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Deacetylation of FOXO4 by Sirt1 stabilizes chondrocyte extracellular matrix upon activating SOX9

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Abstract. – OBJECTIVE: FOXO4 has essential roles in cellular metabolism and prevents cartilage degeneration in osteoarthritis (OA). Here we aim to provide evidence that deacetylated-FOXO4 stabilizes chondrocyte (CH) extracellular matrix (ECM) related to SOX9 activation.

PATIENTS AND METHODS: We used Chromatin immunoprecipitation (ChIP) and Dual-Luciferase reporter assay to verify that the FOXO4 protein activates SOX9 by binding to its promoter. We cultured human CHs with IL-1β to cause degeneration and supplied Sirt1 protein to deacetylate FOXO4. To confirm the function of FOXO4 and SOX9 during CHs degeneration, we also used the FOXO4 and SOX9 silenced CHs by siRNA transfection as a comparison. Western blot assay was used to analyze the protein level of Sirt1, SOX9, and the acetylated condition of FOXO4. Besides, RT-PCR was used to measure the mRNA level of collagen I/II/X, aggrecan, MMP-13, and ADAMTS-5 for determining the ECM states.

RESULTS: FOXO4 protein transcriptionally activates SOX9 expression by binding to its promoter. Under the IL-1β stimulation, FOXO4 acetyl-lysine rate increased, and the SOX9 protein expression decreased, which was alleviated after the supplement of exogenic Sirt1 protein. Meanwhile, Sirt1 overexpression increased the collagen II and aggrecan and reduced the collagen I, collagen X, MMP-13, and ADAMTS-5 mRNA expression. However, the silencing of FOXO4 abolished the Sirt1 induced SOX9 expression and weakened the ECM production stability. Additionally, SOX9 silencing also alleviated the effect of the Sirt1 supplement on the degenerated CHs, though the FOXO4 was highly deacetylated.

CONCLUSIONS: FOXO4 acetylation aggravates during the degeneration of CHs, and the deacetylation of FOXO4 by Sirt1 could activate the SOX9 expression and result in maintaining the ECM stability of cartilage.

Key Words:
FOXO4 deacetylation, SOX9, Osteoarthritis, Sirt1, Extracellular matrix.

Introduction

Articular cartilage is a kind of connective tissue without nerves, blood vessels, and lymph, with the function of autonomously bearing the gravity load and providing almost frictionless joint interface[1]. Osteoarthritis (OA) is the most common cause of the bone and joint system dysfunction, and its pathological features are articular cartilage damage and subchondral bone hyperplasia. The clinical manifestations of the OA patients mainly contain progressive and chronic joint swelling and pain, stiffness, and limited activity[2,3]. Due to the lack of effective methods to reverse the degradation of articular cartilage, the current conservative treatment for early OA is limited, which aims at improving pain symptoms and delaying the progression of the disease[4]. For late severe OA, artificial joint replacement is still the best method. However, surgery is accompanied by many defects, such as high cost, complications, and wear of the prosthesis. How to repair or delay the damage in the early stage of OA has become an essential topic in the research of joint field[5].

The extracellular matrix (ECM) of articular cartilage is mainly composed of type II collagen and aggrecan, and the remolding or loss of ECM is a crucial component of OA cartilage degradation[6]. With the development of OA, the type II

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collagen and aggrecan synthesized by chondrocytes (CHs) are reduced and replaced by collagen I and collagen X, which disorders the environment of cartilage[7]. Meanwhile, it is currently believed that the two main types of enzymes involved in the ECM degradation, matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), are highly activated during the aggravation of cartilage degeneration[8]. Therefore, disruption of the ECM degradation plays a meaningful role in the stability of CHs and the alleviation of OA.

As a transcription factor, FOXO protein participates in many cellular processes, such as autophagy, proliferation, aging, and apoptosis, which maintains cell biology and tissue homeostasis. FOXO4, one of the four FOXO genes in mammals, plays a decisive role in regulating cell differentiation, anti-oxidative stress, anti-inflammatory, aging, and cell apoptosis through transcriptional and signal transduction pathways[9,10]. Matsuzaki et al[11] reported that FOXO4 maintains postnatal cartilage maturation, and homeostasis protects against OA-associated damage. FOXO4 enhances its subcellular localization and transcriptional activity through phosphorylation or deacetylation[12]. Serrano et al[13] announced that the reduction of phosphorylated FOXO4 leads to the dysfunction of CHs and aggravates OA states. However, whether FOXO4 contributes to the stabilization of ECM of CHs remains unknown.

