Sirt1 Protects Cartilage Against Degradation through FoxO1 Nucleo-cytoplasmic Shuttling and Positive Regulation of Autophagy

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Abstract

Background: Sirt1 plays an important role in the pathophysiological process of osteoarthritis (OA) as we reported previously, however, the underlying mechanisms are still poorly addressed.

Methods: The cartilage samples from OA patients' knee joint were collected and detected histologically and immunohistochemistically. The effects of cartilage specific Sirt1 deletion on cartilage homeostasis were evaluated in destabilization of medial meniscus (DMM)-induced OA mice model (n=10). Finally, the underlying regulation of Sirt1 on cartilage was verified by coimmunoprecipitation (CoIP) and chromatin immunoprecipitation (ChIP) *in vitro*.

Results: The expression levels of SIRT1, FoxO1 and autophagy decreased in OA cartilage. We further found that DMM-induced OA in cartilage specific Sirt1 deletion mice displayed an aggravated OA development, with increased chondrocytes hypertrophy and apoptosis while decreased autophagy activity. *In vitro* experiments indicated that FoxO1 regulated Sirt1 expression by nucleo-cytoplasmic shuttling through an auto-feedback loop leading to an increased autophagy level. Consistently, the activation of autophagy activity partly rescued OA-like changes in chondrocytes.

Conclusions: Sirt1 protects cartilage against degradation through FoxO1 nucleo-cytoplasmic shuttling and positive regulation of autophagy, which all play a protective role in OA pathophysiological process, possibly partial adjustment by positive feedback.

Introduction

Osteoarthritis (OA) is a chronic joint disease characterized by degeneration of articular cartilage, synovial hyperplasia and osteophyte formation. It is a common degeneration disease bothering the middle-aged and elderly patients, the increasing prevalence of OA raised individual and state awareness in China[1]. Due to the high disability rate, limited prevention and treatment methods hinder the patients' daily life and occupational ability. There is an urgent need of studying the pathophysiology of OA to develop effective prevention courses and therapeutic methods. A growing body of studies has shown that aging, mechanical injury, metabolic syndrome and obesity contribute to the OA onset[2]. Although extensive studies have revealed age-related molecular changes in cartilage, the molecules that control homeostasis of articular cartilage remain unknown.

Our study has reported that sirtuin 1 (Sirt1) played an important role in ossification process by hyper-activation of mTORC1 signaling[3]. Sirt1 is first identified as an anti-geriatric gene prolonging life span[4]. The loss of Sirt1 in chondrocytes leads to the accelerated development of OA under mechanical stress and aging in mice via activating NF-κB[5], while the underlying mechanisms have not been completely investigated. We further reported that circadian rhythm gene *Bmal1* modulated human cartilage gene expression by cross-talking with *Sirt1*[6]. Moreover, Sirt1 is a repressor of SOST-encoded sclerostin which

rescues ovariectomy-induced bone loss and attenuated biomechanical deterioration[7, 8]. A recently study reported conflicting results of SIRT1 inhibition protected against osteoarthritis by loss of DOT1L activity[9]. Therefore, the underlying mechanisms of loss of Sirt1 in cartilage still remain unclear.

Macroautophagy represents the cellular degradative pathway in mammals, in which phagophores encloses and delivers a portion of cytoplasm to the lysosome[10, 11]. Autophagy related genes (such as Atg5, Atg7 and Atg12) play a vital part among the whole autophagy process. Cartilage-Specific Atg7 deficiency promotes ER stress and impairs chondrogenesis in the PERK-ATF4-CHOP dependent manner[12], likely, knockdown of Atg7 leads to lifespan decrease in the nematode [13]. Meanwhile, the Sirt1 deacetylase is an important regulator of autophagy *in vivo* and provide a link between sirtuin function, and Sirt1 and Atg7 have an apparent interaction *in vitro* and *in vivo* by using mouse embryonic fibroblasts and cancer cells [14, 15].However, the role of Sirt1 in regulating autophagy warrants further investigation.

Forkhead-like box (FoxOs) was found to allow cells to adapt to various stressors, such as oxidative stress and growth factor deprivation by promoting cell cycle arrest, DNA damage repair, autophagy and scavenging free radicals. In particular, SIRT1 regulates FoxO1 expression and FoxO1 transcription responds to SIRT1 changes suggesting a positive feedback loop between *Sirt1* and FoxO1, and FOXO1 transcription factor has unique functions in meniscus development and homeostasis during aging and osteoarthritis, also FoxO1 regulates chondrogenic differentiation through TGFβ1 prompts the irreplaceable role on OA[16–18].

We previously found that SIRT1 regulated P53 activities in Sirt1-knockout mice and aging-related OA cartilage[19]. Sirt1, as an anti-geriatric and anti-apoptosis gene, could be correlate with the nucleocytoplasmic shuttling of transcriptional factor FoxO1 and impair of autophagy activities in Sirt1 CKO mice. To decipher the relationship among "Sirt1, FOXO1 and autophagy" in chondrocytes, we took advantage of cartilage-specific knockout Sirt1 mice and examined the effects of Sirt1 on autophagy-related genes 7 (Atg7) in injury-evoked OA. We found that Sirt1 epigenetically regulated with FoxO1 then increased autophagy in the pathophysiological conditions which suggested that loss of *Sirt1 may* accelerate post-traumatic OA (PTOA).

Methods

OA and normal cartilage sample obtaining and processing

OA cartilage tissues were obtained from distal part of both the lateral and medial femoral condyles of 6 patients (mean age 65.25 years old, Kellgren Lawrence scoring system 3 ~ 4) with medial knee OA during total knee joint replacement surgery. Normal cartilage tissues were obtained from 3 patients undergoing surgery for trauma or ischemic gangrene (mean age 60.67 years old). None of the 3 patients had a history of joint disease, and none of the samples showed macroscopically obvious progressed OA

changes. All OA and normal cartilage samples were obtained in accordance with the World Medical Association Declaration of Helsinki ethical principles.

