Research Paper

TGF-β1 enhances FOXO3 expression in human synovial fibroblasts by inhibiting miR-92a through AMPK and p38 pathways

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ABSTRACT

Osteoarthritis (OA) is an age-related disease marked by synovial inflammation and cartilage destruction arising from synovitis, joint swelling and pain. OA therapy that targets the synovium is a promising strategy for mitigating the symptoms and disease progression. Altered activity of the transforming growth factor-β1 isoform (TGF-β1) during aging underlies OA progression. Notably, aberrant forkhead box class O 3 (FOXO3) activity is implicated in the pathogenesis of various age-related diseases, including OA. This study explored the interaction and cross-talk of TGF-β1 and FOXO3 in human osteoarthritis synovial fibroblasts (OASFs). TGF-β1 stimulated FOXO3 synthesis in OASFs, which was mitigated by blocking adenosine monophosphate-activated protein kinase (AMPK) and p38 activity. TGF-β1 also inhibited the expression of miR-92a, which suppresses FOXO3 transcription. The suppression of miR-92a was effectively reversed with the blockade of the AMPK and p38 pathways. Our study showed that TGF-β1 promotes anti-inflammatory FOXO3 expression by stimulating the phosphorylation of AMPK and p38 and suppressing the downstream expression of miR-92a. These results may help to clarify OA pathogenesis and lead to better targeted treatment.

INTRODUCTION

Osteoarthritis (OA) is marked by synovial inflammation, cartilage destruction, joint swelling and pain. Age-associated inflammation is a key contributor to the pathogenesis of OA [1, 2], as a result of continuous mechanical wear and tear and/or age-related modifications of the cartilage matrix. Cellular apoptosis and senescence potentially also contribute to the progression of OA [3, 4].

The synovium plays an integral role in OA. The synthesis of pro-inflammatory and hydrolytic mediators by the inflamed synovium can induce cartilage erosion, which amplifies synovial inflammation, creating a vicious cycle. OA synovial cells maintain arthritic pathologies by synthesizing the inflammatory mediators and matrix degradation enzymes [5-7]. Thus, research has begun to focus on synovium-targeted therapy in the attempt to halt the progression and lessen the impact of OA symptoms [8, 9].

The homodimeric protein, transforming growth factor beta 1 (TGF-β1), binds to TGF-β type I (TGFBR1) and type II (TGFBR2) serine/threonine kinase receptors on the cell membranes of the target cells via Smad-dependent and Smad-independent pathways [6]. TGF-β1 signaling is altered during aging and is a driving force of OA progression [10, 11]. TGF-β1 concentrations differ substantially between healthy and OA joints, and activate different signaling pathways [10]. To date, the differential signaling pathways between healthy and OA joints have not been fully understood. The forkhead box class O (FOXO) transcription factors are among the major mediators that regulate cellular aging and stress resistance. The mammalian FOXO family (i.e., FOXO1, FOXO3 and FOXO4) [12] regulates the expression of genes governing oxidative defense and DNA repair enzymes, and modulates the activity of the ubiquitin–proteasome system and the autophagic/lysosomal pathway [13-16]. Aberrant FOXO activity is found in the pathogenesis of various age-related diseases, including OA [17-19].

