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Sirt7 protects chondrocytes degeneration in osteoarthritis *via* autophagy activation

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is associated with decreased autophagy activity and imbalance of cell homeostasis in chondrocytes (CHs). Sirtuin 7 (Sirt7) is claimed to have the ability to activate the autophagy response. The aim of this study was to explore the function of Sirt7 in the development of OA involving autophagy by culturing human chondrocytes (CHs).

PATIENTS AND METHODS: We collected knee joint cartilage from patients undergoing traumatic amputation and arthroscopic knee replacement. Protein and mRNA levels of collagen II, Sirt7, ULK1, Lc3, and Beclin1 were analyzed by Western blot and RT-PCR. CHs were isolated from the traumatic cartilage as a control group, and IL-1β was used to induce CHs degeneration. The expression of Sirt7 gene was silenced by siRNA and upregulated by recombinant human Sirt7 protein (rh-Sirt7). An autophagy activator Tat-beclin 1 (Tat) was used to activate autophagy in cultural CHs. Expression of collagen II, Sirt7, ULK1, Lc3, and Beclin1 was determined by immunofluorescence, Western blot, and RT-PCR, respectively.

RESULTS: The protein and mRNA levels of Collagen II, Sirt7, ULK1, Lc3-II, and Beclin1 expressions were decreased in OA cartilage compared with those in healthy cartilage. IL-1β degenerated the CHs resulting in a reduction of collagen II, which also downregulated Sirt7, ULK1, Lc3-II, and Beclin1. Sirt7 deficiency accelerated the catabolism of collagen II and weakened the expression of ULK1, Lc3-II, and Beclin1. On the contrary, exogenous rh-Sirt7 played a positive role in these gene expressions. Finally, Tat alleviated the CHs degeneration by upregulating collagen II and activating ULK1, Lc3-II, and Beclin1, but incapable to mediate Sirt7 expression.

CONCLUSIONS: Overall, these findings suggested that Sirt7 was suppressed in the degenerated cartilage. Sirt7 deficiency does harm to the autophagy level, affecting CHs metabolism, while the upregulation of Sirt7 activated autophagy and protected CHs degeneration. An appropriate increase in autophagy can protect CHs but has no effect on Sirt7 expression.

Key Words:
Osteoarthritis, Chondrocyte degeneration, Sirt7, Autophagy.

Introduction

Osteoarthritis (OA) is mainly characterized by joint swelling and long-term chronic pain, which seriously affects the daily life of patients[1]. Current research indicates that the main pathogenesis of OA is articular cartilage degeneration and chronic inflammation. Articular cartilage degeneration is the central link of the pathogenesis of osteoarthritis. The main pathological manifestations are senescence and apoptosis of chondrocytes (CHs), degradation of extracellular matrix (ECM), poor proliferation leading to cartilage degradation, and osteochondral bone sclerosis leads to osteophyte formation[2]. Therefore, maintaining the activity of articular CHs is one of the key factors in the prevention of OA. Bay-Jensen et al[3] have shown that maintaining the level of autophagy in CHs can be an important way to prevent the degeneration of cartilage.

The process by which eukaryotic cells degrade cytoplasmic proteins and organelles through the lysosomal pathway to maintain survival is called autophagy[4]. Autophagy removes damaged organelles and long-acting macromolecules and is an indispensable mechanism for maintaining cell homeostasis. Green et al[5] found that the occurrence of OA is associated with decreased levels of autophagy in CHs. Therefore, increasing autophagy levels in chondrocytes may be a target for OA therapy. Carames et al[6] found that autophagy may be a protective mechanism against cell death caused by aging and trauma in normal cartilage. Autophagy is a mechanism for clearing the cellular homeostasis of dysfunctional organelles and macromolecules, and enhanced autophagy may serve as a new way to delay joint aging and reduce risk factors for OA[7]. Sasaki et al[8] indicated that autophagy in CHs regulates the production of reactive oxygen species (ROS) and the changes in the expression of OA-related genes (Aggrecan, COL2A1, MMP-13, ADAMTSs). Almonte-Becerril et al[9] detected, by using the animal

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OA model, that the death form of CHs coexisted with autophagy and apoptosis. Bohensky et al[10] suggested that CHs regulate autophagy through HIF-1/AMPK/mTOR signaling pathway.

Jiang et al[11] have found that Sirt7 protects cell death and improves the efficacy of the therapy in lung cancer by regulating autophagic response. Sirt7 is also suggested[12] to promote autophagy in gastric carcinoma progression *via* act in mTOR/IGF2 pathway. However, whether Sirt7 takes part in the development of OA resulting from the mediation of autophagy remains unknown. In this study, we speculate that Sirt7 may participate in cartilage degeneration by regulating autophagy. We compared the expression of Sirt7 between normal articular cartilage and arthritic cartilage of human and explored the relationship among CH degeneration, Sirt7, and autophagy, and then, further elucidated the possible mechanism of Sirt7 regulating autophagy of CHs, which may provide a theoretical basis for therapeutic targets in OA.