Chondrocytes culture

Primary human articular cartilage or chondrocytes isolated from suckling mice knee joints and C28 cell lines (generously gifted from Dr. Peng Hang) were isolated and cultured as described previously[3]. Second or third passage chondrocytes were used in the experiments. Once reached 80% confluence, the chondrocytes were cultured for a further 12 hours in the presence of 10 ng/ml IL-1β, and then another 12 hours with 20 μM Resveratrol or 50 μM EX-527 respectively, also 5 mM 3-MA, 10 nM Rapamycin (Rapa), 10 μM AS1842856 or 2 μg GFP-FoxO1) were treated in each *vitro* experiment.

Mice

Briefly, the inducible $^{TamCart}Sirt1^{flox/flox}$ mice were generated by breeding $Sirt1^{flox/flox}$ mice (a gift from Prof. Gao Xiang, Nanjing University) with FVB-Tg (Col2a1-cre/ERT) KA3Smac/J mice (purchased from The Jackson Laboratory, Bar Harbor, ME; stock no. 006774). Housing in specific pathogen free. $^{TamCart}Sirt1^{flox/flox}$ mice and/or $^{TamCart}Sirt1^{flox/WT}$ mice were IP injected with four doses of tamoxifen (0.2 mg per mouse; Sigma-Aldrich; catalog no. WXBC3371V) at postnatal days 5,7, 9, and 11. Henceforth, we refer to the $^{TamCart}Sirt1$-/- mice as conditional knockout (CKO) mice, and the $Sirt1^{flox/flox}$ control littermates as control mice. For Genotyping and determination of cartilage-specific deletion of Sirt1. PCR analyze was utilized. $Sirt1^{flox/flox}$ mice are as follows: two forward primers, 5'-GGT TGA CTT AGG TCT TGT CTG-3' and 5'-AGG CGG ATT TCT GAG TTC GA-3', and a reverse primer, 5'-CGT CCC TTG TAA TGT TTC CC-3'. The 550-bp PCR product was detected in WT mice, and the 750-bp PCR product was detected in homozygous $Sirt1^{flox/flox}$ mice. The Cre transgene used the primers 5'-CAC TGC GGG CTC TAC TTC AT-3' (forward) and 5' -ACC AGC AGC ACT TTT GGA AG-3' (reverse) to amplify a 358-bp DNA product. At 8 weeks of age, 10 tissue-specific deletion of the Sirt1 gene and control mice per group were anesthetized and instability of the right knee joint was induced by transection of the anterior attachment of the medial meniscus to the tibial plateau. The sham operation involved the same surgery, except that the anterior medial meniscus ligament was visualized but left intact. This experiment was approved by the animal ethics committee of Xi'an Jiaotong University.

Histopathological assessment

Apoptotic cells in the growth plate were identified by an *In Situ* Cell Death Detection Kits (Roche, Germany). Sections were immunostained with SPlink Detection Kits (Beijing zhongshanjinqiao technology, China), followed by standardized development in DAB chromogen. Mice joints were counterstained by methyl green. For immunofluorescence staining, after using primary antibodies, at 4 °C overnight, then we use Goat Anti-Rabbit IgG Cy3 or Goat Anti-Mouse IgG H&L (FITC) 1:500 in PBS, 2 h at 37 °C. Images are captured at 40X magnification with a Nikon E800 microscope. Primary SIRT1, COL2A1, COL10A1, SOX9, MMP13, ADAMTS5 and VEGF antibodies were purchased from Abcam, USA. Primary ATG7, LC3, P62 and FoxO1 antibodies were purchased from Cell Signaling Technology, USA. The second antibodies or immunofluorescence antibodies were purchased from Xi'an Zhuangzhi Technology, China.

The positive cells were stained DAB or immunofluorescence cells, and all the cells in the pictures were taken for total cells.

Real-time PCR

The cDNA products were directly used for PCR or stored at − 80 °C for later analysis. Real-time quantitative PCR was performed in a MJ Mini Real-Time PCR Detection System using SYBR Premix Ex Taq II. The primers were: Gapdh, Forward 5'-TGACGCTGGGGCTGGCATTG-3', Reverse 5'-GCT CTT GCT GGG GCT GGT GG-3', *Sox9*, Forward 5'-AGG CAA GCA AAG GAG ATG AA-3', Reverse 5'- TGG TGT TCT GAG AGG CAC AG -3', *Col2a1*, Forward 5'- GAG CCC TGC CGG ATC TGT-3', Reverse 5'-GAG GCA GTC TTT CAC GTC TTC-3', *Mmp13*, Forward 5'- GCT GCG GTT CAC TTT GAG AA-3', Reverse 5'-GGC GGG GAT AAT CTT TGT CCA-3', *Adamts5*, Forward 5'-TTC AAC GTC AAG CCA TGG CAA CTG-3', Reverse 5'-TGA CGA TAG GCA AAC TGC ACT CCT-3', *Sirt1*, Forward 5'-AGG CGG ATT TCT GAG TTC GA-3', Reverse 5'-CGT CCC TTG TAA TGT TTC CC-3', *FoxO1*, Forward 5'-GCT TAG AGC AGA GAT GTT CTC ACA TT-3', Reverse 5'-CCA GAG TCT TTG TAT CAG GCA AAT AA-3', *Atg7*, Forward 5'-ATT GTG GCT CCT GCC CCA GT-3', Reverse 5'-CAG GAC AGA GAC CAT CAG CTC CAC-3'.

Western blotting

Proteins were separated by 6–12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE); separated proteins were then electronically blotted onto polyvinylidene difluoride membranes. The membranes were then blocked and subsequently incubated with designated primary antibodies (all primary antibodies were purchased from Cell Signaling Technology or Abcam, USA) overnight at 4 °C. At last, the blots were visualized by an enhanced chemiluminescence (ECL) detection system with a horseradish peroxidase-conjugated secondary antibody (Xi'an Zhuangzhi Technology, China).

Coimmunoprecipitation and chromatin immunoprecipitation

Cytoplasmic lysate (200 μg) was incubated for 2 h at 4 °C with the corresponding antibodies coupled to 20μL of packed protein A + G Sepharose beads. Immune complexes were resolved by means of SDS-PAGE and immune-blotted with the indicated antibodies, negative control used the same species IgG, input was the whole cell lysate. For chromatin immunoprecipitation, purified DNA was analyzed by real time PCR methods using SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, USA), according to the manufacturer's instructions. The amount of immunoprecipitation DNA in each sample is represented as signal relative to the total amount of input chromatin.