SRY-box 9 (SOX9) is one of the pivotal transcription factors for maintaining CHs phenotype and cartilage homeostasis[14,15]. It has a close relationship with the ECM metabolism of cartilage, which regulates collagen II, aggrecan, MMPs, and ADAMTSs expression[16]. Suppression of SOX9 results in chondrodysplasia and negatively regulates chondrogenesis[17]. On the contrary, SOX9 upregulation protects CHs against inflammatory injury and promotes the expressions of collagen II and aggrecan[18]. One article mentioned the dephosphorylation of FOXO4 decreased the levels of SOX9 gene and aggrecan mRNA expression[13]. However, the interaction of FOXO4 and SOX9 during the OA is uncertain. In this study, we found that FOXO4 binds to the promoter regions of SOX9, and the deacetylation of FOXO4 by Sirt1 protein supplement could amplify SOX9 expression, which contributes to the stabilization of ECM production of CHs.

Patients and Methods

CHs Source and Culture

This study was approved and registered by the Ethics Committee of the Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology. The source of the CHs was from the isolation of the knee joint cartilage from the patients undergoing distal femoral amputation caused by osteosarcoma. We recruited five patients (from 32 years to 54 years old) without OA history and visible joint degeneration. All patients signed an informed consent form before the operation. All the procedure was under the guidance of the Declaration of Helsinki.

We collected the CHs from the digestion of the cartilage. Briefly, after washing by the sterile PBS, cartilage was cut into small pieces and digested with the solution containing 0.25% trypsin and type XI collagenase (Beyotime, Shanghai, China) overnight. CHs were re-suspended in culture medium (DMEM/F12 medium containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, Gibco, Rockville, MD, USA) after centrifuge. The medium was changed every three days. For inducing CHs degradation, we cultured cells with IL-1β (10 ng/mL, R&D Systems, Minneapolis, MN, USA) for three days. Besides, the recombinant human Sirt1 protein (50 μg/mL, ab101130, Abcam, Cambridge, UK) in the medium was used to upregulate the cellular Sirt1 expression of CHs.

siRNA Transfection

The FOXO4 and SOX9 gene expression were silenced *via* siRNA transfection by Lipofectamine 2000 (Beyotime, Shanghai, China) according to the manufacturer's advice. CHs were seeded in 24-well culture plates and the Opti-MEM (Sigma-Aldrich, St. Louis, MO, USA) and siRNA targeting FOXO4 or SOX9 (Assay ID: s8836 or 106059, Thermo Fisher Scientific, Waltham, MA, USA) were added to Lipofectamine reagent. CHs were transfected with the mixture for 48 h and changed the medium.

Chromatin Immunoprecipiation (ChIP)

We used the ChIP assay to confirm FOXO4 binds to SOX9 promoter regions. We searched the 2000-bp upstream promoter region of the SOX9 from the National Center for Biotechnology Information database. We used the JASPAR core database to predict the DNA-binding sites for the FOXO4 in the SOX9 promoter. We found over ten

predicted binding sites from JASPAR and chose top four highest scores of which for verification *via* ChIP reaction (#26157, Invitrogen, Carlsbad, CA, USA). Briefly, CHs were crosslinked, quenched, and sonicated to extract the chromatin. Then, a goat anti-FOXO4 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) linked beads were used for immunoprecipitation. Normal goat IgG linked beads were used as a control. After purifying the DNA from immunoprecipitated chromatin, PCR using ChIP DNA sample as a template was performed to compare the binding efficiency with the following program: 94°C for 5 min, 40 cycles of 94°C for 15 s, 65°C for 5 s and 72°C for 5 s. The primer used for PCR was designed by Primer 5 software (Table I). The whole chromatin without immunoprecipitation was used as Input DNA templates. The amplificated DNA regions from Input, FOXO4, and IgG antibodies immunoprecipitated were imaged by agarose gels electrophoresis.

Cell Transfection and Luciferase Assays

To prove that FOXO4 promotes SOX9 expression at a transcriptional level, we used Dual-Luciferase assays to verify that FOXO4 activates SOX9 promoter expression. We designed and purchased the vector with empty or FOXO4 coding, pGL6-TA plasmid with empty or WT/Mut SOX9 promoter sequence, and pRL-Renilla plasmid from Genechem (Shanghai, China). For vector or plasmid transfection, the cells were seeded in 24-well plates and transfected using Lipofectamine 2000 (Beyotime, Shanghai, China) according to the manufacturer's advice. After 24 h transfection, firefly and Renilla Luciferase activities were finally determined by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot (WB)

We determined the protein level of Sirt1, FOXO4, and SOX9 of CHs by WB. Total protein was isolated with the radioimmunoprecipitation assay (RIPA) buffer containing inhibitors of Class I, II, and III deacetylases (Beyotime, Shanghai, China) and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) as previously described[19]. The membranes were incubated with rabbit anti-Sirt1 (Abcam, Cambridge, UK), rabbit anti-SOX9 (Abcam, Cambridge, UK), or rabbit anti-FOXO4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK) for loading control. Finally, the membranes were incubated with desired secondary antibodies and exposed in enhanced chemiluminescence (ECL) substrate (Beyotime, Shanghai, China). The measurements of the band intensities were made using Image J software (NIH, Bethesda, MD, USA).