Patients and Methods

Patient Tissue Samples Collection

Our project was approved by the Liyi People's Hospital Ethics Committee. A total of 10 patients who underwent traumatic amputation of the knee and arthroscopic knee replacement surgeries from August 2018 to April 2019 participated in this project. We divided the joint cartilage tissues into two groups: 1) healthy group (from traumatic amputation with no degeneration), 2) OA group (from arthroscopic knee replacement with evident degeneration). These tissues were used to protein and mRNA extraction and CHs isolation in the following experiments.

CHs Isolation and Culture

Cartilage samples obtained from the operating room were immersed in low-sugar medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), stored at low temperature, and transferred to the laboratory as soon as possible. In the ultra-clean workbench, the specimen was cut into approximately 0.5×0.5 cm^2 cartilage pieces with tissue scissors, and the cartilage pieces were cut into asbestos fragments of about 1 mm^2 by ophthalmic scissors. The fragments were digested by 0.25 % trypsin (Sigma-Aldrich, St. Louis, MO, USA), and then, washed with phosphate-buffered saline (PBS), and incubated with DMEM medium containing 0.2 % type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) in 37°C for 40 min. After the supernatant was discarded, CHs were obtained *via* centrifugation. The passage 1 CHs were used in the following experiment groups: 1) control group (isolated from healthy cartilage); 2) IL-1β group (CHs treated with 10 or 20 ng/mL); 3) null-siRNA group (CHs transferred with null-siRNA); 4) Sirt7-siRNA group (CHs transferred with Sirt7-siRNA); 5) Sirt7 group (CHs treated with recombinant human Sirt7 protein[13], rh-Sirt7, 500 ng/mL, TP305658, Origene, Rockville, MD, USA); 6) Tat-beclin1 group (CHs treated with Tat-beclin1, Tat, 5 μM, Selleck, Houston, TX, USA).

Western Blot Analysis

The cellular protein of CHs was isolated with the radioimmunoprecipitation lysis buffer (Beyotime, Shanghai, China) and the concentration was measured by bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Then, the protein was separated by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with 5% milk, and then, incubated with primary antibodies against collagen II, Sirt7, ULK1, LC3II/I, Beclin1, and β-Actin (as a loading control) overnight at 4°C (all the antibodies were purchased from Abcam, Cambridge, MA, USA). Then, the protein was incubated again with secondary antibody for 1 h at room temperature. Finally, enhanced chemiluminescence (ECL) substrate (Beyotime, Shanghai, China) was used to expose the bands on membrane.

Immunofluorescence (IF)

CHs were seeded on coverslips. Before staining, CHs were fixed with 4% paraformaldehyde for and permeabilized by 0.1% Triton-X for 15 min, respectively. Following, 5% bovine serum albumin was used to block the CHs. The sections were incubated with primary antibodies overnight at 4°C, including anti-collagen II, anti-Sirt7 (purchased from Abcam, Cambridge, MA, USA). The coverslips were subsequently incubated with Alexa Fluor488 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI) for 1 h in the dark. The staining intensity was determined with the Image-Pro Plus software (Silver Springs, MD, USA).

Table I. Primer sequences of the genes for RT-PCR.

Gene name	Forward (5'>3')	Reverse (5'>3')
Sirt7	GACCTGGTAACGGAGCTGC	CGACCAAGTATTTGGCGTTCC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
ULK1	GGCAAGTTCGAGTTCTCCCG	CGACCTCCAAATCGTGCTTCT
Lc3-II	GATGTCCGACTTATTCGAGAGC	TTGAGCTGTAAGCGCCTTCTA
Beclin1	CCATGCAGGTGAGCTTCGT	GAATCTGCGAGAGACACCATC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

CHs were cultured in 6-well plate until the cell density up to 60%, and the RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). The nucleic acid-protein analyzer was used to detect RNA content and purity. The RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit (Roche, Basel, Switzerland). ULK1, Beclin1, and LC3 mRNA were detected according to the PCR kit instructions (Toyobo, Osaka, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalization. The corresponding gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method, and 3 replicates were set for each sample. The primer sequences are shown in Table I.

RNA Interference

For the silencing of Sirt7 gene in the CH, CHs were transfected with small interfering RNA targeting human Sirt7 (Sirt7-siRNA) or negative control siRNA (null-siRNA) (purchased by GenePharma, Shanghai, China) using Lipofectamine 2000 (Beyotime, Shanghai, China) following the manufacturer's instructions.

Statistical Analysis

We analyzed the data in the study using the Statistical Product and Service Solutions (SPSS) software package (Version 22.0, IBM Corp., Armonk, NY, USA) and all the data were represented as mean ± standard deviation. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). All experiments were repeated at least three times. It is indicated statistically significant on the condition of p-value<0.05 between groups.