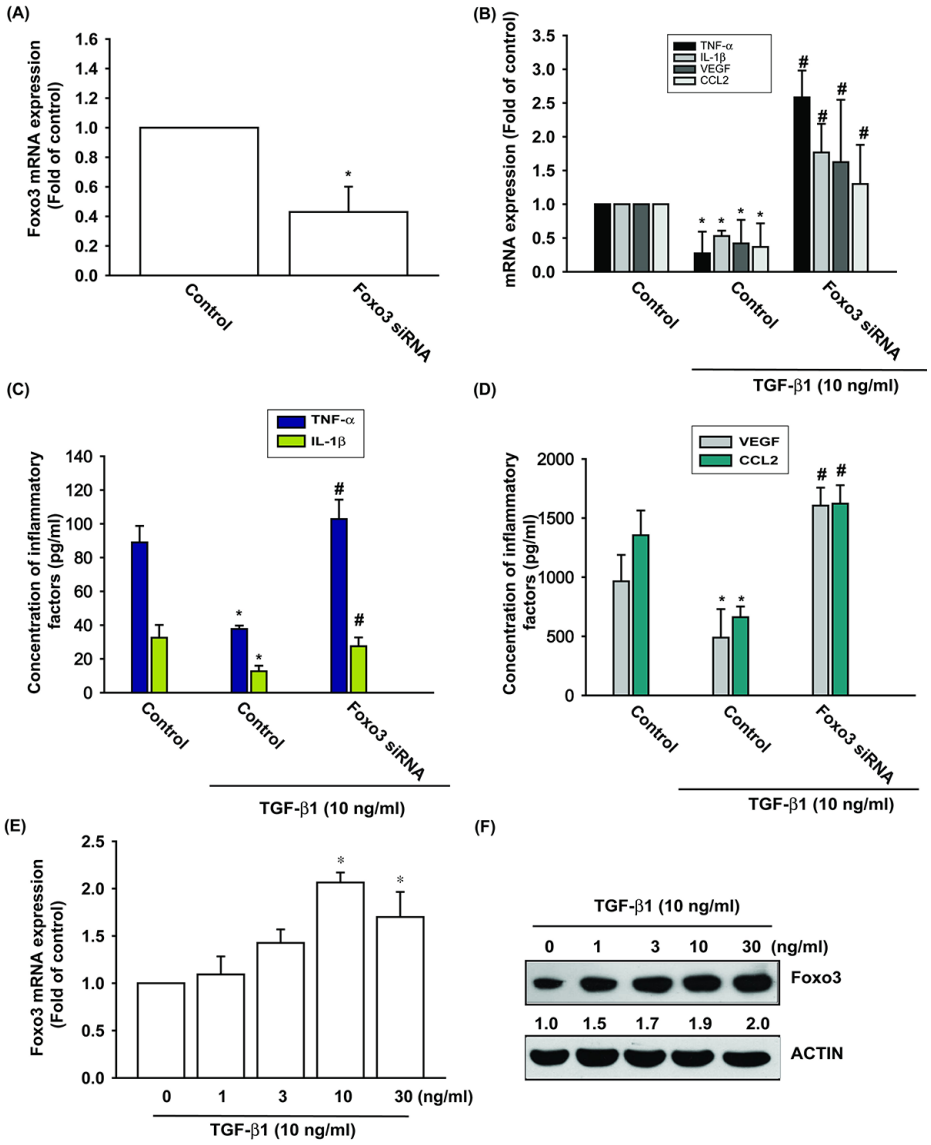

Figure 1. TGF-β1 promotes anti-inflammatory effects via FOXO3-dependent expression in OASFs. (**A**) OASFs were transfected with FOXO3 siRNA and the FOXO3 mRNA expression was examined by qPCR. (**B-D**) OASFs were transfected with FOXO3 siRNA, then incubated with TGF-β1 (10 ng/ml). The mRNA and protein levels of TNF-α, IL-1β, VEGF and CCL2 was examined by qPCR and ELISA. OASFs were incubated with 0, 1, 3, 10, and 30 ng/ml of TGF-β1 for 24 h; FOXO3 mRNA and protein expression were examined by qPCR (**E**) and Western blot (**F**). Results are expressed as the mean ± SEM. *$p < 0.05$ as compared with the control group; #$p < 0.05$ as compared with the TGF-β1-treated group.

Non-coding, single-stranded micro-ribonucleic acids (miRNAs) modulate the expression of target genes at the post-transcriptional level [20, 21]. By base pairing with the seed sequence of target mRNA molecules, the 3'-untranslated region (3'-UTR), miRNAs inhibit the expression of target genes. Various miRNAs are involved in OA pathogenesis [22, 23]. Despite TGF-β1 and FOXO3 activity being similarly involved in the pathogenesis of OA as well as the aging process, no investigations to date have evaluated any possible correlations between TGF-β1 and FOXO3. In view of the importance of synovial cells in OA pathogenesis, we explored the interaction and cross-talk between TGF-β1 and FOXO3 in human osteoarthritis synovial fibroblasts (OASFs). We hypothesized that TGF-β1 upregulates FOXO3 expression by modulating intermediate miRNA expression in OASFs.

RESULTS

TGF-β1 stimulates the expression of FOXO3 in human OASFs

We have already established that TGF-β1 promotes levels of anti-inflammatory enzyme HO-1 expression in OASFs [6]. We therefore examined the anti-inflammatory effects of TGF-β1. Stimulation of OASFs with TGF-β1 diminished the mRNA and protein expression of inflammatory mediators, including TNF-α, IL-1β, VEGF and CCL2 (Fig. 1B-D). Both TGF-β1 and FOXO3 have been shown to be involved in the pathogenesis of OA, as well as the aging process [10, 19]. However, the interplay between TGF-β1 and FOXO3 in the pathogenesis of OA and their impact on OASFs is unclear. Transfection of cells with FOXO3