WB Analysis of Endogenous FOXO4 Acetylation

FOXO4 was immunoprecipitated using a goat anti-FOXO4 antibody from the total protein extracted from CHs. We determined the acetylation of FOXO4 by WB using a rabbit anti-acetyl-lysine antibody (Cell Signaling Laboratory, Danvers, MA, USA). The total immunoprecipitation of FOXO4 protein expression was verified *via* blotting with a rabbit anti-FOXO4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the above description.

Real Time-PCR Analysis

Total RNA was extracted from CHs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary deoxyribose nucleic acid (cDNA) was reverse-transcribed from RNA by Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA). PCR

Table I. Predicted binding sites for FOXO4 to SOX9 promoter.

Predicted sequence	Start	End	Score	PCR primer sequences for ChIP
GTCAATA	76	82	8.17	Sense: 5'-CTGTGCAGCCTCGGAGAATC-3'; Anti-sense: 3'-GAACAGGTGGGTGACGGAA-5'
GGAAACA	307	313	7.47	Sense: 5'-TCGGGGCAGTTCTGAGTTTT-3'; Anti-sense: 3'-TGAAATCGGGCACCCACTGT-5'
AAAAACA	1426	1432	7.14	Sense: 5'-AACTGGGCCAACTTGTGAGA-3'; Anti-sense: 3'-TGTGGGAAGTAAGTAAACGA-5'
GTAAACA	1870	1876	12.71	Sense: 5'-GCCAGGACCATTCCGACAGC-3'; Anti-sense: 3'-AAACGAGGAGCACGGAACCC-5'

analysis was performed using SYBR Green Master Mix (TOYOBO, Osaka, Japan) to determine the collagen II, aggrecan, collagen I, collagen X, MMP13, and ADAMTS-5 RNA expressions were performed using Green Master. The method of $2^{-\Delta\Delta Ct}$ was used to calculate the relative gene expression by normalization to GAPDH as a calibrator sample. The primers used for PCR are listed in Table II.

Statistical Analysis

Data were analyzed by the Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) for Windows and presented as the means ± standard error of the mean (S.E.M.). The differences between the two groups were analyzed using the Student's *t*-test. A comparison between multiple groups was made using a one-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated a significant difference.

Results

FOXO4 Activates the Promoter Regions of SOX9 in CHs

We predicted that FOXO4 protein regulated the SOX9 expression *via* its DNA promoter. We searched 2000bp upstream of the promoter of SOX9 from the Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) and used the JASPAR core database (http://jaspar.genereg.net/) to predict the DNA-binding sites for the FOXO4 in the 2000 bp regions. From the several putative binding sites, we chose the four highest ratings (P1-P4) to ensure reliability (Figure 1A). To verify that FOXO4 regulated SOX9 expression in the transcriptional level, we used the ChIP assay to confirm the specific binding sites. We pulled down the cellular FOXO4 protein by the anti-Runx2 antibody linked beads from the cell lysis, and the IgG linked beads were used as a negative control. We also isolated the full DNA from the cell lysis without immunoprecipitation as a positive control. After purifying the DNA from immunoprecipitated chromatin, clear DNA was amplificated by PCR and imaged by agarose gels electrophoresis. As shown in Figure 1B, all the PCR primers could amplify the predicted promoter regions (lane 1), and no nonspecific binding site was observed (lane 3). However, only the P4 putative binding site was verified to be amplificated as SOX9 DNA regions (lane 2), which was the specific binding site (Figure 1C).