Statistical analysis

Data was presented as mean ± SD and statistically analyzed by using Graph Pad Prism 6.0. Student's *t* test and ANOVA with *post hoc* tests were used for pair wise and multiple comparisons, respectively, after normal distribution was confirmed using the *Shapiro–Wilk* test. Data quantified based on the OARSI ordinal grading system were analyzed using nonparametric statistical methods. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

Decreased Sirt1 and autophagy-related gene expression in human OA cartilage

To study the effects of Sirt1 on human cartilage, we collected joint samples from healthy and OA patients. The superficial articular cartilage was rough and fibrotic, the thickness of cartilage layer thinned and the total number of chondrocytes reduced in patients with osteoarthritis compared to normal-looking cartilage. The hypertrophic differentiation of articular chondrocytes increased, H&E and Safranin O staining showed that the cartilage of tibia plateau severely degenerated and loss of Safranin O staining in OA patient (Figure.1A). The expression of SIRT1 and ATG7 localized in the three zones of cartilage and expression levels significantly decreased in articular cartilage of OA patients, so did the levels of Beclin-1, ULK1 and LC3, while in contrast the expression of P62 increased (Figure.1B-E). Our results suggested that OA exhibited a declined expressions of the SIRT1 and autophagy-related markers.

IL1β-responsive SIRT1 promoted anabolic matrix synthesis in human primary chondrocytes

To determine the effects of SIRT1 on catabolic activities in human chondrocytes, the isolated primary chondrocytes were treated with interleukin 1β (IL1β), SIRT1 agonist Resveratrol (Res), and SIRT1 inhibitor EX-527. 10 ng/ml IL1β induced an OA-like changes and down-regulated the mRNA levels of *Col2a1* and up-regulated *Mmp13* (Supplemental Figure.1A). The expression level of SIRT1 decreased significantly under the stimulation of IL1β (Figure.2A). Meanwhile, IL1β caused an increase of ADAMTS5, MMP13 and VEGF, indicating the hypertrophic differentiation of cartilage chondrocytes. Strikingly, EX-527 inhibited IL-1β-responsive SIRT1 expression, with such effect being nullified by Resveratrol (Figure.2B).

To determine the effects of autophagy on OA-like changes in cartilage, we detected the expression of autophagy-related genes. Our data showed that expression of ATG7 and LC3 decreased, while expression of P62 increased in response to IL-1β, whereas EX-527 and Resveratrol had an adverse effect compared to the above results (Figure.2B). Consistent with the protein level, the mRNA levels had the similar change, EX-527 aggravated OA-like changes, while Res alleviated OA-like changes in part. (Figure.2D, E). All of these results indicated that SIRT1 plays the protective role on matrix proteoglycan synthesis via autophagy. Thus, Resveratrol ameliorated OA-like changes while EX-527 exacerbated OA pathogenesis in vitro.

Disruption of Sirt1 in cartilage caused accelerated chondrocytes apoptosis in mice

To confirm the specificity and efficiency of gene excision in the CKO mice, we took advantage of PCR analysis, immunofluorescence, and western blot at the end of the study period, as shown in Supplemental Figure.1B-E. No difference was found with respect to the body weight or body length of mice at birth between CKO and control littermates (data not shown).

Safranin-O fast green (SO) staining was used to evaluate the articular cartilage of the medial femoral condyle and the tibial plateau. DMM mice exhibited significant cartilage degradation as indicated by a higher OARSI score compared with sham mice (Figure.3C). Cartilage was thinner in Sirt1-CKO mice than that in control mice at one months and two months post-DMM respectively, and the OARSI scores showed higher scores of tibia plateau and summed score in CKO OA mice versus control DMM mice (Figure.3A-D). H&E staining also showed less and irregular chondrocytes in CKO mice (Supplemental Figure.2A). 12months post-DMM mice demonstrated that articular cartilage of CKO DMM-mice was almost deprived, the OARSI scores also demonstrated higher scores of tibia plateau and summed score in CKO OA mice versus control DMM mice (Figure.3E, F). Next, the effects of Sirt1 ablation on OA chondrocyte apoptosis by in situ cell death was evaluated, when two months after DMM as a representative study period (Figure.3G). The number of TUNEL-positive cells was increased in the CKO DMM mice (Figure.3H), so did the levels of Caspase3 (Figure.3G, H). Therefore, our data suggested that disruption of *Sirt1* caused chondrocytes apoptosis in mice.

Sirt1 deletion accelerated chondrocytes hypertrophy and cartilage damage in PTOA

Firstly, efficiency of Sirt1 knockout in cartilage was confirmed (Supplement Fig. 2A). To determine the effects of Sirt1 on matrix biosynthesis, we detected the expression of both anabolism markers (COL2A1 and SOX9) and hypertrophic markers (COL10A1 and VEGF). Our results showed that COL2A1 and SOX9 decreased while hypertrophy markers increased in CKO-DMM mice in contrast to CON-DMM mice (Figure.4.A, B). Also, the catabolism markers MMP13 and ADAMTS5 were analyzed, like the hypertrophic markers, MMP13 and ADAMTS5 both up-regulated in CKO-DMM mice (Figure.4.C, D). In consideration of previous study on autophagy in OA, we then examined ATG7, P62, LC3, Beclin-1 and ULK1 in mice tibia plateau. The levels of ATG7, LC3, Beclin-1 and ULK1 reduced in CKO-DMM mice (Figure.4.C, D and supplemental Figure.2B), while the expression of P62 was up-regulated in CKO-DMM mice on the contrary (Supplemental Figure.2B), which showed a more severe OA-like changes. Taken together, deletion of Sirt1 in cartilage exacerbated cartilage degeneration.

