrated in Fig. 3A and B, IL-1β induced NO and PGE2 production, however, the induction was mitigated by si1-FOXM1 transfection. Besides, we found that the mRNA and protein levels of iNOS and COX-2 were significantly increased in IL-1β-induced chondrocytes. However, the expressions of iNOS and COX-2 were reduced in FOXM1 knockdown chondrocytes when compared to the IL-1β-induced chondrocytes (Fig. 3C–E).

### 3.4. Knockdown of FOXM1 suppressed the production of TNF-α and IL-6 in IL-1β-stimulated human OA chondrocytes

It is well known that inflammatory cytokines such as TNF-α and IL-6 play important role in OA. Next, we determined the secretion of TNF-α and IL-6 in cultured medium. The results of ELISA proved that the IL-1β-stimulated production of TNF-α and IL-6 were significantly inhibited by knockdown of FOXM1 (Fig. 4A and B).

### 3.5. Knockdown of FOXM1 decreased production of MMP-3 and MMP-13 in IL-1β-stimulated chondrocytes

Matrix metalloproteinases (MMPs) including MMP-3 and MMP-13 are crucial for the pathogenesis of OA [5]. Subsequently, we assessed the effect of si1-FOXM1 on production of MMP-3 and MMP-13 in chondrocytes. Compared with the control cells, the production of MMP-3 and MMP-13 were obviously elevated in IL-1β-stimulated chondrocytes. However, knockdown of FOXM1 suppressed the IL-1β-induced production of MMP-3 and MMP-13 (Fig. 5A and B).

### 3.6. Knockdown of FOXM1 prevented IL-1β-induced NF-κB activation in chondrocytes

NF-κB is an important inflammation-related signaling pathway. In order to investigate the effect of si1-FOXM1 on NF-κB signaling pathway, the expressions of NF-κB p65, p-p65 and IκBα were measured using Western blot. As shown in Fig. 6A–C, IL-1β stimulation significantly increased the level of p-p65 and IκBα degradation in chondrocytes, suggesting IL-1β resulted in activation of NF-κB signaling pathway. Knockdown of FOXM1 blocked the IL-1β-induced NF-κB activation in chondrocytes.

## 4. Discussion

OA is the most common form of arthritis with the major characteristic, articular cartilage breakdown [11,12]. It is believed that inflammation is involved in the development and progression of OA, even in the early stages of the disease [4]. Proinflammatory cytokines are critical mediators of the disturbed metabolism and enhanced catabolism of joint tissue involved in OA [1]. FOXM1 is a transcription factor that has been demonstrated to be involved in inflammatory responses. IL-1β signaling has been found to play a central role in regulating chondrocyte inflammation in OA [5]. Our results revealed that IL-1β induced the expressions of FOXM1 in chondrocytes. A number of studies proved that the effect of IL-1β on chondrocytes can be generally described as anabolic and catabolic, which involves the induction of aggrecanases and MMPs, as well as the downregulation of the chondrogenic extracellular matrix (ECM) synthesis [1]. In a previous in vitro study, treatment with IL-1β reduces the expression of type II collagen, the major component of ECM, in chondrocytes [13]. IL-1β has been found to stimulate chondrocytes to release MMPs, including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1) and MMP-13