To further indicate the efficiency of the P4 site, we used a Dual-Luciferase reporter gene to verify that the overexpression of FOXO4 upregulates SOX9 expression *via* the binding site. We cloned a DNA sequence containing the WT or Mutated P4 region into the pGL6-TA plasmids (WT-pGL and Mut-pGL) (Figure 1D), which was used to transiently transfected into CHs to overexpress P4 region. The empty plasmid was used as a negative control. Besides, FOXO4 coded vector was used to upregulate the cellular FOXO4 expression, and the null-vector was used as control. The Luciferase activity was normalization to the density of pRL-Renilla plasmid (pRL). From the results of Figure 1E, we noticed that transfection with FOXO4-vector + WT-pGL significantly increased the level of Luciferase activity than which transfected with null-vector + WT-pGL plasmid. In contrast, the Luciferase activity was significantly lower when the P4 sequence was mutated even under the upregulation of FOXO4. Therefore, it is clear that P4 is the efficient cite for FOXO4 binding to the SOX9 promoter, which is the anchor for FOXO4 to activate the SOX9 expression.

Sirt1 Overexpression Deacetylates FOXO4 and Promotes SOX9 Expression

To understand the interaction of FOXO4 and SOX9 in the degenerated CHs, we cultured the

Table II. Predicted binding sites for FOXO4 to SOX9 promoter.

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
Collagen X	ATGCTGCCACAAATACCCTTT	GGTAGTGGGCCTTTTATGCCT
Aggrecan	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
ADAMTS-5	GAACATCGACCAACTCTACTCCG	CAATGCCCACCGAACCATCT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

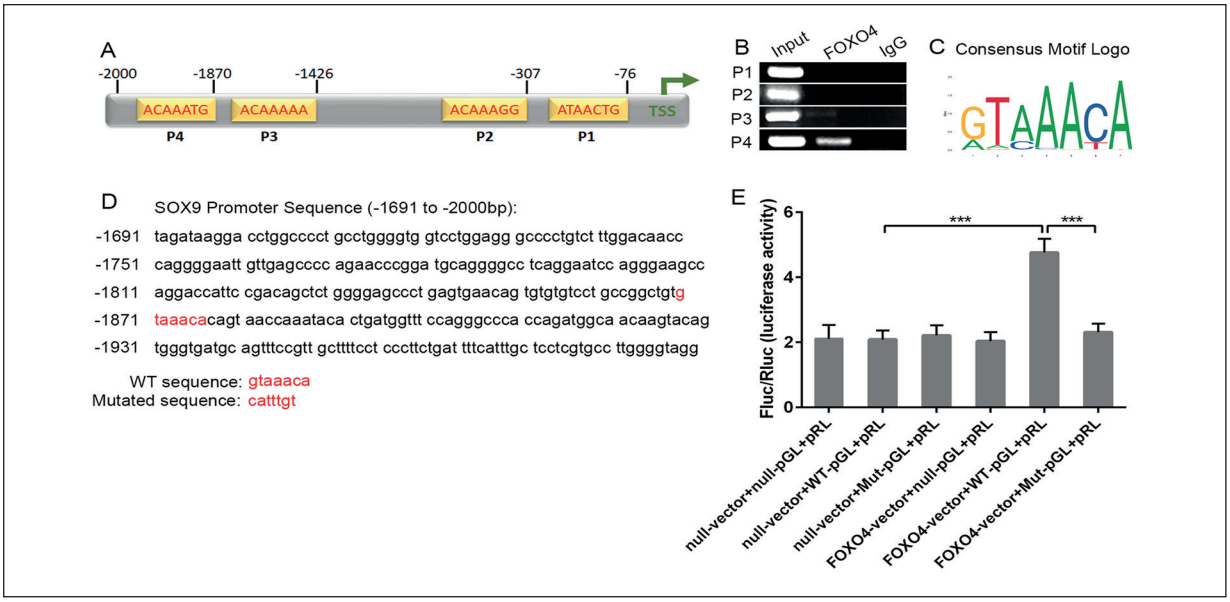

Figure 1. FOXO4 activates the promoter regions of SOX9. **A,** Four putative sites for FOXO4 were predicted to bind with the SOX9 promoter. **B,** Agarose gels electrophoresis for the PCR after immunoprecipitation. Lane 1 is the positive control of total DNA fragments; lane 2 is the amplification of the DNA from the FOXO4 linked beads; lane 3 is the amplification of the DNA from the IgG inked beads. **C,** Consensus motif logo for the specific binding site. **D,** The WT and Mutated sequence for inserting into the pGL reporter plasmid. **E,** Luciferase activity was more dramatic following FOXO4-vector transfection following the WT-pGL overexpression, and no significant difference in luciferase activity was observed with empty-vector or mutated sequence transfection. Results are expressed as mean ± SEM. (***p<0.001).