SIRT1 imported and relocated FoxO1 from cytoplasm to nucleus

To determine the crosstalk between *Sirt1* and FoxO1, we cultured human C28 cell line *in vitro* and used co-immunoprecipitation (Co-IP) to detect the interaction between SIRT1, FoxO1 and ATG7. As noted in Figure.5A, by using an antibody directed against endogenous ATG7 and FoxO1, we co-precipitated endogenous SIRT1 from C28 cell lines. Also, co-precipitation of endogenous ATG7 or FoxO1 showed that three proteins had obvious interactions with each other (Figure.5B, C). To further understand the nucleo-cytoplasmic shuttling of FoxO1 under the Sirt1 regulation, we employed the Laser Scanning Confocal Microscope to track the translocation in C28 treated with EX-527 or Res. Increased SIRT1 activity in

nucleus contributed to the FoxO1 transcriptional activity from cytoplasm to nuclear (Figure.5D). IHC staining was used to detect the FoxO1 protein expression levels in knee joint cartilage samples from human and mice. Consistently, FoxO1 was observed in OA patients and CKO DMM-mice, in keeping with the levels of SIRT1 and ATG7, FoxO1 decreased in OA or deletion of Sirt1 as well (Figure.5E,F).

Rapamycin induced autophagy by inhibiting mTOR, 3-Methyladenine (3-MA), a selective PI3K inhibitor, also blocks the formation of autophagosomes. In the OA-like changes primary cultured mice chondrocytes, 3-MA aggregated these changes, as Rapa rescued them partially, as observed by the expression of MMP13, ADAMTS5 and VEGF (Supplemental Figure.3A, B). Meanwhile the results showed a reduction in SIRT1, LC3 conversion, and FoxO1 expression, with such effects being rescued by Rapamycin. In contrast, P62 expression was up-regulated (Supplemental Figure.3C, D). These data suggest that SIRT1, FoxO1 and autophagy all participated in osteoarthritis.

Sirt1 regulated autophagy via Sirt1/FoxO1/autophagy pathway

To further understand the positive interaction between SIRT1, FoxO1 and autophagy in osteoarthritis, chondrocytes were transfected with graded concentrations of GFP-FoxO1 plasmid and AS1842856. Then the levels of SIRT1 and ATG7 were detected, FoxO1 over expression up-regulates SIRT1 and ATG7 expression, in contrast, AS1842856-treated cells showed a dose-dependent reduction of SIRT1 and ATG7 expression (Fig. 6A). Indeed, different concentrations of Res and EX-527 also were used to detect the levels of FoxO1 and ATG7. The levels of FoxO1 and ATG7 increased significantly in increased concentrations Res-treated cells, which were in contrast to the EX-527-treated cells (Figure.6B). We further used Sirt1 siRNA to examine its effect on IL-1β-induced chondrocytes. Sirt1 siRNA decreased SIRT1 and FoxO1 expression while increased MMP13 and ADAMTS5 expression in IL-1β-induced OA-like changes (Figure.6C). RT-PCR was further applied to detect the expression of FoxO1 mRNA, which showed that inhibition of FoxO1 exacerbated the OA-like changes while p-FoxO1 reversed the effects partially (Figure.6D). As a transcription factor, the function of FoxO1 was elaborated using chromatin immune-precipitation (ChIP). RT-PCR results showed that FoxO1 bonds both Sirt1 and Atg7 promoters (Figure.6E). Overall, FoxO1 may mediate an auto-feedback loop regulating SIRT1 expression and autophagy activity in cartilage.

Discussion

We previously identified that both Sirt1 and autophagy were important regulators in cartilage development, growth and health including endochondral ossification and aging-related OA. In this work, we aimed to investigate the role of Sirt1 in the regulation of chondrocyte autophagy in the pathogenesis of OA. The OA cartilage samples showed reduced Sirt1-FoxO1 expression. Based on this observation we teased the Sirt1's role in chondrocyte autophagy using a novel Col2-ERT-Cre; $Sirt1^{flox/flox}$ mice. SIRT1 remarkably decreased in the advanced OA stage of mouse OA model. Meanwhile, the expression levels of

FoxO1 and autophagy also reduced. Our data further revealed that Sirt1 regulated autophagy via nucleo-cytoplasmic shuttling of FoxO1.

FoxO1, as a transcription factor, mediates an auto-feedback loop regulating SIRT1 *in vitro*[16], while FoxO1 antagonist suppresses autophagy and lipid droplet growth in adipocytes[20]. Moreover, FoxO1-mediated autophagy imbalanced anabolic and catabolic in human OA-like changes chondrocytes[21]. However, the potential mechanism of autophagy activity in arthritis has not been fully elucidated. Autophagy played a diphasic role in the pathogenesis of articular cartilage degeneration [22]. It was reported that autophagy may protect cartilage from matrix degradation[23]. Previous literature that SIRT1, FoxO1 and autophagy are highly expressed in normal cartilage, while those decrease in severely degenerated cartilage respectively [24, 25]. While the direct relationship between FoxO1 and autophagy is not clearly defined. We observed increased cartilage-degrading enzymes, hypertrophic proteins and damaged cartilage-anabolism in human and mice. One possible mechanism for these observations is the decrease autophagy regulated by Sirt1 and FoxO1.

Sirt1 deacetylase is an important regulator of autophagy both *in vivo* and *in vitro*[14, 26]. Increased Sirt1 expression is beneficial for OA osteoblasts and attenuates the progression of osteoarthritis in mice OA models [27, 28]. To strengthen our hypothesis that Sirt1 is chondro-protective, the Sirt1 activator Res and inhibitor EX-527 were utilized to test the benefits on primary human OA-like chondrocytes. Our results suggested that Sirt1 was involved in cellular stress, especially in the early cartilage degeneration process through SIRT1/P53/P21 pathway[19]. To further answer the question that how Sirt1 modulates the autophagy to achieve the preventive effects in the OA development and progression, our previous work showed that Sirt1 or Atg7 deletion in cartilage delay the progress of endochondral ossification[3, 12]. These findings consist with precious studies that cartilage-specific FoxO1 or autophagy deficiency impaired[29, 30]. Moreover, our results revealed that FoxO1 and autophagy decreased in OA patients and Sirt1-CKO mice after surgery. Taken our results and previous studies together, FoxO1 may bridge the gap of how SIRT1 regulates autophagy in OA. Thus, decreased autophagy activity in Sirt1-CKO mice might be related to the Sirt1 deficiency or FoxO1 inhibition.