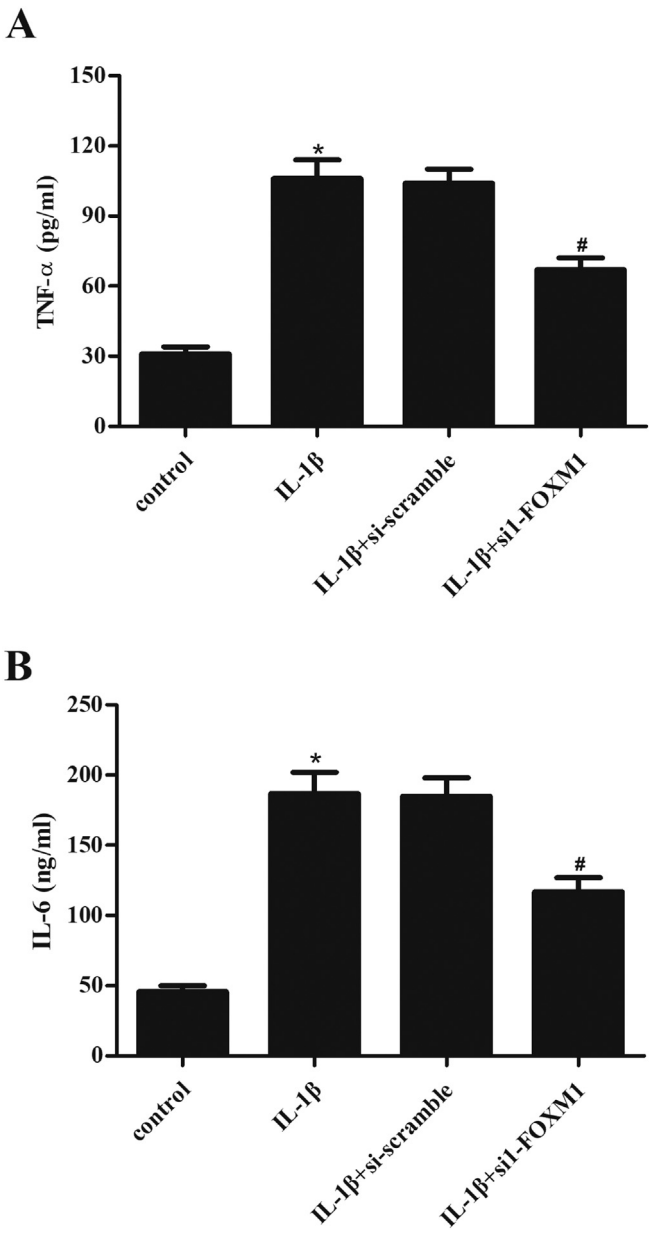


**Fig. 4.** Production of TNF-α and IL-6 in IL-1β-stimulated chondrocytes were inhibited by knockdown of FOXM1. (A and B) The levels of TNF-α and IL-6 in the cultured medium were assessed using ELISA. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. IL-1β-induced chondrocytes.

(collagenase 3), which are key regulators of cartilage destruction in OA [14]. In the present study, we found that IL-1β induced the production of MMP-3 and MMP-13 in chondrocytes.

IL-1β also has crucial effect on inflammatory responses through upregulation of further proinflammatory cytokines, such as IL-6, and chemokines such as IL-8, monocyte chemoattractant protein 1 and CC-chemokine ligand 5 [1]. Treatment of chondrocytes with these cytokines has the ability to stimulate the production of inflammatory mediators such as soluble phospholipase A2, iNOS, COX-2 and microsomal prostaglandin E synthase 1 (MPGES-1), NO and PGE2, which are implicated in OA pathology [1]. Previous studies have demonstrated that IL-1β, TNF, and IL-6 are major cytokines in OA pathophysiology owing to the magnitude of their involvement in OA. Our results showed that IL-1β induced the production of TNF-α and IL-6 in chondrocytes. Moreover, our results also denoted that IL-1β induced the expressions of iNOS and COX-2 in chondrocytes. Lim et al. [15] reported that FOXM1