CHs with IL-1β to induce the degeneration. After three days of stimulation, the full expression of FOXO4 protein was not significantly changed. However, the acetylated FOXO4 of which was markedly increased compared to the control. To offend the acetylation of FOXO4, we added the human active Sirt1 protein in the medium with the presence of IL-1β. Compared to the control group, IL-1β suppressed the Sirt1 protein expression, and the exogenic Sirt1 protein supplement was functional to up-regulate the cellular Sirt1 level. Additionally, Sirt1 protein stimulation also rescued the IL-1β-induced acetylated-FOXO4 overexpression. Furthermore, in contrast to the control, SOX9 expression was also suppressed, which was increased after the supplement of Sirt1 (Figure 2A and 2B). Therefore, during CHs degener-

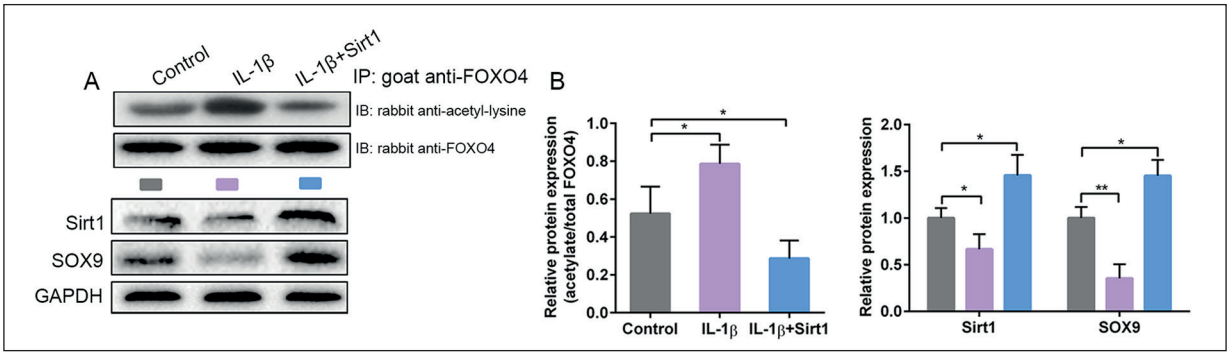

Figure 2. Sirt1 overexpression deacetylates FOXO4 and promotes SOX9 expression. CHs were cultured with IL-1β (10 ng/mL) for three days with or without the supplement of human Sirt1 protein (50 μg/mL). **A,** We immunoprecipitated the FOXO4 protein from the total cell lysates and analyzed by WB using an anti-acetyl-lysine antibody to determine the acetylated rate of FOXO4 and anti-FOXO4 antibody to determine the total FOXO4 level. WB is also used to determine the cellular Sirt1 and SOX9 protein expression. **B,** Quantification of WB measured by Image J software. Results are expressed as mean ± SEM. (*p < 0.05, **p < 0.01).

ation, the deacetylation of FOXO4 might promote the SOX9 upregulation, which relies on the presence of Sirt1.

FOXO4 Is Necessary for Sirt1-Induced SOX9 Expression and ECM Stability

To further determine whether the upregulation of SOX9 in the degenerated CHs depends on the increased FOXO4 deacetylation, we compared the normal CHs with the FOXO4-silenced CHs in the IL-1β medium with or without the presence of Sirt1. After the supplement of the human Sirt1 protein, the cellular Sirt1 protein level of the FOXO was also increased. However, when blocked the FOXO4 expression, Sirt1 upregulation did not increase the SOX9 level compared to the treatment of IL-1β without the Sirt1 protein supplement (Figure 3A and 3B). Apart from these, we also analyzed the mRNA expression of the representative ECM markers, containing collagen II, aggrecan, collagen I, and collagen X, and the catabolic enzymes of ECM, such as MMP-13 and ADAMTS-5. Under the culture with IL-1β, Sirt1 protein treatment increased the collagen II and aggrecan expression and decreased the collagen I, collagen X, MMP-13, and ADAMTS-5 expression (Figure 3C). Silencing of FOXO4 improved the chondrogenic ECM production, and the effect of Sirt1 upregulation was alleviated excepting the collagen X expression. Therefore, the presence of FOXO4 is necessary for the Sirt1 mediated SOX9 upregulation and the improvement of ECM stability.