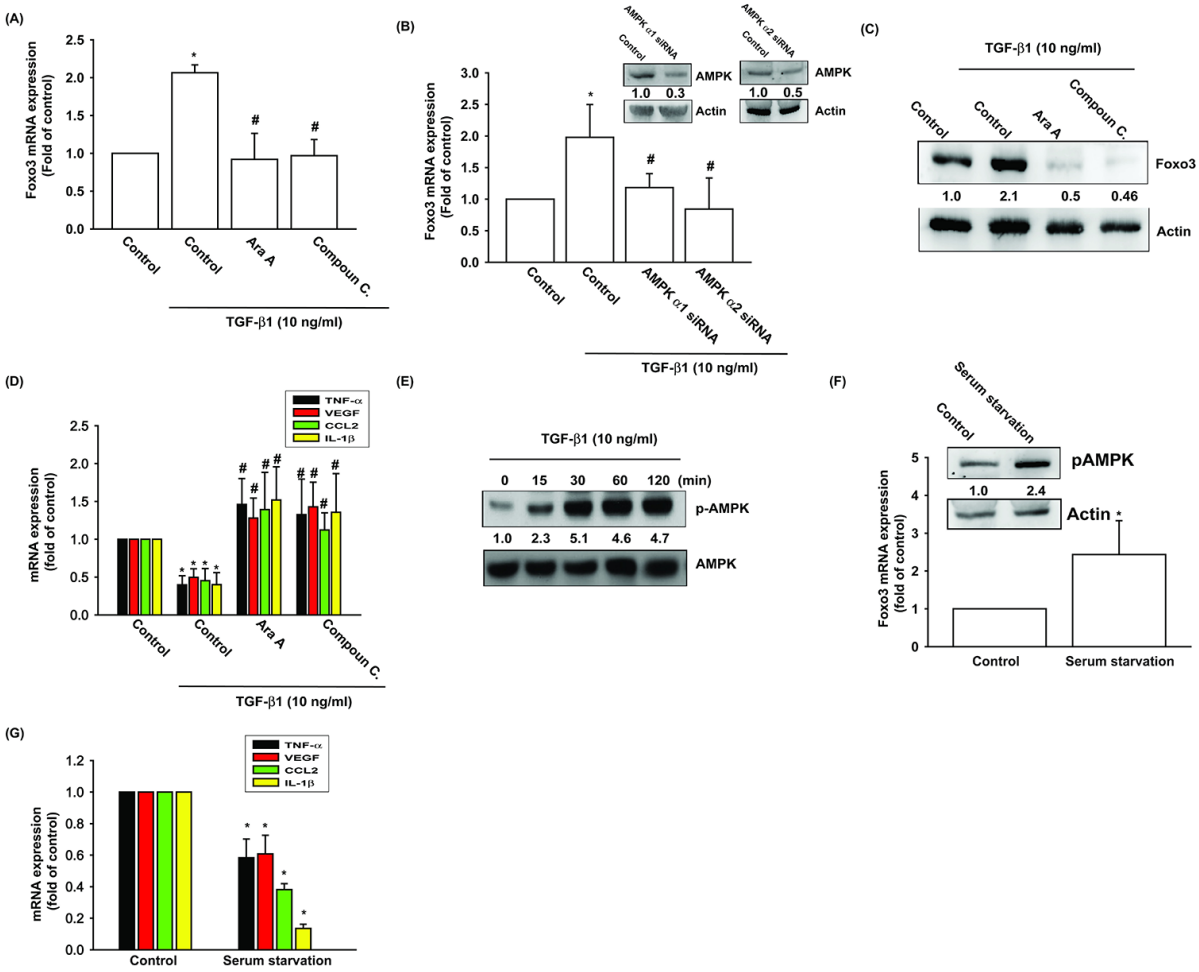

Figure 2. AMPK activation is involved in TGF-β1-induced FOXO3 synthesis. (**A-D**) OASFs were pretreated with AMPK inhibitors (Ara A and compound C) or transfected with AMPKα1 and α2 siRNAs, then incubated with TGF-β1 (10 ng/ml). The mRNA and protein levels were examined by qPCR and Western blot. (**E**) OASFs were incubated with TGF-β1 for the indicated time intervals, and the extent of AMPK phosphorylation was examined by Western blot. (**F**) Cells were serum starvation for 24 h, the AMPK phosphorylation and indicated mRNA expression were examined by Western blot and qPCR. Results are expressed as the mean ± SEM. *$p < 0.05$ as compared with the control group; #$p < 0.05$ as compared with the TGF-β1-treated group.

siRNA suppressed FOXO3 expression (Fig. 1A) and reversed TGF-β1-inhibited the mRNA and protein expression of TNF-α, IL-1β, VEGF and CCL2 (Fig. 1B-D). We also found that TGF-β1 (0–30 ng/ml) stimulated the synthesis of FOXO3 mRNA and protein in a concentration-dependent manner (Fig. 1E and 1F). According to these data, TGF-β1 promotes anti-inflammatory effects through FOXO3 expression.

TGF-β1 stimulates FOXO3 expression via the phosphorylation of AMP activated protein kinase (AMPK) and p38

The AMP activated protein kinase (AMPK) is regulated by various stimuli, including TGF-β1 [24]. To validate the role of AMPK in TGF-β1-enhanced FOXO3 production, we pretreated OASFs with AMPK inhibitors (Ara A and compound C) or transfected them with AMPK α1/α2 siRNAs. The qPCR and Western blot assay confirmed significant mitigation of TGF-β1-enhanced FOXO3 synthesis in OASFs after the administration of AMPK inhibitors and AMPK α1/α2 siRNAs (Fig. 2A-C). AMPK inhibitors also reversed TGF-β1-inhibited the expression inflammatory mediators (Fig. 2D). TGF-β1-induced stimulation of OASFs led to a time-dependent increase in the phosphorylation of AMPK, as shown by Western blot (Fig. 2E). Increasing the AMP:ATP ratio leads to activate AMPK signaling. We also found serum starvation increased AMPK phosphorylation and

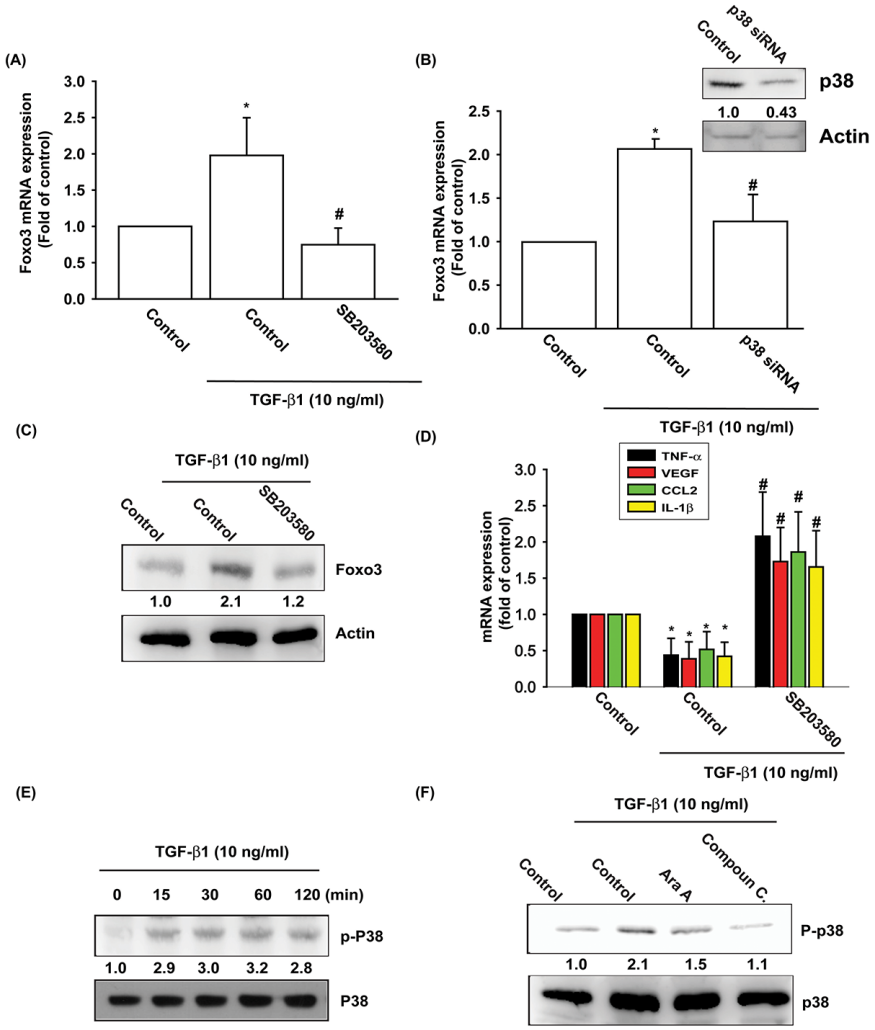

Figure 3. The p38 pathway is involved in TGF-β1-induced FOXO3 production. (**A-D**) OASFs were pretreated with a p38 inhibitor (SB203580) or transfected with p38 siRNA for 24 h, then incubated with TGF-β1 (10 ng/ml) for 24 h. The mRNA and protein levels were examined by qPCR and Western blot. (**E**) OASFs were incubated with TGF-β1 for the indicated time intervals; the extent of p38 phosphorylation was examined by Western blot. (**F**) OASFs were pretreated with AMPK inhibitors for 24 h, then incubated with TGF-β1 (10 ng/ml). The p38 phosphorylation was examined by Western blot. Results are expressed as the mean ± SEM. *$p < 0.05$ as compared with the control group; #$p < 0.05$ as compared with the TGF-β1-treated group.