Results

Sirt7 and Autophagy Levels in Human OA Cartilage

To determine the difference of Sirt7 expression between healthy cartilage and OA cartilage, we collected the tissues from the knee joint undergoing operation due to trauma and OA. Healthy cartilage from knee traumatic operation was chosen as control, and total protein and mRNA were isolated from the samples in these two groups. As shown in Figure 1A, collagen II expression in the control group was significantly higher than OA condition, which meant the cartilage in OA degenerated much severally. As expected, the expression of Sirt7 was also higher in the control group compared with OA. The levels of the autophagic gene, containing ULK-1, Lc3, and Beclin-1were measured. The data indicated that ULK-1, Lc3-II, and Beclin1 were significantly decreased in OA condition compared to the controls (Figure 1A and 1B). In addition to this, the result associated with mRNA levels was parallel with Western blot (Figure 1C). These results suggested that the expression of Sirt7 was suppressed in the degenerated cartilage, and the level of autophagy was decreased in the OA condition.

IL-1ß Induced CHs Degeneration Inhibits the Expression of Sirt7 and Autophagy In Vitro

To determine the expression of Sirt7 and autophagy levels in the degenerated CHs *in vitro*, the CHs were isolated from the healthy cartilage, and different concentration of IL-1β (10 ng/mL and 20 ng/mL) was used to induce CH degeneration. We found that the expression of collagen II decreased under the stimulation of IL-1β, as well as the Sirt7 level (Figure 2A and 2B). As demonstrated in Figure 2C and 2D, the expression of ULK-1, Lc3-II, and Beclin1 were all reduced with the presence of an increased concentration of IL-1β. mRNA lev-

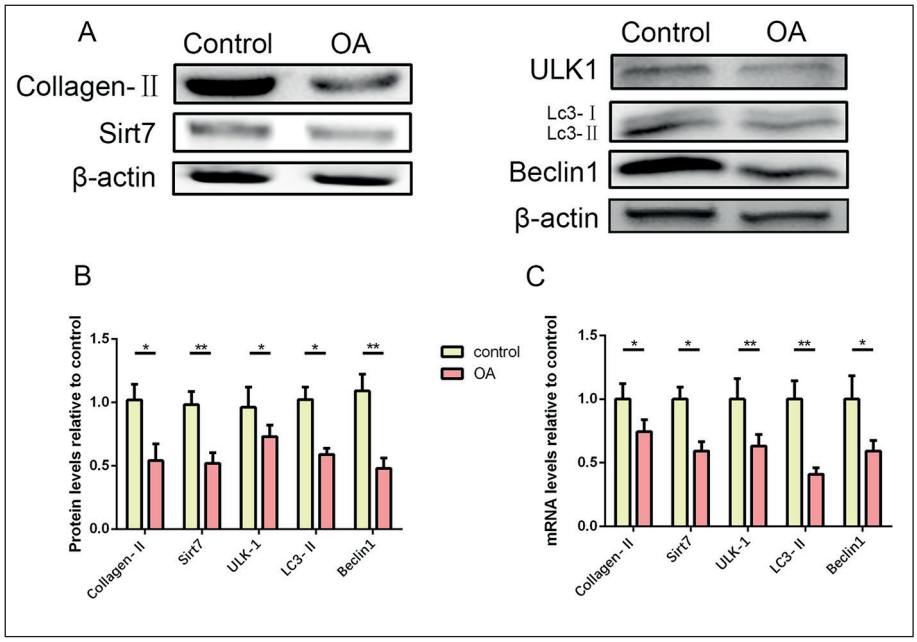

Figure 1. Sirt7 and autophagy levels in healthy and OA cartilages. Total protein and mRNA were extracted from the cartilage tissue collected from the patients. **A**, The protein levels of Sirt7, collagen II, ULK1, Lc3, and Beclin1 were determined by Western blot and (**B**) quantification analysis. **C**, mRNA levels of Sirt7, collagen II, ULK1, Lc3- II and Beclin1 and were determined by RT-PCR. The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01).

els of these genes were also analyzed by RT-PCR, and it was found that IL-1β played a negative role in the development of OA degeneration with the decline in expression of Sirt7 and ULK-1, Lc3-II, and Beclin1 (Figure 2E). Therefore, Sirt7 was downregulated in the IL-β induced degenerated CHs, suppressing the autophagy.

Sirt7 Silencing Promotes CHs Degeneration Via Autophagy Inhibition In Vitro

To determine whether Sirt7 deficiency affects the autophagy levels in the degenerated CHs *in vitro*, we used siRNA to silence the Sirt7 gene expression in CHs *in vitro*, and null-siRNA was used as a negative control. The IF result indicated that null-siRNA did not affect the Sirt7 level, but Sirt7-siRNA successfully reduced the expression of Sirt7 expression (Figure 3A and 3B). The protein expression of collagen II was significantly decreased under the silencing of Sirt7. Besides, the deficiency of Sirt7 also suppressed the activity of ULK1 and Beclin1 protein compared with the control groups (Figure 3C and 3D). Apart from these, RT-PCR was performed to detect the mRNA differences after Sirt7 silencing. The data indicated that silenced Sirt7 promoted the downregulation of collagen II mRNA in CHs as well as ULK1, Lc3-II, and Beclin1 (Figure 3E). After collecting these data, we found that silencing of Sirt7 accelerated the development of CHs degeneration by the suppressed progression of autophagy.