We planned to determine whether Sirt1 regulated autophagy through FoxO1 in chondrocytes. Given that there is an interaction between SIRT1 and ATG7 in Hela cells and mouse embryonic fibroblasts [14], and FoxO1 was significantly mediated by SIRT1 and other histone deacetylases in neutrophils [31, 32]. We also investigated the interaction between ATG7, SIRT1 and FoxO1 in chondrocytes, unlike in the cancer cells or liver, and the involvement of autophagy and FoxO1 in OA progress. Previous study shows FoxO1 and FoxO3 deletion exhibit more severity cartilage degradation than FoxO4 [29, 31, 32], which suggested that FoxO transcription factors modulate autophagy and proteoglycan 4 in cartilage homeostasis and OA. By using C28 cell lines, it is confirmed that ATG7, FoxO1 and SIRT1 have an apparent interaction, which is supported by precious studies in other organs[14, 33, 34]. Our study demonstrated that Sirt1 regulated FoxO1 transcriptional activity, and then FoxO1 mediated autophagy to maintain cartilage homeostasis via cartilage hypertrophy and apoptosis.

To further verify the direct connection between Sirt1 and autophagy, it is important to determine the function of autophagosome formation in Sirt1 deleted mice. To target the Sirt1-FoxO1 axis in an autophagy-dependent manner in arthritis, several potential drugs could be beneficial to arthritis treatment. Except Resveratrol used in this study, the old drug metformin may alleviate oxidative stress and enhances autophagy in diabetic kidney disease via AMPK/SIRT1-FoxO1 pathway[35], and by enhancing autophagy flux and decreasing the release of vWF and P-selectin. The Sirt1/FoxO1 pathway is also a promising target to prevent atherosclerosis (AS) and arterial thrombosis [36]. Similar to aging and mechanical stress-induced chronic inflammatory diseases in kidney and cardiovascular system, our previous reports[19] and current observations suggested that Sirt1 possibly protected against injury-induced OA with aging via SIRT1/FoxO1/autophagy during OA progress.

There are some limitations to our study. The connection between autophagy and OA progression, and Sirt1's role in this mechanism was reported by other groups to some extent, while we attempted to make a leap conceptually and serves to validate previous findings regarding Sirt1's protective role in cartilage supporting autophagy in normal chondrocytes. To examine whether deletion of Sirt1 in mice is dependent on age and/ or the surgical method, we selectively delete Sirt1 gene in cartilage. We further explored the role of SIRT1 in tamoxifen-induced cartilage-specific deletion of Sirt1 male mice with trauma-induced OA followed by a 12-month duration, which differed from that the small sample size (n = 4) in young mice used for the surgical induction of OA[5, 17]. Another concern of previous reports may not represent OA pathology in humans because the observation lasted only for 8 weeks after surgery with insignificant OA scores between Sirt1-CKO and control mice. Lastly, the direct association between Sirt1/FoxO1/autophagy requires further investigation.

Conclusions

In conclusion, cartilage-specific Sirt1-deficient accelerates the OA progression under mechanical instability condition which suggest the chondro-protective role of Sirt1 against the development of OA via Sirt1/FoxO1/autophagy pathway. Further studies will provide novel and deep insight into pathomechanisms of OA that may develop new therapeutic approaches.

Declarations

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Authors contribution

Study conception and design: Mao Xu and Shufang Wu. Acquisition of data: Mao Xu, Zhuang Qian, Ying Zhang, Xin Gao, Zhengmin Ma. Analysis and interpretation of data: Mao Xu, Xiaomin Kang, Xinxin Jin. All

authors made substantial contribution service, this manuscript for intellectual content and approved the final version to be published. Shufang Wu had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal studies were approved by the animal ethics committee of Xi'an Jiaotong University.

Consent for publication

The data presented in this manuscript have not been submitted or published elsewhere.

Conflict of interest

The authors declare that they have no competing interests.

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Figures

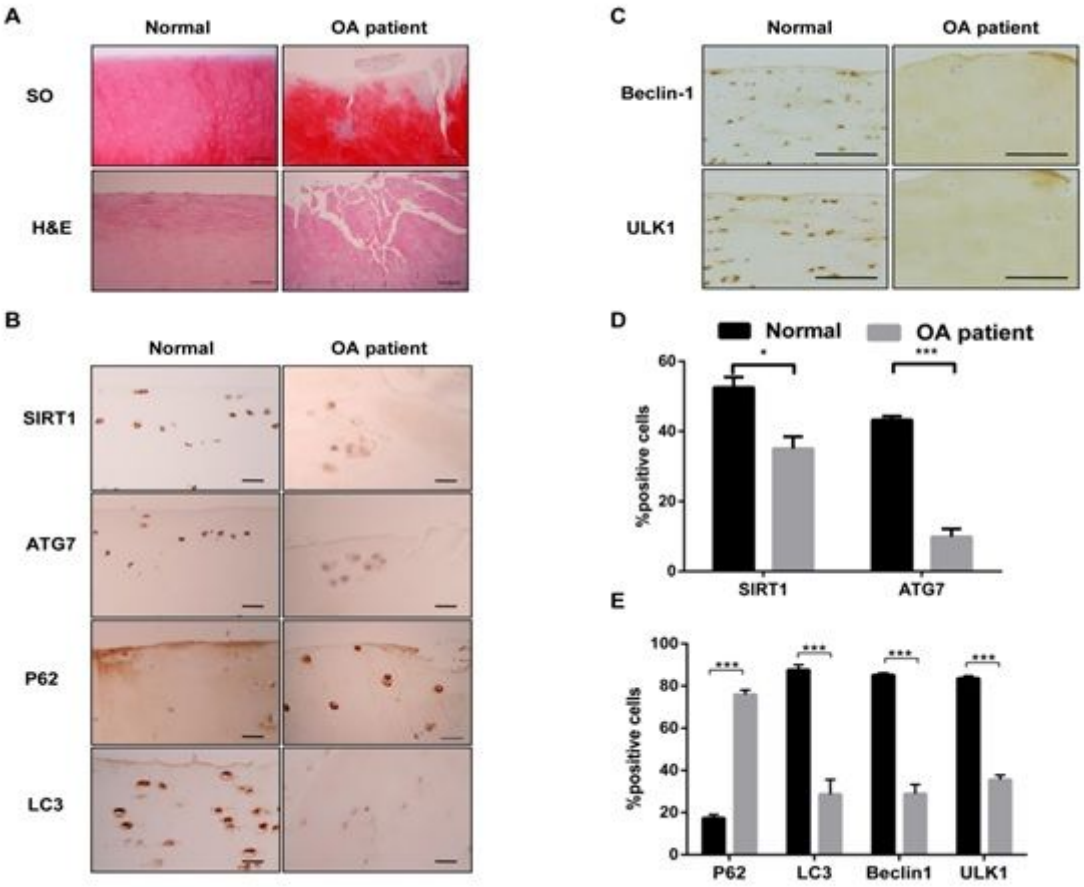

Figure 1

Decreased Sirt1 and autophagy-related genes in human OA articular cartilage. (A) The H&E and Safranin-O (SO) staining on articular cartilage in normal and OA patient. (B-C) The representative immunohistochemistry images in normal and OA patient knee joints (B, Scale bar=25μm; C, Scale bar=100μm). (D-E) Quantitative analysis of positive cells. Data are presented as mean ± SD (n=6/group, Student t test).