FOXO3 synthesis as well as suppressed the expression of inflammatory mediators (Fig. 2F, G).

The p38, a mitogen-activated protein kinase involved in cell differentiation, aging and autophagy, regulates chondrocyte apoptosis and is involved in OA pathogenesis [25, 26]. AMPK stimulates downstream p38 activity [27, 28]. We pretreated OASFs with a p38 inhibitor (SB203580) and p38 siRNA prior to TGF-β1 administration. As shown in Fig. 3A-D, pretreatment with SB203580 or transfection with p38 siRNA significantly mitigated TGF-β1-enhanced FOXO3 synthesis and TGF-β1-inhibited the expression of inflammatory mediators. Under Western blot assay, TGF-β1 time-dependently stimulated the phosphorylation of p38 (Fig. 3E), and the TGF- β 1-induced p38 phosphorylation mitigated by AMPK inhibitors (Fig. 3F). These data demonstrate that TGF-β1 enhances FOXO3 expression through AMPK and p38 signaling pathways.

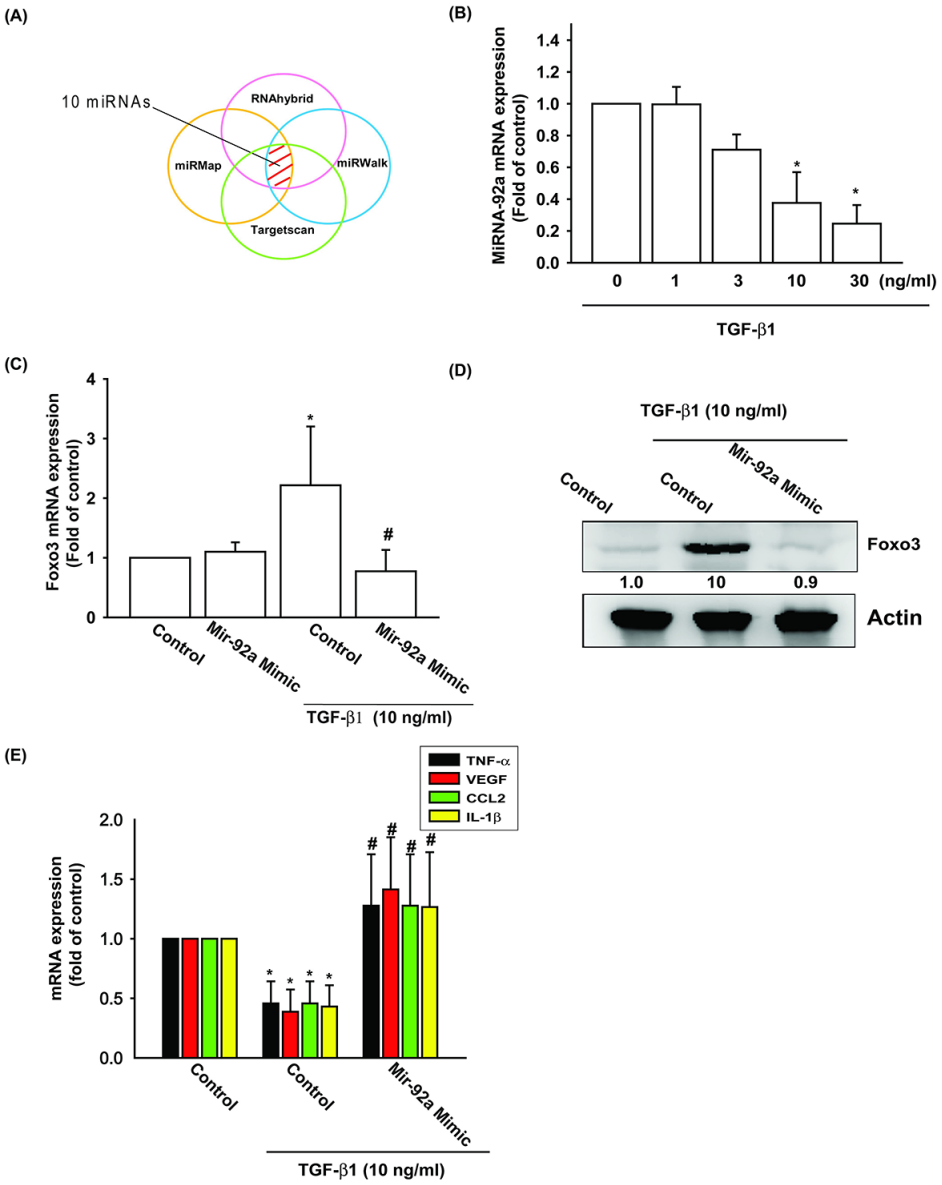

Figure 4. TGF-β1 suppression of miR-92a enhances FOXO3 production. (**A**) Open-source software (TargetScan, miRDB, and miRWalk) was used to identify which miRNAs could possibly interfere with FOXO3 transcription. (**B**) OASFs were incubated with TGF-β1 at concentrations of 0, 1, 3, 10, and 30 ng/ml. miR-92a expression levels were examined by qPCR assay. (**C-E**) OASFs were transfected with miR-92a mimic and then stimulated with TGF-β1. The mRNA and protein levels were examined by qPCR and Western blot. Results are expressed as the mean ± SEM. *$p < 0.05$ as compared with the control group; #$p < 0.05$ as compared with the TGF-β1-treated group.