Sirt7 Stimuli Attenuates IL-1ß Induced CHs Degeneration Via Autophagy Activation In Vitro

To determine whether the exogenous supply of Sirt7 can reverse the IL-1β induced CHs degeneration and active autophagy *in vitro*, CHs were treated with rh-Sirt7 protein *in vitro*. As shown in Figure 4A and 4B, the expression of Sirt7 was suppressed by IL-1β, which however, was reversed by rh-Sirt7 protein stimulation. The result of Western blot indicated that rh-Sirt7 played a positive role in the development of CHs degeneration and upregulated the protein levels of ULK1 and Beclin1 (Figure 4C and 4D). We also measured the mRNA expression of this gene, and the results were consistent with the protein aspect (Figure 4E). To sum up, the exogenous supply of Sirt7 slowed down the progress of IL-β induced CHs degeneration which could be associated with the promotion of autophagy.

Autophagy Alleviates IL-1ß Induced CHs Degeneration but Not Upregulate Sirt7 Expression

According to the above results, we thought the expression of Sirt7 resulted in the changes in autophagy levels, but whether the upregulation of autophagy also could affect the Sirt7 expression in CHs was still unknown. To find out the answer to this question, we used autophagy activation Tat-Beclin 1[14] (Tat) to treat CHs under the