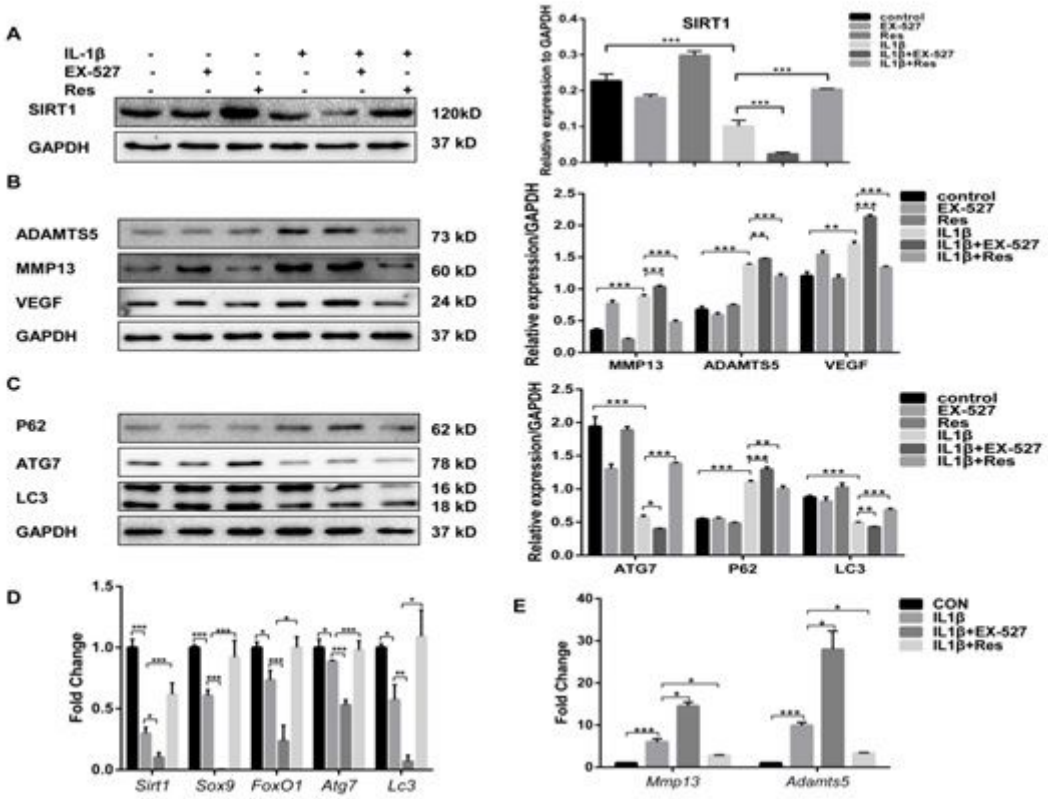

Figure 2

IL1β-responsive SIRT1 promoted anabolic matrix synthesis in human primary chondrocytes. (A) The protein level of SIRT1 in human primary chondrocytes. (B) The protein level of ADAMTS5, MMP13 and VEGF. (C) The protein level of ATG7, P62 and LC3. (D-E) qRT-PCR analysis of chondrocytes anabolic and catabolic genes. Data are presented as mean ± SD. (n=3/group, one-way ANOVA).