TGF-β1 enhances FOXO3 expression by inhibiting miR-92a synthesis

Several miRNAs exhibit differential expression patterns between osteoarthritic and normal cartilage and are involved in the inflammatory and catabolic processes of OA [29]. However, the exact roles of miRNAs in OA pathogenesis are little understood. We used open-source software (TargetScan, miRDB, and miRWalk) to identify miRNAs that could possibly interfere with FOXO3 transcription (Fig. 4A). Among the 10 candidate miRNAs that could possibly bind to the 3'UTR region of FOXO3 mRNA, the expression level of miR-92 was most significantly suppressed after TGF-β1 administration (Supplementary Fig. S1). To confirm these findings, we compared levels of miR-92a expression in OASFs with different TGF-β1 dosages. We found that TGF-β1 (1–30 ng/ml) inhibited miR-92a expression in a concentration-dependent manner (Fig. 4B). To further determine whether TGF-β1 stimulates FOXO3 expression by inhibiting miR-92a synthesis, we transfected OASFs with the miR-92a mimic, which reduced TGF-β1-enhanced FOXO3 mRNA and protein synthesis (Fig. 4C and 4D). In addition, miR-92a mimic also antagonized TGF-β1-suppressed the expression of inflammatory mediators (Fig. 4E). These findings suggest that TGF-β1 promotes FOXO3 expression by suppressing miR-92a expression in OASFs.

We also used the luciferase reporter vector, including the wild-type 3'UTR of FOXO3 mRNA (wt-FOXO3-3'UTR) and the vector harboring mismatches in the predicted miR-92a binding site (mt-FOXO3-3'UTR), to determine whether miR-92a regulates transcription of the *FOXO3* gene (Fig. 5A). We showed that miR-92a mimic reduced TGF-β1-enhanced luciferase activity in the wt-FOXO3-3'UTR plasmid, but not in the mt-FOXO3-3'UTR plasmid (Fig. 5B and 5C). In addition, the AMPK inhibitors (Ara A and compound C) and the p38 inhibitor (SB203580) reversed TGF-β1-inhibited miR-92a expression (Fig. 5D). It appears that miR-92a directly suppresses FOXO3 transcription by binding to the 3'UTR region of the human FOXO3 mRNA, and that miR-92a expression is negatively regulated by AMPK and p38 phosphorylation induced by upstream TGF-β1 signaling.

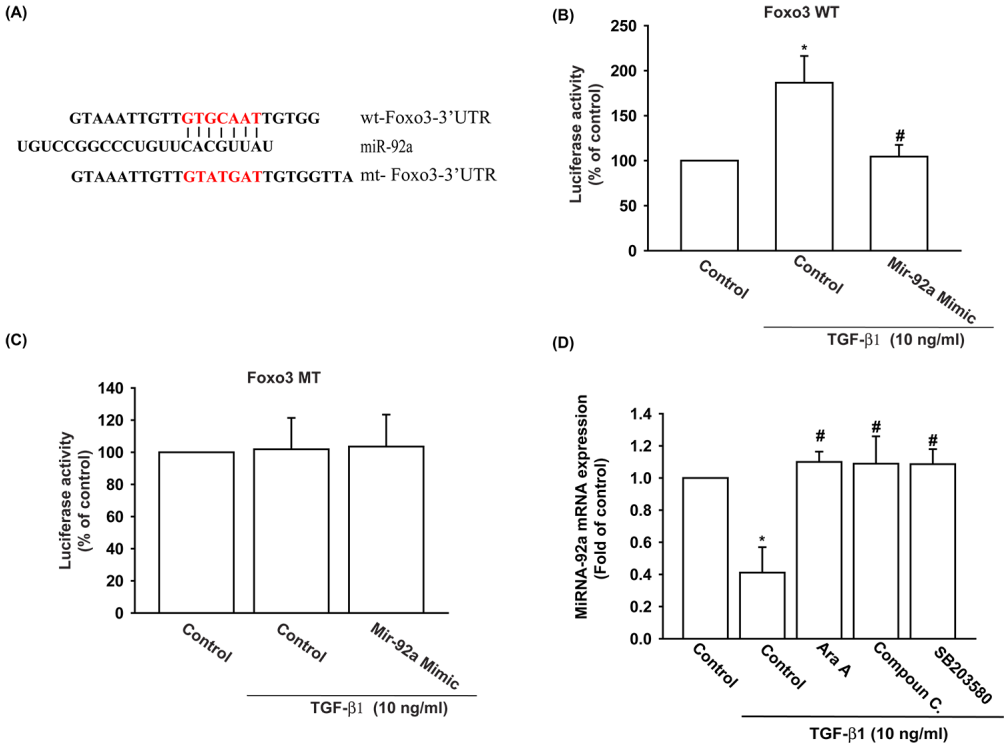

Figure 5. The binding of miR-92a to FOXO3 3' UTR mitigates TGF-β1-induced increases in FOXO3 expression. (**A**) Diagram of the miR-92a binding site in the wild-type and mutant FOXO3 3'UTRs. (**B, C**) OASFs were transfected with the wt-FOXO3-3'UTR (**B**) or mt-FOXO3-3'UTR (**C**) plasmid with or without miR-92a mimic, then stimulated with TGF-β1. FOXO3 promoter activity was expressed as the relative luciferase activity. (**D**) OASFs were pretreated with Ara A, compound C and SB203580 for 30 min, then incubated with TGF-β1 (10 ng/ml) for 24 h. The expression of miR-92a was examined by qPCR. Results are expressed as the mean ± SEM. **p* < 0.05 as compared with the control group; #*p* < 0.05 as compared with the TGF-β1-treated group.

