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ORIGINAL ARTICLE

Estrogen receptor is involved in the osteoarthritis mediated by ATG16L1-NLRP3 activation

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ABSTRACT

Objectives: This study aims to explore the mechanisms of dual regulation of osteoarthritis (OA) progression by the involvement of estrogen receptor (ER) in autophagy and inflammation.

Materials and methods: Bioinformatics methods were used to explore the relationship among associated genes. Western blot assays were used to detect related protein expression of OA in C28I2 and induced OA cellular model. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis were used to detect OA related gene expression in C28I2 and induced OA cellular model. Co-immunoprecipitation (CO-IP) analysis were used to verify the direct interaction between ER and NOD-like receptor thermal protein domain associated protein 3 (NLRP3).

Results: The C28I2 cellular model of OA was induced by interleukin-1β (IL-1β). The small interfering ribonucleic acid (SiRNA)-mediated knockdown of autophagy-related 16 like 1 (ATG16L1) in C28I2 decreased the expression of MAP1LC3B (LC3B) and NLRP3. Besides, ER-beta (ERβ) agonist changed the gene expression of NLRP3 and ATG16L1. Moreover, CO-IP analysis indicated the direct interaction between ER and NLRP3.

Conclusion: Our study results revealed that ATG16L1, NLRP3, and IL-1β interacted closely and ERβ was involved in OA process by affecting autophagy and inflammatory activation.

Keywords: ATG16L1, estrogen receptor, interleukin-1 beta, NLRP3, osteoarthritis.

Osteoarthritis (OA) is a degenerative joint disease caused by cartilage degeneration, resulting in weight-bearing joint dysfunction,[1] high disability rate, and poor postoperative results, affecting more than 300 million individuals worldwide.[2,3] Currently, early treatment of OA is focused on symptomatic relief, while the preferred treatment option for patients with end-stage disease is total knee arthroplasty. However, one-third of patients still have residual symptoms after surgery and fail to achieve a satisfactory clinical outcome. Recent studies have demonstrated that the pathogenesis of OA is cartilage degeneration caused by long-term loading of the joint, genetics and low-intensity inflammation.[4,5] Clarifying the relevant molecular roles involved in the degenerative process is of utmost importance for the effective prevention and treatment of OA.

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Chondrocytes, as the unique type of cell in cartilage tissue, can express a variety of receptor proteins associated with innate immunity, and produce inflammatory and catabolic mediators, which promote local chronic inflammation in arthrosis, and ultimately induce cartilage aging and degeneration.[6] However, the refined molecular mechanism of the degeneration development is not acclaimed. Interleukin-1 beta (IL-1β) is important in the process of cartilage senescence and degeneration, as it not only directly participates in cartilage

degeneration, but also drives the chondrocyte inflammatory cascade.[7] Nevertheless, in clinical and animal experiments, local treatment of OA with IL-1β inhibitors has not achieved convincing results yet.[8,9]

The NOD-like receptor thermal protein domain associated protein 3 (NLRP3) expression was identified to be enhanced in human and mouse OA cartilage, and by blocking NLRP3 expression in mice, cartilage degeneration and chondrocyte damage could be attenuated and cartilage degeneration prevented.[10] The NLRP3 inflammasome is a key cytoplasmic sensor in innate immunity, which is a filamentous signaling platform composed of the sensor protein NLRP3, the adaptor protein apoptosis-associated speck-like protein (ASC) and the protease Caspase-1.[11,12] The NLRP3 and ASC is combined to recruit pro-caspase-1 forming a complex and generates biologically active caspase-1, which acts on the pro-IL-1β inflammatory factor precursor to activate IL-1β. Therefore, activation of the NLRP3 inflammasome may be a capable marker of OA development.

Recently, autophagy may be involved in the entire process of chondrocyte senescence and related to NLRP3 inflammatory vesicles, in which the autophagy-like protein autophagy-related 16 like 1 (ATG16L1) as s major participants in autophagy encoded by the autophagy gene is a pivotal component in the formation of the ATG16L1-Atg5-Atg12 complex, which can also recruit MAP1LC3 (LC3) protein to regulate the initiation and maturation of autophagosomes.[13,14] Estrogen receptor (ER) α and β genes have shown expression in human chondrocytes, which affect osteoblasts through the NLRP3/Caspase-1 pathway, and the risk of OA occurrence and progression and improves its clinical symptoms can be reduced by estrogen replacement therapy.[15,16] Self-reported OA showed 46% and 21% of women and men aged 45 years and over in the region, respectively indicating the sexual dimorphism effect of OA and the potential effectiveness of estrogen therapy.[17] However, dual regulation of ER in OA process through autophagy and inflammasome activation has not been reported, which needs further investigation.