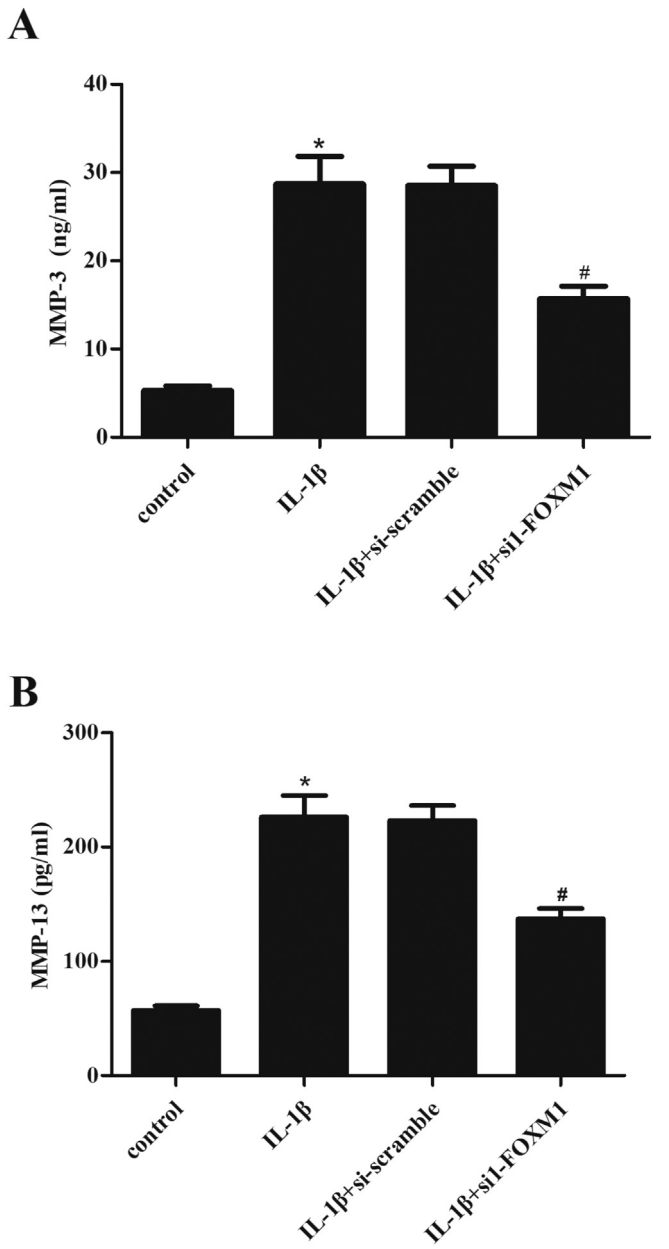


**Fig. 5.** Production of MMP-3 and MMP-13 in IL-1β-stimulated chondrocytes were decreased by knockdown of FOXM1. (A and B) The levels of MMP-3 and MMP-13 in the cultured medium were assessed using ELISA. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. IL-1β-induced chondrocytes.