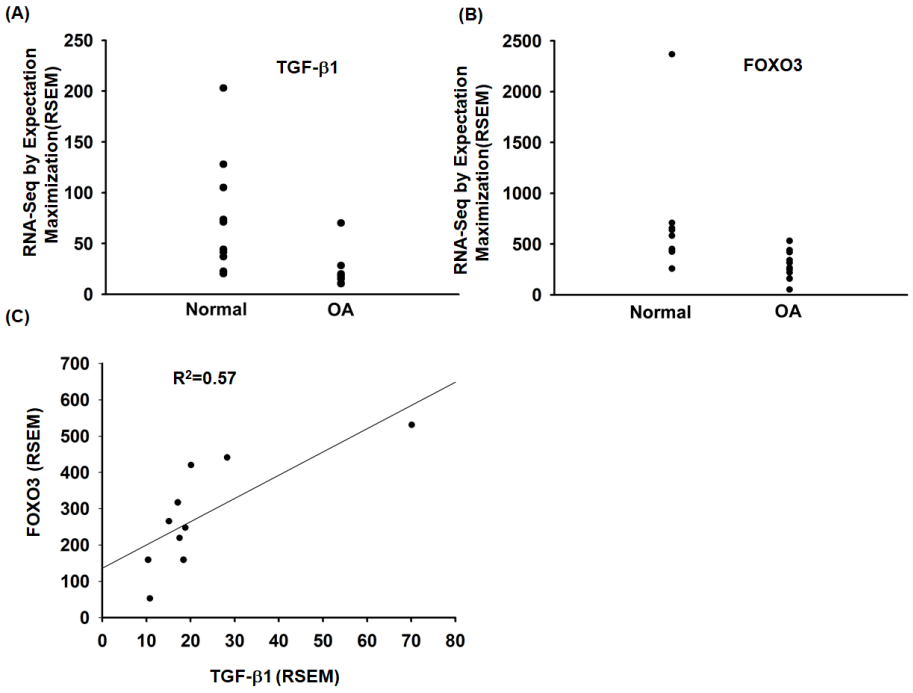

Figure 6. The TGF-β1 and FOXO3 expression in OA patients. (**A**, **B**) Expression levels of TGF-β1 and FOXO3 in paired normal and OA tissues retrieved from the GEO dataset records (GDS5403). (**C**) Correlation between TGF-β1 and FOXO3 expression levels in OA specimens retrieved from the GEO dataset.

TGF-β1 expression is positively correlated with FOXO3 expression in OA patients

To investigate the role of TGF-β1 and FOXO3 in OA patients, we analyzed the GEO database, which revealed lower levels of TGF-β1 and FOXO3 expression in OA tissue compared with normal tissue (Fig. 6A and 6B). Under immunohistochemistry staining, the expression of FOXO3 is substantially lower in OA tissue (Supplementary Fig. S2). In addition, TGF-β1 expression was positively correlated with FOXO3 expression in OA patients (Fig. 6C). Otherwise, the expression level of miR-92a was higher in OA tissue (Supplementary Fig. S3).

DISCUSSION

TGF-β1 is a pleiotropic cytokine that is pivotal in the pathogenesis of OA. TGF-β1 concentrations differ substantially between healthy and OA joints, leading to the activation of different signaling pathways [10]. TGF-β1 can mediate the activities of chondrocytes, synovial cells and subchondral osteoblasts, and thus exert a global impact on the knee [30]. In spite of the global influence of TGF-β1 in the pathogenesis of OA, the impact of TGF-β1 on OASFs has not yet been fully delineated. Our team has previously discovered that TGF-β1 stimulates the expression of HO-1 in OASFs via the stimulation of PKCα phosphorylation and suppression of the downstream expression of miR-519b [6]. We found that TGF-β1 can stimulate anti-inflammatory gene FOXO3 expression in OASFs by stimulating AMPK and p38 phosphorylation and suppressing the downstream expression of miR-92a. These results support the existing literature on OA pathogenesis.

The miRNAs are small, non-coding RNA fragments that suppress the translation of or induce the degradation of target mRNAs [31]. Many miRNAs are involved in OA pathogenesis [22, 32]. We used open-source software (TargetScan, miRDB, and miRWalk) to evaluate which candidate miRNAs can possibly interfere with the transcription of FOXO3. Among the selected miRNAs, miR-92a was suppressed to the greatest extent by TGF-β1 in a qPCR assay. We have shown that transfection of OASFs with miR-92a mimic mitigates TGF-β1-stimulated FOXO3 expression. These findings underscore the importance of miR-92a in the process of TGF-β1-stimulated FOXO3 expression.

The role of the AMPK and p38 in OA pathogenesis has been discussed previously. AMPK is a eukaryotic heterotrimeric serine/threonine protein kinase [33].

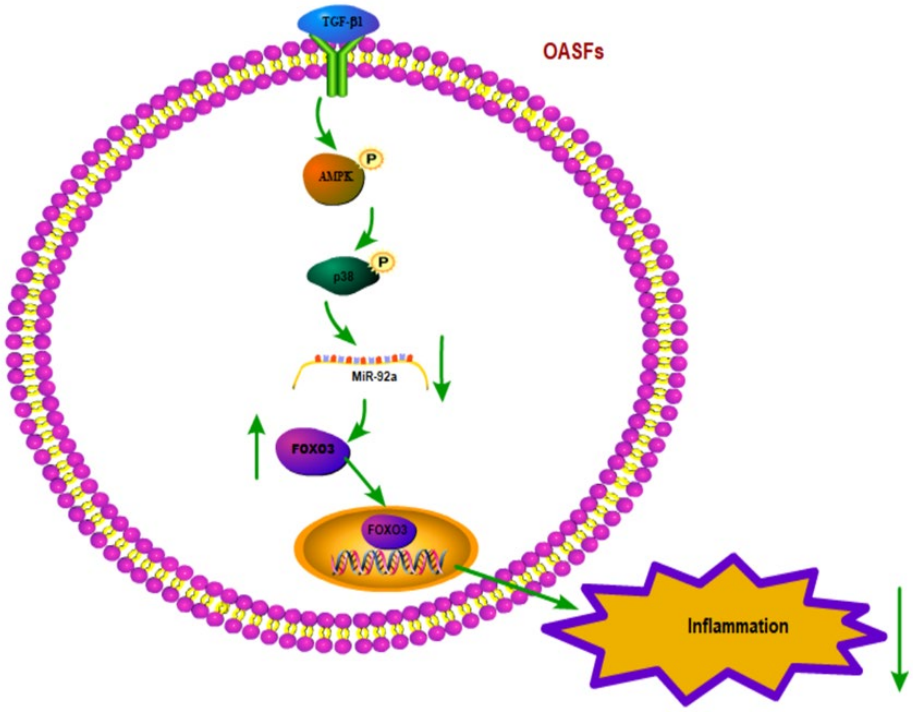

Figure 7. Schematic diagram summarizes the mechanism whereby TGF-β1 promotes FOXO3 expression in OASFs. TGF-β1 promotes anti-inflammatory FOXO3 expression in OASFs by downregulating miR-92a through the AMPK and p38 signaling pathways.