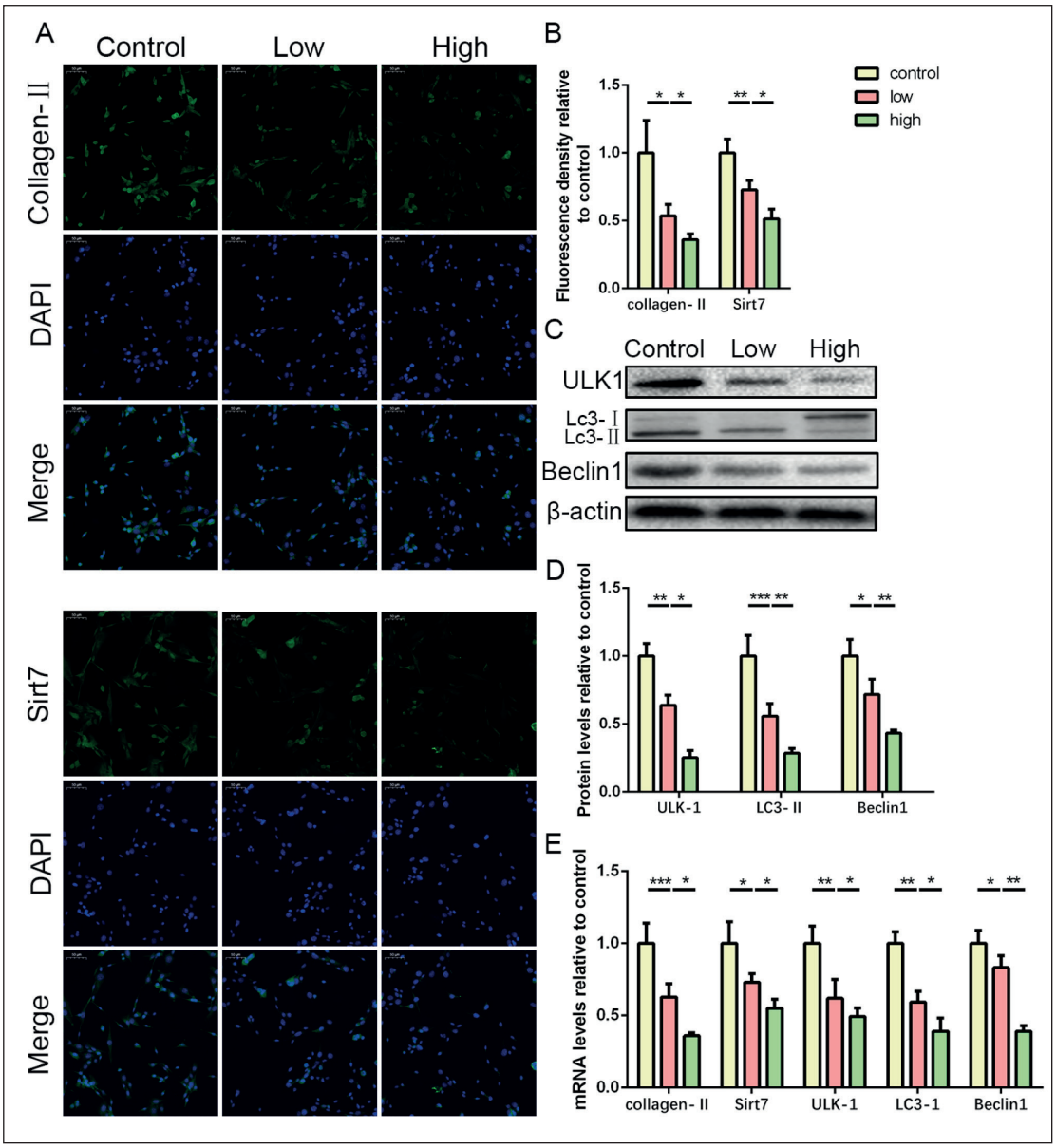

Figure 2. Sirt7 and autophagy levels in IL-1β induced CHs *in vitro*. CHs of healthy cartilage were pretreated with a low or high concentration of IL-1β (10 ng/mL or 20 ng/mL) for 24 h. The protein expression level of collagen II and Sirt7 were determined by immunofluorescence and quantification analysis (**A**, **B**) (magnification: 400×). The protein expression levels of ULK1, Lc3 and Beclin1 were determined by Western blot and quantification analysis (**C**, **D**). The mRNA expression levels of collagen II, Sirt7, ULK1, Lc3- II and Beclin1 were assayed by RT-PCR (**E**). The values are mean ± SD of three independent experiments (n=3). (**p*<0.05, ***p*<0.01, ****p*<0.001).

stimulation of IL-1β. As shown in Figure 5A and 5B, the protein expression of collagen II was decreased in the treatment of IL-1β and upregulated again resulting from the stimuli of Tat, which meant autophagy protected the degeneration of CH. The protein level of ULK1 and Beclin1 significantly upregulated with the treatment of Tat compared with the IL-1β group (Figure 5C and 5D). We used PT-PCR to analyze the mRNA levels and found that Tat promoted the expression of collagen II, ULK1, Lc3-II, and Beclin1 but make no effect on Sirt7 expression (Figure 5E). The result suggested that autophagy could protect CHs degeneration by upregulation ULK1, Lc3-II, and Beclin1 expression but made no sense to the Sirt7 level.