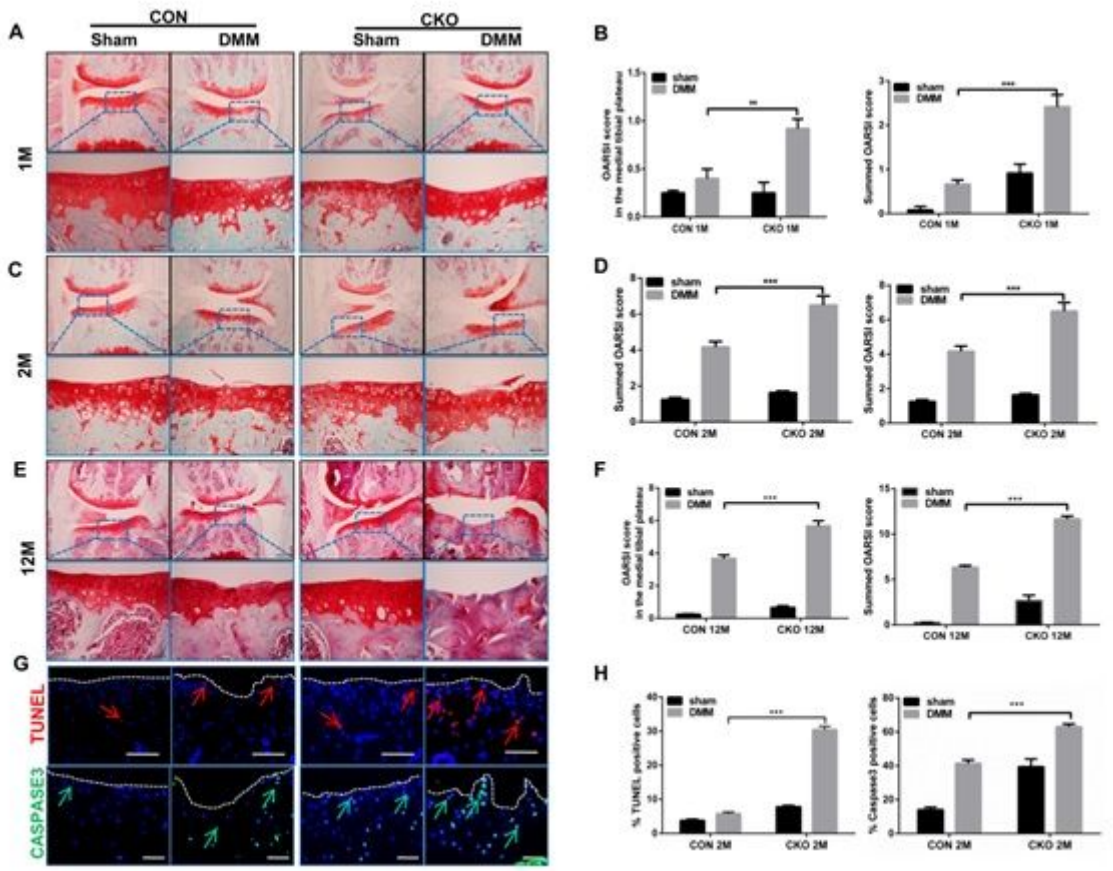

Figure 3

Disruption of Sirt1 in cartilage caused accelerated chondrocytes apoptosis in mice. Safranin-O fast green staining in mice articular cartilage after DMM and sham surgery in (A) One month, (C) Two months and (E) Twelve months (upper scale bar=100μm, under scale bar=25μm). The OARSI score of medial tibial plateau (MTP) and summed score in (B) One months , (D) Two months and (F) Twelve months. (G) Representative images of the TUNEL and CASPASE3-positive cells two months after post-DMM (upper scale bar=50μm, under scale bar=25μm). (H) Quantitative analysis of % positive cells. Arrows indicate positive cells. Data are presented as mean ± SD (1M=one months, 2M=two months, 12M=12months, n=10/group, two-way ANOVA).

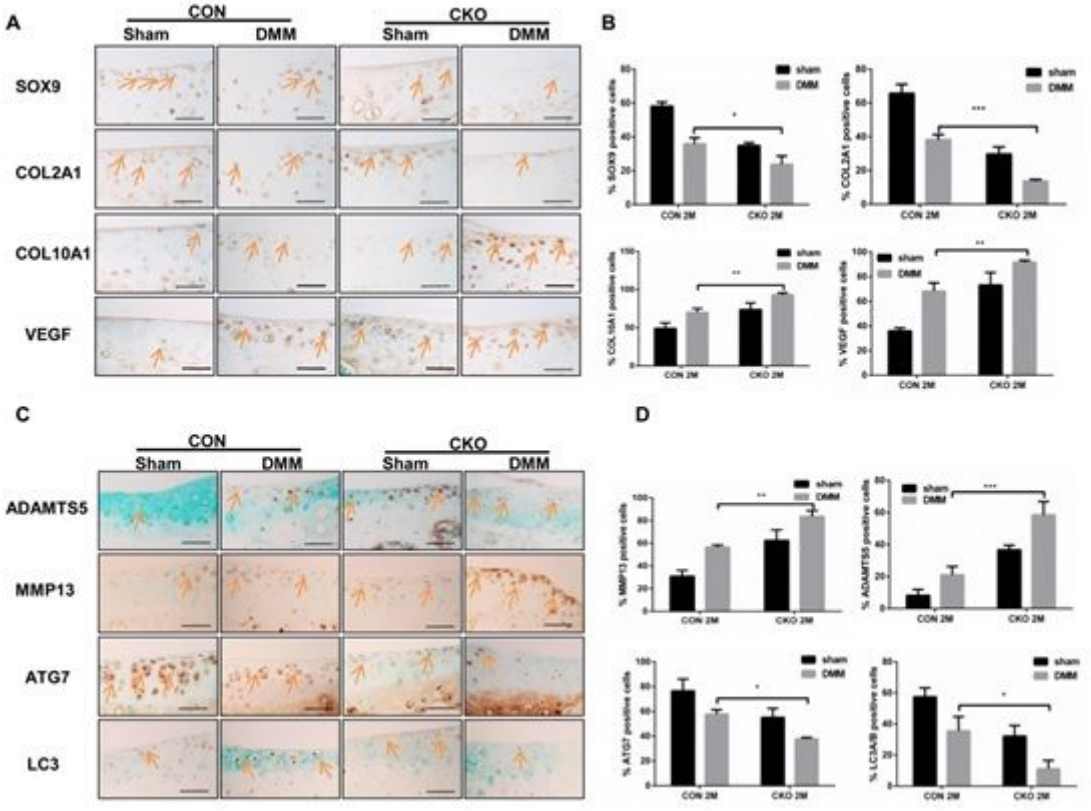

Figure 4

Sirt1 deletion accelerated chondrocytes hypertrophy and cartilage damage in PTOA. Representative images of immunohistochemistry for (A) COL2A1, SOX9, COL10A1 and VEGF, (B) MMP13, ADAMTS5, ATG7 and LC3 of articular cartilage in the control and CKO articular cartilage after DMM in two months (scale bar=25μm). (C-D) Quantitative analysis of %positive cells. Arrows indicate positive cells. Data are presented as mean ± SD (n=10/group, two-way ANOVA).