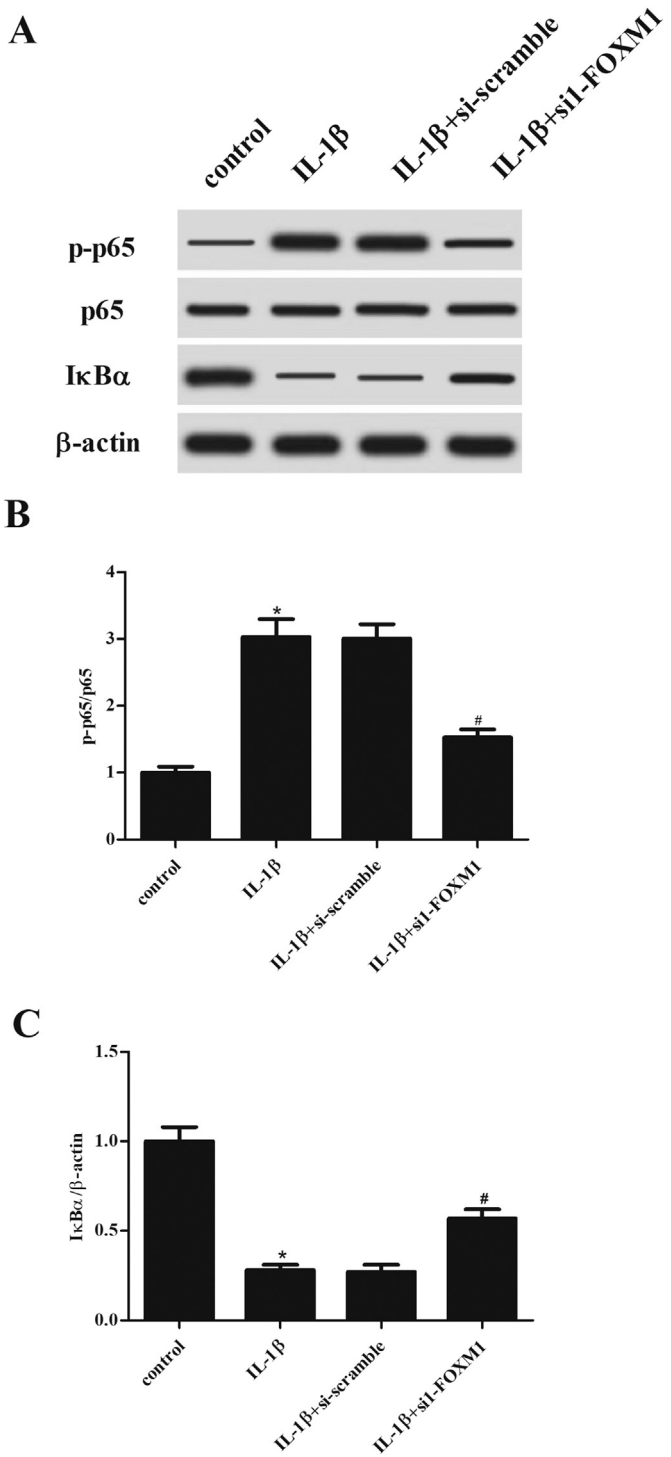


**Fig. 6.** The activation of NF-κB in IL-1β-stimulated chondrocytes was prevented by knockdown of FOXM1. (A) The expressions of NF-κB p65, p-p65 and IκBα were measured using Western blot. Quantification analysis of (B) p-p65/p65 and (C) IκBα/β-actin. $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$ vs. IL-1β-induced chondrocytes.

silencing increased IL-1β-induced production of pro-inflammatory cytokines (IL-6 and IL-8), expressions of COX-2 and MMP-9, as well as release of PGE2 and PGF2α in primary amnion cells. The current study proved that knockdown of FOXM1 suppressed the IL-1β-induced inflammatory responses, which is evidenced by the decreased production of NO, PGE2, TNF-α and IL-6, as well as the expressions iNOS and COX-2 in chondrocytes.

Increasing evidences have proven that the proinflammatory and catabolic effects of IL-1β are mediated by the activation of several signaling pathways including the p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathways, Wnt-β-catenin, most importantly, NF-κB signaling pathway [16–18]. NF-κB has been found to be activated in OA and plays crucial roles in the pathogenesis of OA [19]. Activation of NF-κB induces the expressions of several inflammatory mediators such as iNOS, COX-2 and chemokines, and also promotes the expressions of proteins in cartilage degradation including MMP-1, MMP-9, MMP-13 and a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS)-4 [19]. Therefore, the NF-κB signaling pathway is considered as a potential therapeutic target for OA treatment. Actually, several pharmacological inhibitors of NF-κB have been proven to exhibit protective properties in animal and cell models of OA [20–22]. In the current study, the NF-κB signaling pathway was markedly activated after IL-1β stimulation, while knockdown of

FOXM1 blocked the NF-κB activation in IL-1β-induced chondrocytes.

## 5. Conclusion

In summary, our work demonstrated that FOXM1 was up-regulated in IL-1β-induced chondrocytes. Knockdown of FOXM1 improved cell viability and attenuated inflammatory response in IL-1β-induced chondrocytes. The underlying mechanism of the protective effects of FOXM1 silencing on chondrocytes might be mediated by the inhibition of NF-κB activation. Our findings might provide new insight for the role of FOXM1 in the pathogenesis of OA, indicating that FOXM1 might be a potential target for the OA therapy.

## Conflict of interest

The authors declare no conflict of interest.

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