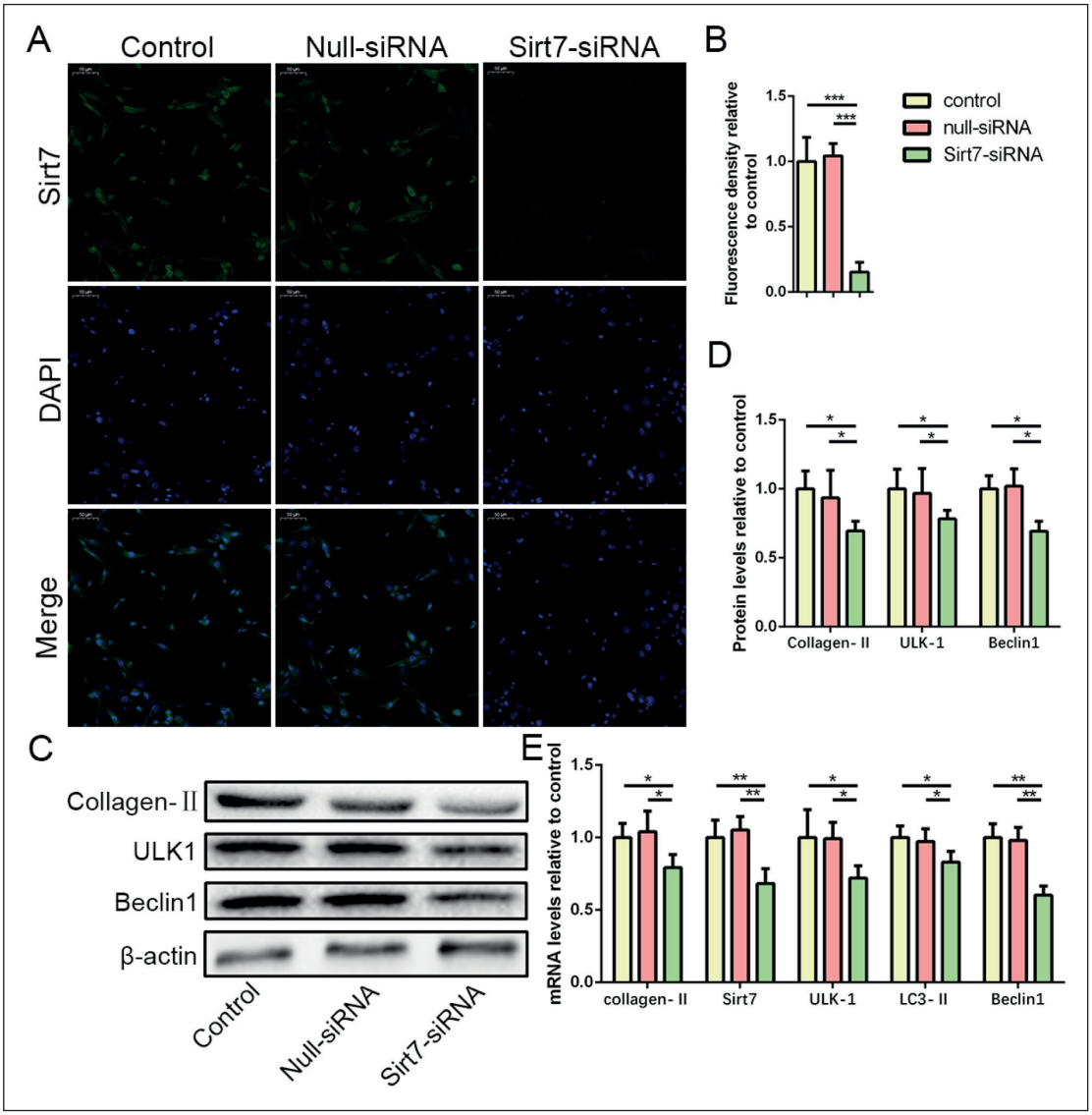

Figure 3. Sirt7 deficiency weakens autophagy and accelerates human CHs degeneration. CHs were transfected with null-siRNA and Sirt7-siRNA. The protein expression level of Sirt7 was determined by immunofluorescence and quantification analysis (**A**, **B**) (magnification: 400×). The protein expression levels of collagen II, ULK1, and Beclin1 were determined by Western blot and quantification analysis (**C**, **D**). The mRNA expression levels of collagen II, Sirt7, ULK1, Lc3- II and Beclin1 were assayed by RT-PCR (**E**). The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

Discussion

OA is a chronic degenerative joint disease whose major pathological changes are articular CHs reduction and cartilage matrix degradation. In recent years, with the deepening of research on the pathogenesis of OA, it is found that the imbalance of CH homeostasis is closely related to the occurrence and development of OA[7]. Autophagy, as a protective mechanism of cells, is widely involved in many processes, such as cell development, proliferation, and apoptosis, which also plays an important role in the process of CH maturation and homeostasis[15]. Autophagy can regulate the adaptive response of cells to external stimuli, and timely remove damaged organelles and macromolecular substances, thereby avoiding the accumulation of damage, and promoting cell survival. The autophagy-related genes ULK1, Beclin1, and Lc3 in mammals acted as inducers, regulators, and performers, respectively, and they participate in the process of protein processing modification[16].