SOX9 Is Necessary for FOXO4 Deacetylation-Induced ECM Stability

Finally, to confirm FOXO4 regulates the ECM synthesis through the activation of SOX9, we established the degenerated CHs with SOX9 deficiency. As shown in Figure 4A, the Sirt1 protein treatment deacetylated the FOXO4 compared to the non-Sirt1 treatment, no matter SOX9

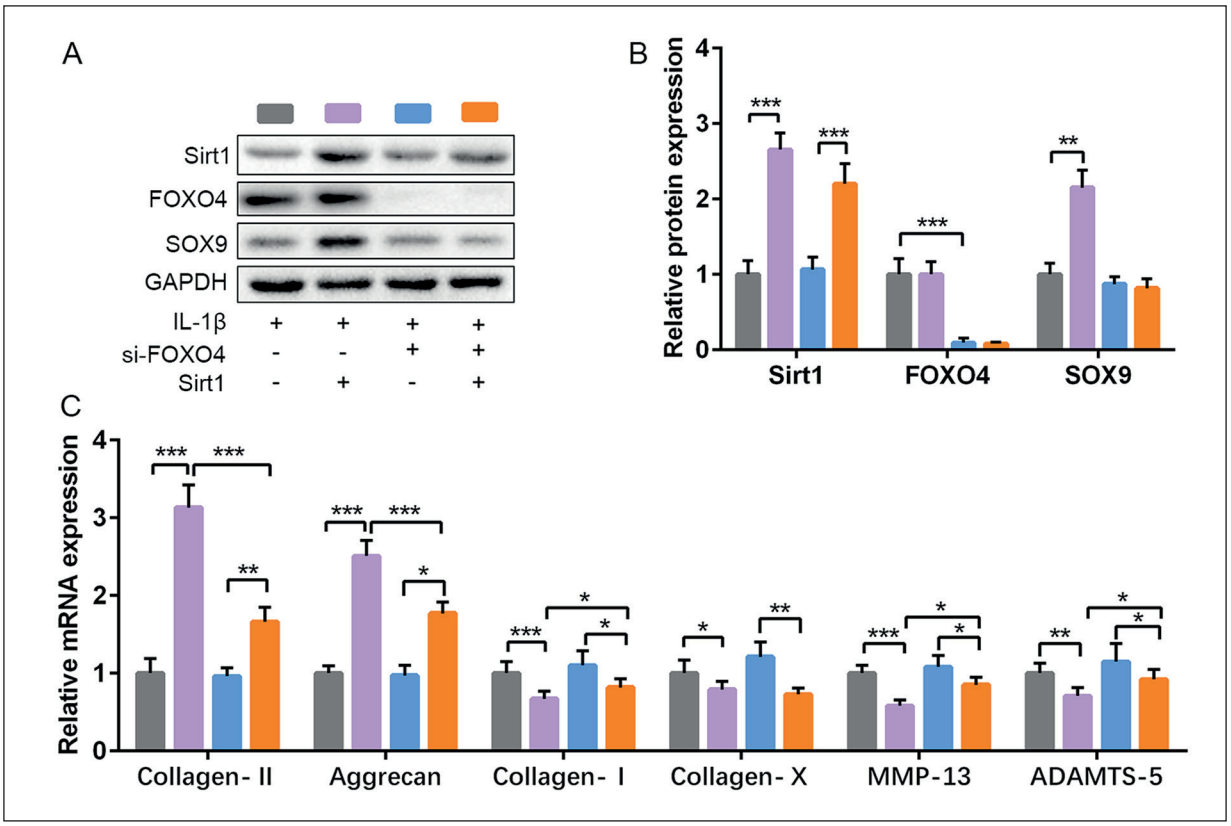

Figure 3. FOXO4 silencing weakens Sirt1-induced SOX9 expression and ECM stability. The FOXO4 silenced CHs, and non-silenced CHs were subjected to 10 ng/ml IL-1β for 3 days, with or without the presence of Sirt1 protein. **A,** WB analysis for the protein expression of Sirt1, FOXO4, and SOX9, and **B,** its quantification measured by Image J software. **C,** RT-PCR analysis for collagen II/X/I, aggrecan, MMP-13, and ADAMTS-5 by normalization to GAPDH expression. Results are expressed as mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