AMPK is activated by the increased intracellular AMP:ATP ratio, which enables cells to accommodate to changes in energy demands [33]. Decreased AMPK activity in articular chondrocytes as a result of aging, joint injury and chronic inflammation, leads to mitochondrial dysfunction, increased oxidative stress and matrix loss due to inflammation, as well as poor cellular quality control due to perturbed autophagy. These events typically lead to cell death and cartilage destruction, which enables the development and progression of OA [33, 34]. Our previous research revealed that the phosphorylation of AMPK and p38 is involved in the adiponectin-enhanced interleukin-6 expression in OASFs [35]. In the present study, we show that TGF-β1 stimulates FOXO3 expression via AMPK and p38 phosphorylation. Our data emphasize the importance of AMPK and p38 in OA pathogenesis. It has been reported that transcriptional and posttranscriptional regulation play key role in miRNA activation and inhibition [36]. In this study, OASFs treatment with AMPK and p38 inhibitor antagonized TGF-β1-inhibited miR-92a expression indicating TGF-β1 reduced miR-92a expression through AMPK/p38 pathway. Whether AMPK/p38 regulate miR-92a expression through transcriptional or posttranscriptional regulation is needs further examination.

CONCLUSION

In summary, our study shows that the TGF-β1 in OASFs triggers the phosphorylation of AMPK and p38 and contributes to a decline in miR-92a synthesis. The decreased miR-92a expression enhances synthesis of the anti-inflammatory gene *FOXO3* (Fig. 7). These results improve our understanding about the role of OASFs in the pathogenesis of aging and OA and may result in more effective therapy for OA patients.

MATERIALS AND METHODS

Antibodies against FOXO3, AMPK, and p38 were all purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies against p-AMPK and p-p38 were purchased from Cell Signaling (Cell Signaling, UK). ON-TARGETplus siRNAs for FOXO3, AMPKα1, AMPKα2, p38 and the control were bought from Dharmacon (Lafayette, CO, USA). Ara A, Compound C and SB203580 were supplied by Calbiochem (San Diego, CA, USA). Cell culture supplements were purchased from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase® Reporter Assay System was bought from Promega (Madison, WI, USA). The qPCR primers and probes, as well as the Taqman® one-step PCR Master

Mix, were supplied by Applied Biosystems (Foster City, CA). All other chemicals not described above were supplied by Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Synovial tissue from the suprapatellar pouch of the knee were obtained from 20 patients diagnosed as having stage IV OA. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg/mL streptomycin, as previously described [37, 38]. The protocol was approved by the Institutional Review Board (IRB) of China Medical University Hospital and all methods were carried out in accordance with the IRB's guidelines and regulations. Informed written consent was obtained from all patients.

Real-time quantitative PCR of mRNA and miRNA

Total RNA was extracted from human synovial fibroblasts by TRIzol; reverse transcription used 1 μg of total RNA transcribed into cDNA by oligo (dT) primers [39, 40]. Real-time quantitative PCR (RT-qPCR) used the Taqman® One-Step RT-PCR Master Mix. qPCR assays were then detected by StepOnePlus system.

Western blot analysis

Cell lysate was separated by SDS-PAGE electrophoresis then transferred to polyvinylidene difluoride (PVDF) membranes, according to the method described in our previous studies [39, 41]. After blocking the blots with 4% bovine serum albumin, the blots were treated with primary antibody and then secondary antibody. Enhanced chemiluminescent imaging of the blots was visualized with the UVP Biospectrum system (UVP, Upland, CA, USA).

Analysis of the gene expression omnibus (GEO) dataset

Data for normal healthy controls and OA patients (n=10 in each group) were retrieved from the GEO (accession code; GDS5403) dataset, which contained mRNA sequencing.

Plasmid construction and luciferase assays

Wild-type and mutant FOXO3 3′-UTRs were generated on the miR-92a target recognition sites (seed sequences). The wild-type 3′-UTRs of FOXO3 were cloned into the pmirGLO-luciferase reporter vector using NheI and BglII restriction sites. The primer sequences used were defined as FOXO3 forward primer: CGGCTAGCTGCGCCTTGGCTTTATAACT; the reverse primer: GGCTCGAGCCCTCCTTCACT GCTACTGG. All constructs were sequenced to verify that they contained the 3′-UTR inserts. The mutant 3′UTR region of FOXO3 mRNA (mt-FOXO3- 3′-UTR) was purchased from Invitrogen. Luciferase activity was assayed using the method described in our previous publications [42, 43].

Statistics

All values are given as the mean ± S.E.M. Between-group differences were assessed for significance using the Student's *t*-test. The statistical difference was considered to be significant if the p value was <0.05.

CONFLICTS OF INTEREST

The authors have no financial or personal relationships that could inappropriately influence this research.

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SUPPLEMENTARY MATERIAL

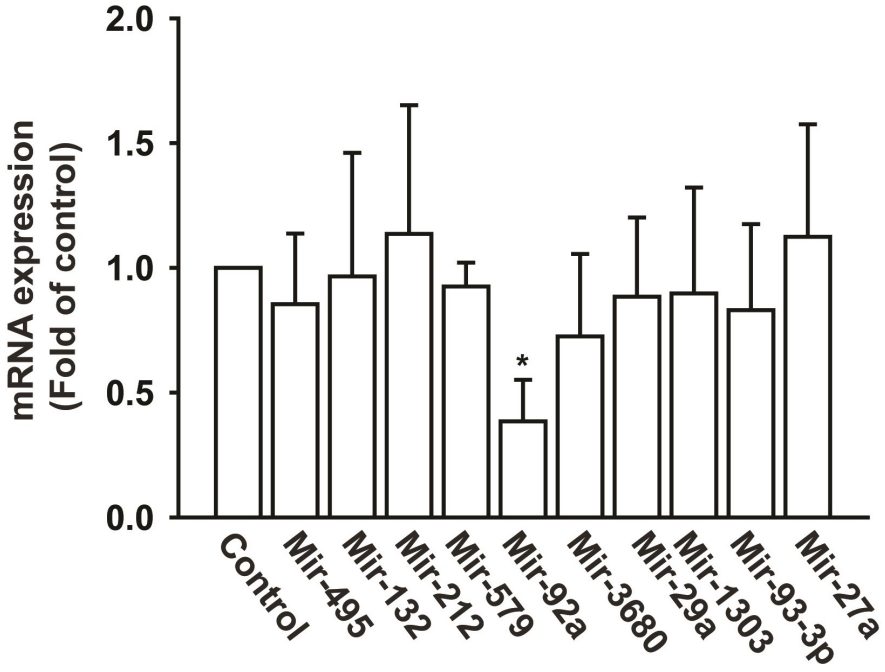

Figure S1. The TGF-β1 inhibits miR-92a expression. OASFs were incubated with TGF-β1 (10 ng/ml) for 24 h. The miRNA expression was examined by qPCR. Results are expressed as the mean ± SEM. *$p < 0.05$ as compared with the control group.