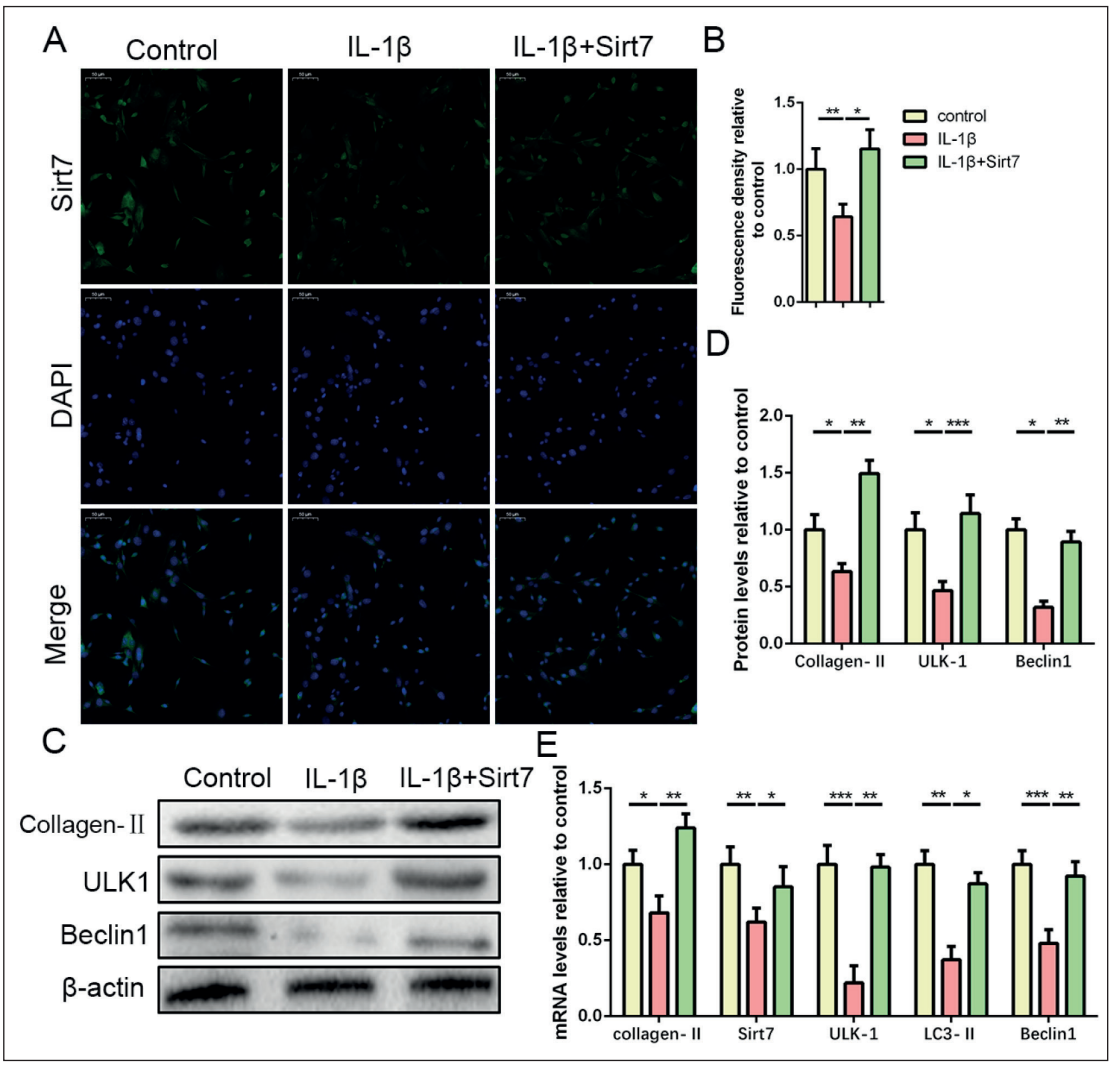

Figure 4. Sirt7 overexpression activates autophagy and protects human CHs degeneration. CHs were pretreated with IL-1β with or without rh-Sirt7 (500 ng/mL). The protein expression level of Sirt7 was determined by immunofluorescence and quantification analysis (**A**, **B**) (magnification: 400×). The protein expression levels of collagen II, ULK1, and Beclin1 were determined by Western blot and quantification analysis (**C**, **D**). The mRNA expression levels of collagen II, Sirt7, ULK1, Lc3-II and Beclin1 were assayed by RT-PCR (E). The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

ULK1 is required for the formation of autophagic vacuoles, and the inactivation of ULK1 kinase inhibits the formation of Lc3-II and autophagy. When ULK1 activity is enhanced, autophagy will be initiated[17]. Lc3 is widely expressed in soluble proteins of mammalian tissues and cultured cells, that are called cytosolic Lc3-I. When autophagy occurs, the cytosolic form of Lc3-I binds to phosphatidylamine *via* an ubiquitin-like enzymatic reaction and is converted to Lc3-II in the form of a phospholipid conjugate compound, which is continuously recruited to autophagic vacuoles for autophagy[18]. Beclin1 is a yeast ATG6 homolog which plays a positive regulated role in autophagy[19]. In adult healthy articular cartilage, autophagy maintains CH homeostasis, and CHs in the superficial layer of articular cartilage highly express Beclin1, ATG5, and Lc3-II[20]. In addition, Sasaki et al[8] found that the expression of Lc3-II and Beclin1 mRNA in CHs of OA is increased in the initial stage of cartilage degeneration.

Sirt7 is verified as an important regulator of cartilage homeostasis and participates in the development of OA[21]. In our study, we firstly elucidated that Sirt7 expression decreased significantly in OA joint cartilage which is consistent with the auto-

phagic gene expression. Additionally, it has been discovered[22-25] that sirtuin family induced autophagy is involved in different cell types. Although the previous report[26] highlights Sirt7 as a mediator of autophagy involved in the tissue repair process, the association between Sirt7 and autophagy in the process of OA has not been declared so far. As a consequence, our study suggested that Sirt7 protected the cartilage degeneration *via* promoting CHs autophagy. Secondly, our data suggested Sirt7 was downregulated in the IL-β induced degenerated CHs. The transient increase of autophagy is a compensatory response to cell survival, but sustained autophagy will cause CHs damage and apoptosis[27]. In the middle and late stage of OA, autophagy of CHs decreases, apoptosis increases, cartilage degeneration becomes severe, and ECM synthesis decreases. Inflammation can inhibit the level of Sirt7 and autophagy. Conversely, Sirt7 can also affect autophagy. In this present study, silencing Sirt7 decreased the level of autophagy, while exogenous Sirt7 protein activated the autophagy, both of which affected the collagen II expression of CHs. However, we still wondered whether autophagy can affect Sirt7 expression in turn. After using the activator of autophagy, it was found that