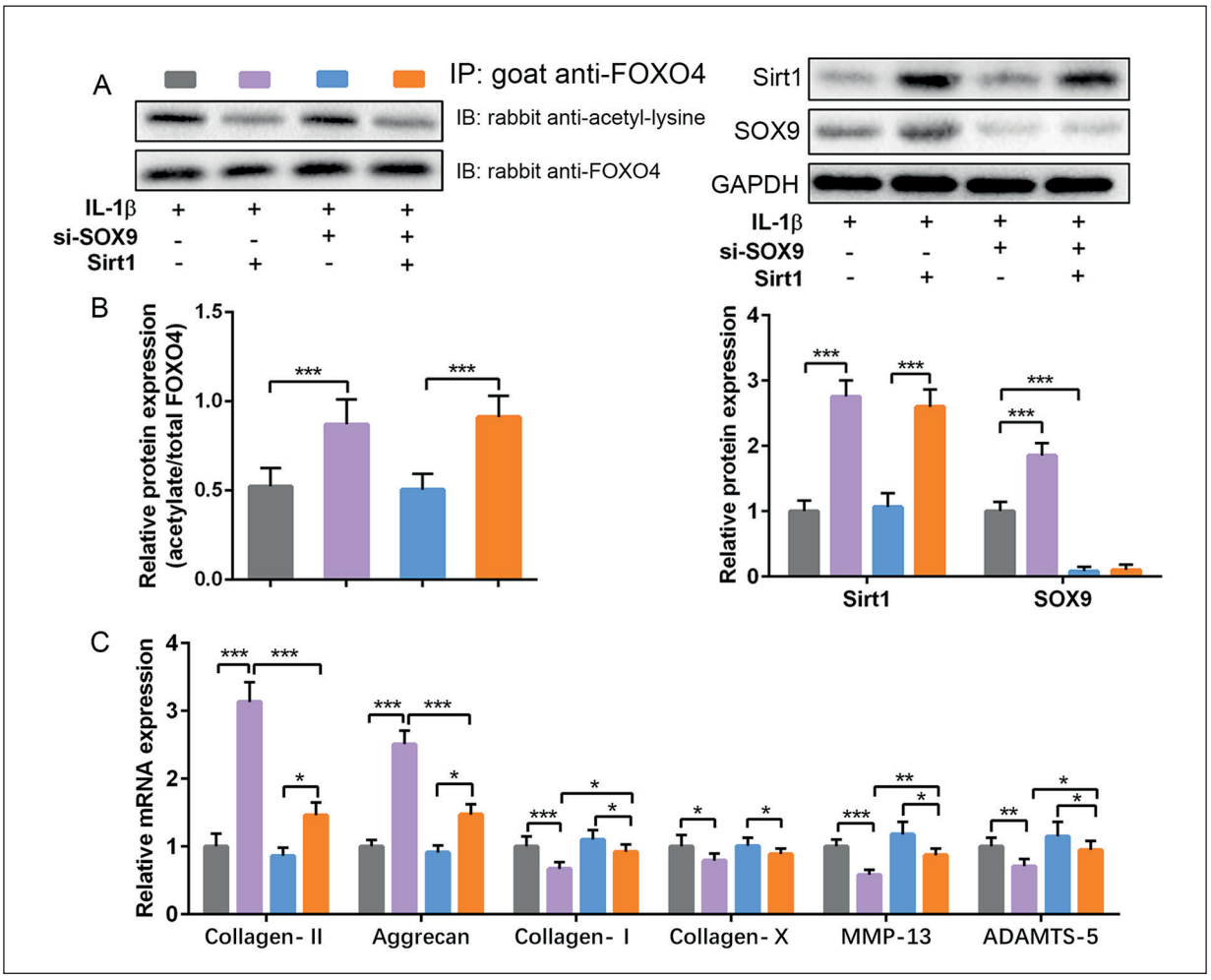

Figure 4. SOX9 silencing weakens FOXO4 deacetylation-induced ECM stability. The SOX9 silenced CHs, and non-silenced CHs were subjected to 10 ng/ml IL-1β for 3 days, with or without the presence of Sirt1 protein. **A,** We immunoprecipitated the FOXO4 protein from the total cell lysates and analyzed by WB using an anti-acetyl-lysine antibody to determine the acetylated rate of FOXO4 and anti-FOXO4 antibody to determine the total FOXO4 level. WB is also used to determine the cellular Sirt1 and SOX9 protein expression. **B,** Quantification of WB measured by Image J software. **C,** RT-PCR analysis for collagen II/X/I, aggrecan, MMP-13, and ADAMTS-5 by normalization to GAPDH expression. Results are expressed as mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

was silenced or not. In the SOX9-blocked CHs, the SOX9 protein was also low expressed and even the Sirt1 was upregulated (Figure 4A and 4B). Similar to the effect of FOXO4 silencing, even in the SOX9-deficient CHs, Sirt1 protein overexpression contributed to the generation of collagen II and aggrecan mRNA expression. It decreased the collagen I, collagen X, MMP-13, and ADAMTS-5 expression. However, without SOX9, the effect of the Sirt1 was partly rejected except the collagen X expression (Figure 4C). Therefore, the positive impact of the deacetylated FOXO4 in the degenerated CHs is somehow dependent on the mediation of SOX9.