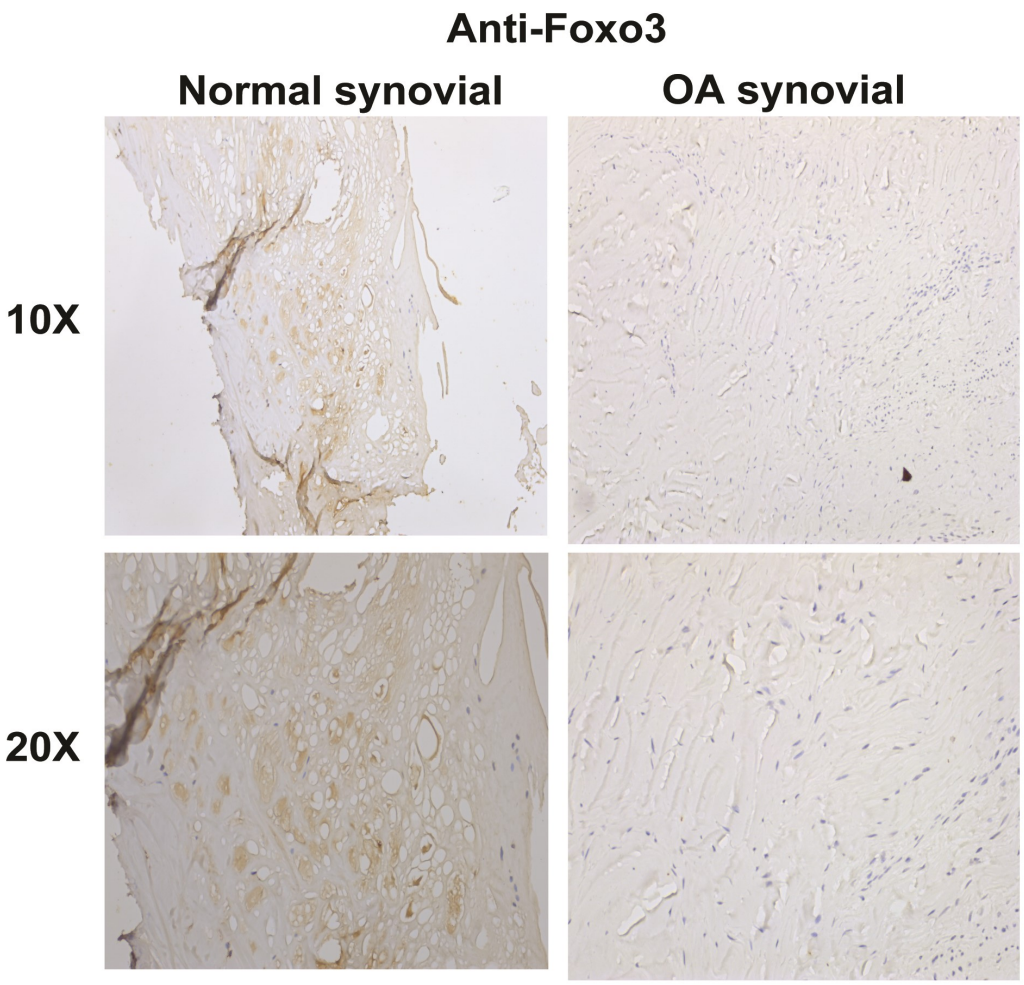

Figure S2. The FOXO3 expression in normal and OA patients. The normal and OA specimens were immunostained (IHC) with anti-FOXO3 antibody.

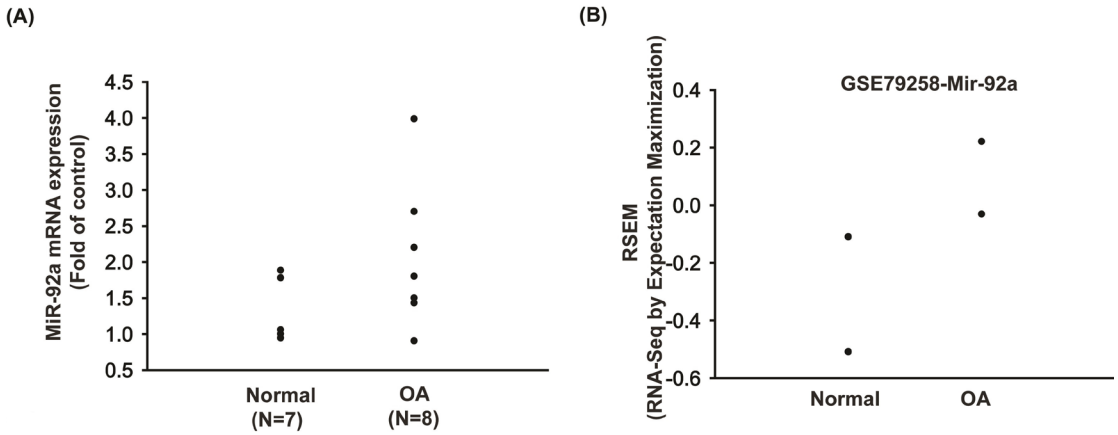

Figure S3. The miR-92a expression in normal and OA patients. (**A**) The miR-92a expression in normal and OA specimens were examined by qPCR. (**B**) Expression levels of miR-92a in paired normal and OA tissues retrieved from the GEO dataset records.