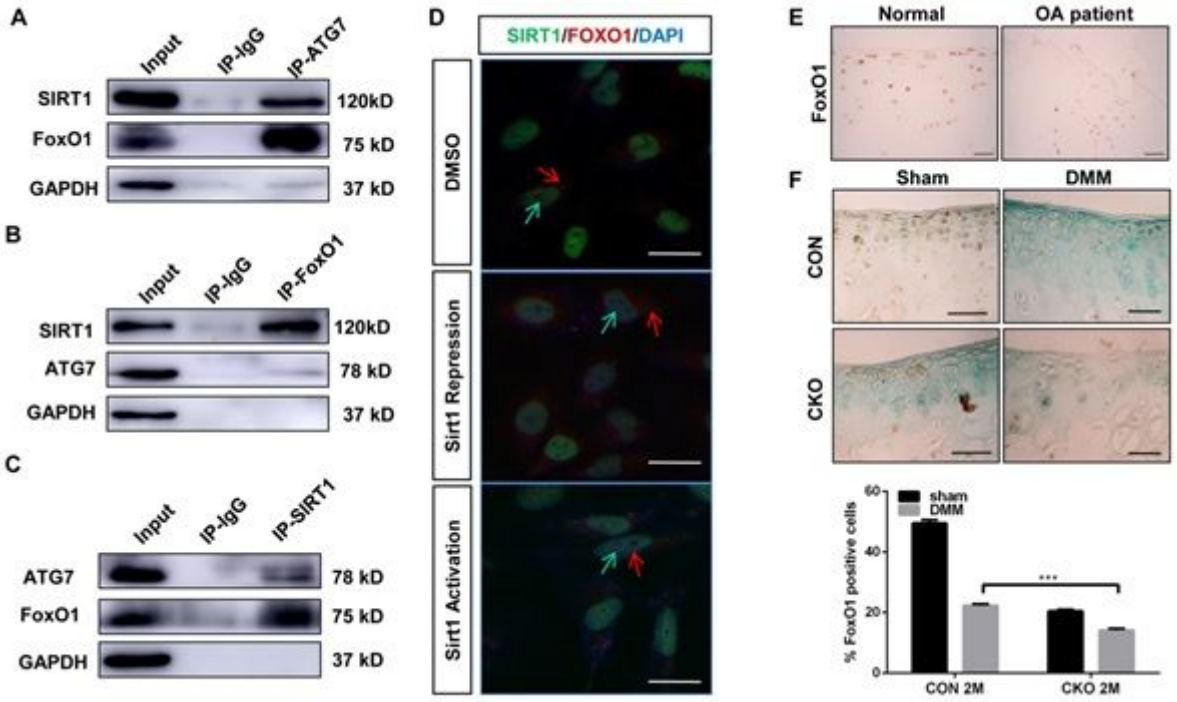

Figure 5

Sirt1 imported and relocated FoxO1 from cytoplasm to nucleus. (A-C) CoIP results of ATG7, SIRT1 or FoxO1 in C28 cells. (D) The representative image of immunohistochemistry for FoxO1 of the normal and OA patient (scale bar=25μm). (E) The representative image of immunohistochemistry for FoxO1 of control and CKO DMM articular cartilage (scale bar=25μm). (F) Quantitative analysis of FoxO1-positive cells. (G) SIRT1 and FoxO1 in situ expression by Laser Scanning Confocal Microscope in C28. Green arrows indicate SIRT1, Red arrows indicate FoxO1.

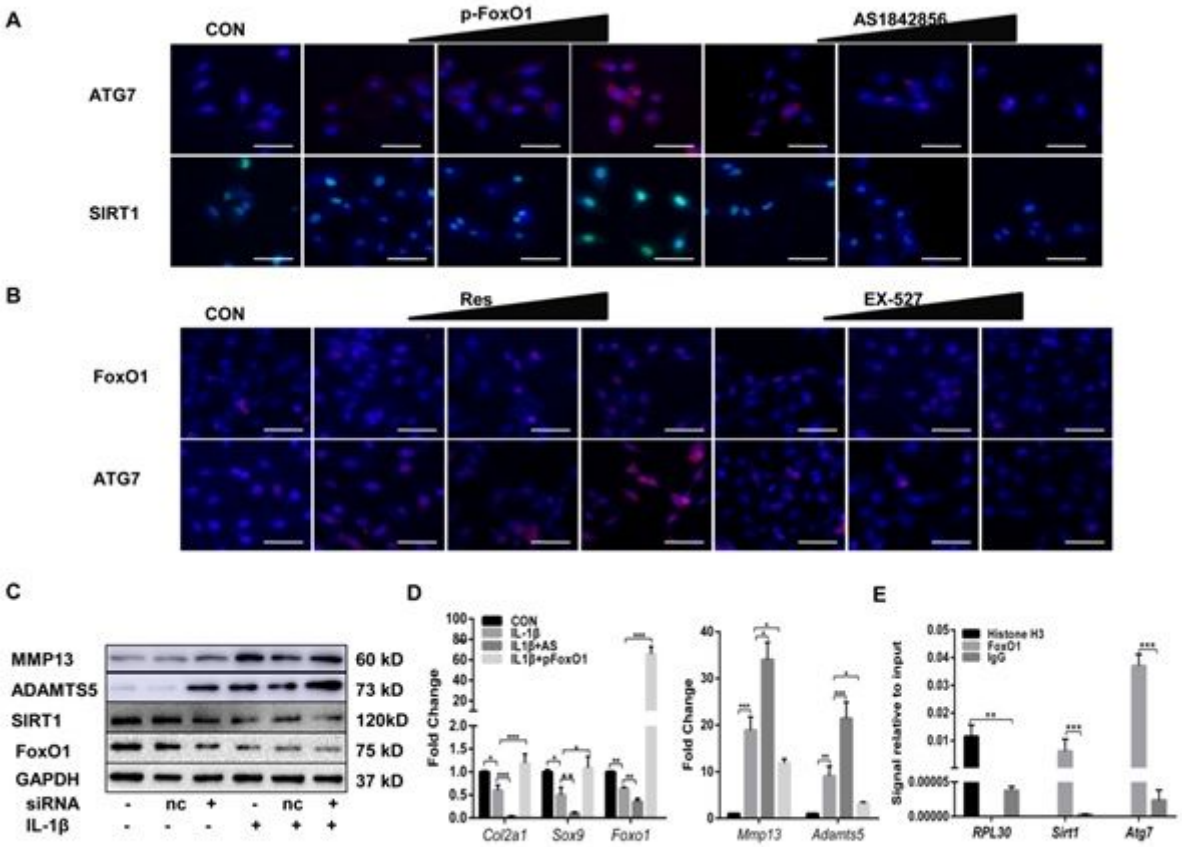

Figure 6

Sirt1 regulated autophagy via SIRT1-FoxO1-Autophagy pathway. Representative images of immunofluorescence for (A) ATG7 and SIRT1 (B) ATG7 and FoxO1 in C28 cells. (C) Western blot analysis after treated with negative control or SiRNA. (D) qRT-PCR analysis of chondrocytes differentiation and hypertrophy genes. (E) Purified DNA was analyzed by RT-PCR methods using ChIP. Data are presented as mean ± SD. (n=3/group, *P< 0.05).

Supplementary Files

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