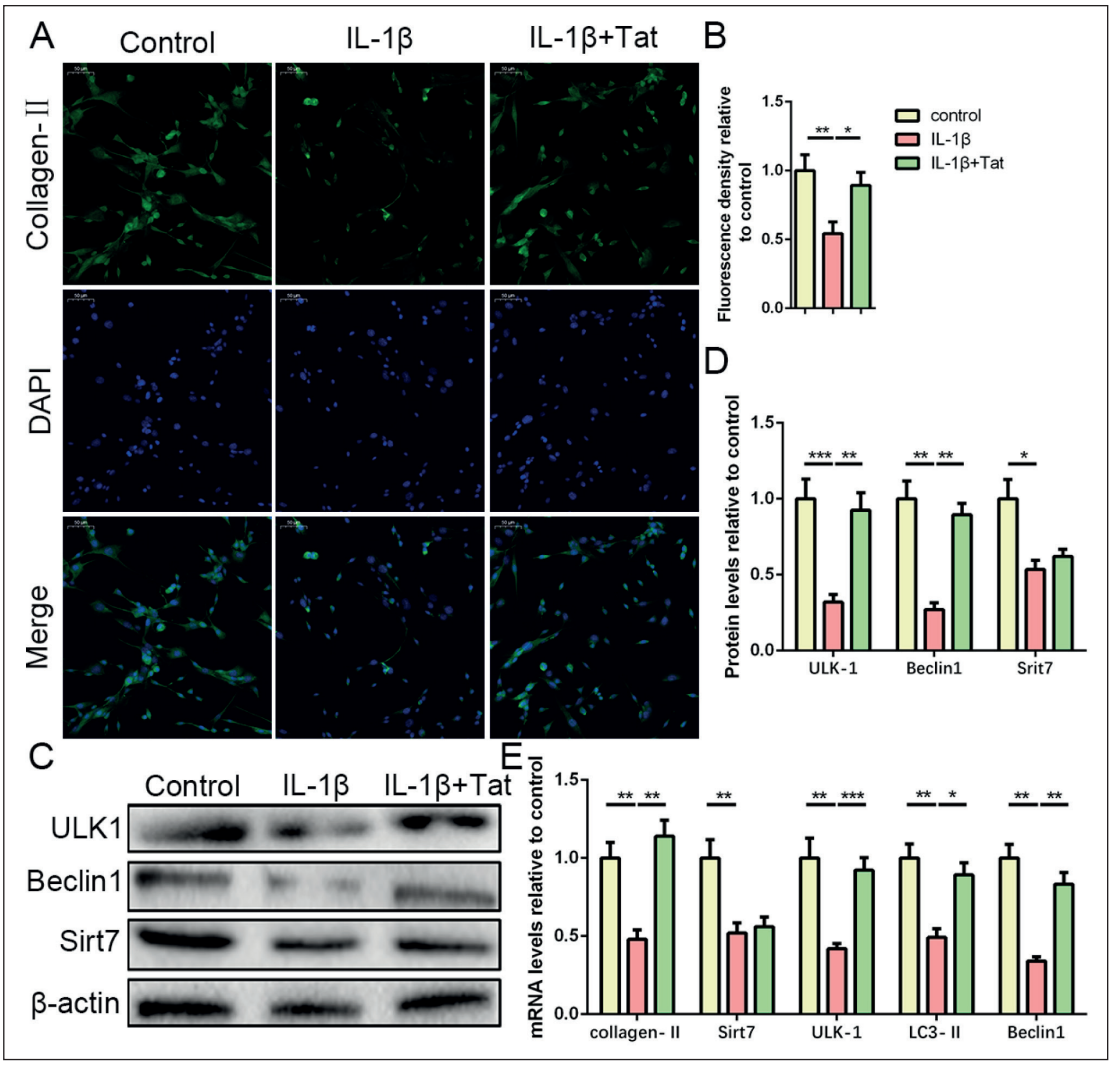

Figure 5. Tat upregulates autophagy and promotes protects human CHs degeneration but fails to mediate Sirt7. CHs were pretreated with IL-1β with or without Tat (5 μM). The protein expression level of collagen II was determined by immunofluorescence and quantification analysis (**A**, **B**) (magnification: 400×). The protein expression levels of Sirt7, ULK1, and Beclin1 were determined by Western blot and quantification analysis (**C**, **D**). The mRNA expression levels of collagen II, Sirt7, ULK1, Lc3- II and Beclin1 were assayed by RT-PCR (E). The values are mean ± SD of three independent experiments (n=3). (*p<0.05, **p<0.01, ***p<0.001).

autophagy could protect the degenerated CHs by upregulation of ULK1, Lc3-II, and Beclin1. As the existence of interaction between autophagy and apoptosis during the progression of OA, we are also interested about the effect of Sirt7 on the apoptosis response to CHs. Therefore, our further research will focus on the relationship among Sirt7, autophagy, as well as the apoptosis in OA.

Conclusions

Overall, the expression of Sirt7 and autophagy was reduced in degenerated CHs. Inhibition of Sirt7 expression reduces autophagy levels and promotes the degeneration of cartilage. Exogenous supplementation of Sirt7 will upregulate autophagy levels and inhibit CH degeneration. However, if the expression of autophagy is activated, it can protect chondrocytes, but it does not affect the expression of Sirt7. A further study will explore which receptors or pathways are involved in the regulatory process of Sirt7 on autophagy in the progression of OA. We hope that Sirt7 will be another target for future OA treatment.

Conflict of Interests

The authors declare that they have no conflict of interest.

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