Discussion

SOX9 is known as the regulator of chondrogenesis, which plays a vital role in the formation of ECM and the maintenance of chondrogenic phenotype[20]. We hypothesized that FOXO4 prevents the degradation of ECM during OA through the mediation of SOX9. Therefore, we predicted the putative binding sites for FOXO4 in the promoter of SOX9 to verify that FOXO4 transcriptionally regulates SOX9 expression. Fortunately, the overexpression of FOXO4 in the CH was effective in activating one putative binding site of the SOX9 promoter regions. For the first time, we detected

the transcriptional regulation of SOX9 involves the participation of FOXO4. The activity of FOXOs is regulated in many ways, including gene expression levels, post-translational modifications, and protein-protein interactions[21]. Post-transcriptional modification mainly includes three categories: phosphorylation, acetylation, and ubiquitination, which change the stability, nuclear import and export status of FOXOs binding to DNA, and the transcriptional activity for specific target genes as well[22]. The acetylation of FOXOs is regulated by histone acetyltransferase and histone deacetylase. Usually, acetylation affects the affinity of FOXOs with DNA, and deacetylation will increase this affinity[23,24]. Kim et al[25] found that the acetylation of FOXOs protein is reversible. Sirt1 can respectively bind to FOXO1, FOXO3a, and FOXO4 proteins to explicitly remove the acetyl group of FOXOs, thereby upregulating the DNA binding ability of FOXOs protein to specific target genes and increasing its transcriptional activity. After cultured with IL-1β, we found the total amount of FOXO4 remained with an increase of acetyl-lysine, which affected its transcriptional activity. The supplement of Sirt1 of the degenerative CHs reversed the acetylation of FOXO4, which promoted the expression of SOX9.

To elucidate how FOXO deacetylation keeps the stability of ECM synthesis, we respectively silenced the FOXO4 and SOX9 gene expression, and both of which weakened the protective effect of Sirt1-overexpression on ECM during CHs degeneration. IL-1β stimulation decreased the amount of deacetylated FOXO4, as well as the SOX9 expression. We have no evidence that the acetylation of FOXO4 contributes to the reduction of SOX9. However, the FOXO4 deacetylation is reasonable for the upregulation of SOX9, because the effect of Sirt1 was abolished when FOXO4 was silenced. The deacetylated function of Sirt1 is essential for the activation of SOX9. Independent with the regulation by FOXO4, the level of acetylated SOX9 protein also rises in degenerated articular cartilage[26]. Under the deacetylation effect of Sirt1, SOX9 can enter the nucleus to exert transcriptional regulation by increasing the affinity with the transport receptor Importin-β[27]. Unfortunately, Sirt1 expression is reduced in OA, further reducing the content of deacetylated SOX9. Therefore, the deacetylated effect of Sirt1, on the one hand, increases the expression of SOX9 through FOXO4. On the other hand, it may also directly improve the transcriptional activity of SOX9, which promotes ECM stability.

In our study, the supplement of Sirt1 upregulated the SOX9, collagen II, and aggrecan expression, and reduced the collagen I/X, MMP-13, and ADAMTS-5 levels. Collagen II is the primary component in the ECM of cartilage, and its change trend is highly consistent with SOX9. Multiple pieces of evidence indicate that SOX9 promotes its expression at the transcription level by binding to the enhancer of the collagen II gene[28]. Cartilage degeneration of OA comes from the remodeling of ECM, including disturbance of the secretion of CHs and increased matrix degradation. The enzymes involved in the deterioration of the ECM mainly include MMP-13 and ADAMTS-5, which is significantly overexpressed in the joints and articular cartilage of patients with OA, but is almost undetectable in normal articular cartilage tissues[29]. It has been confirmed that the activity of SOX9 has a negative regulatory effect on the expression of MMP-13 and ADAMTS-5[30,31]. Apart from this, collagen X is one of the markers of CH hypertrophy, which promotes the change of matrix components. Runx2 is reported to bind the proximal promoter of collagen X and regulate its expression[32]. The HMG domain at the N-terminus of SOX9 protein can directly interact with the Runt domain of Runx2, which reduces the particular Runx2 binding site on the collagen X promoter, thereby inhibiting the transcription of collagen X[33]. Therefore, the upregulation of SOX9 is meaningful to the maintains of ECM stability during cartilage degeneration. Without SOX9, the efficiency of FOXO4 on ECM stability is alleviated.

Conclusions

To sum up, for the first time, we showed that the FOXO activation increases the SOX9 expression through binding to its promoter. During the process of CHs degeneration, preventing the acetylation of FOXO4 by Sirt1 presents a contribution to SOX9 activity resulting in the resistance against the ECM degradation. Maintaining a high level of FOXO4 deacetylation helps to keep the stability of the cartilage ECM, which could be a novel direction for the prevention and treatment of OA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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