In the present study, we hypothesized that whether ER acted on autophagy-like protein ATG16L1 to activate NLRP3 inflammasome directly and affected the process of OA cartilage degeneration. We, therefore, aimed to explore the mechanisms of dual regulation of OA progression by the involvement of ER in autophagy and inflammation.

MATERIALS AND METHODS

Study design

The Western blot, real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence assays were performed in Human Chondrocyte Cell Line (C28/I2) and its induced OA cell models. Experiments were performed in triplicate and repeated three times with similar results.

Pathway analysis

The STRING database collected multiple public databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG), National Center for Biotechnology Information (NCBI) and Gene Ontology, integrated these data to generate a comprehensive protein-protein interaction network database. The STRING database provides visualization of protein interaction networks, and information on protein families, pathways, and subcellular localization. The Welch's t-test was applied to investigate the significantly interactions between ATG16L1, NLRP3 and IL-1β. Cytoscape 3.9.1 was used for network diagrams creation of functional pathway, possible extracellular and extracellular signaling pathways. Imported STRING database interaction analysis data, where node1 and node2 are a pair of nodes and the combined score is the interaction score. Then, perform node and network analyzed of imported data were selected. When the size of node as a continuous variable is changed with more interaction lines between nodes, the node becomes larger. Besides, the strength of interaction between nodes is reflected by the combined score.

Cell culture and treatment

The C28/I2 cell line was a kindly gift from Doctor Huiyao Lan. This cell line was cultured in 10 cm culture dishes with F12 medium (GIBCO, USA) with 10% fetal bovine serum (Sijiqing Biological Engineering Materials Co., Hangzhou, China). Cells were stimulated with the indicated amount of recombinant human IL-1β (PeproTech, DE, USA) for 12 h with serum-starved, when approximately 50% confluence was reached, and followed by incubation with recombinant TGF-β1 (10 ng/mL; R&D Systems, Minneapolis, MN, USA) for the indicated time period.

Western blot and immunoprecipitation

The cells were washed with ice-cold phosphate-buffered saline (PBS) and were solubilized in lysis buffer complemented with protease inhibitors and centrifuged at 12,000 rpm for 15 min at 4°C.

The protein concentration of the supernatant was determined by bicinchoninic acid assay (BCA) assay kit (Epizyme Biotech, Shanghai, China). For Western blotting, 20 to 30 µg of protein was separated in 7.5 to 12% acrylamide Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) gel (Epizyme Biotech, Shanghai, China), and proteins were detected using the primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Protein expression levels were visualized by enhanced chemiluminescence. Antibody information is summarized in Table I.

For immunoprecipitation, the sample with 700 µg of protein was diluted to 1 mg/mL in non-denaturing lysis buffer with protease inhibitors, of which 60 µL was taken out as input. A total of 20 µL of protein A/G Magnetic Beads was washed by washing buffer three times and mixed with respective antibodies on a rotating platform at room temperature for 30 min. The precipitate was incubated with remaining lysate overnight at 4°C. Beads were, then, washed three times with washing buffer and boiled for 5 min with 1x loading dye. The supernatant obtained after centrifugation was analyzed using the Western blot assay.

RT-qPCR

Total ribonucleic acid (RNA) was converted to complementary deoxyribonucleic acid (cDNA) using a one-step reverse transcription PCR kit according to the manufacturer's instructions. The cDNA was subject to qPCR amplification using the CFX96 RT-PCR detection system (Bio-Rad, Hercules, California, USA). Primer sequences are included in Table II.

Immunofluorescence

The cells in the treated and untreated groups were fixed in 4% paraformaldehyde solution for 20 min, washed three times with PBS, permeabilized

TABLE I
List of antibodies used in this experiment

Antibodies	Source	Dilution
NLRP3	Abcam (ab15101)	1:1000 for WB, 1:100 for IF
ER-α	Proteintech (21244-1-AP)	1:800 for WB, 1:100 for IF
ER-β	Proteintech (14007-1-APC)	1:800 for WB
LC-3B	Abcam (ab192890)	1:1000 for WB
MMP	Abcam (ab134184)	1:1000 for WB
ATG16L1	Abcam (ab187671)	1:1000 for WB
TNF-α	Abcam (ab66579)	1:1000 for WB
GAPDH	Cell Signaling Technology (5174)	1:1000 for WB

NLRP3: NOD-like receptor thermal protein domain associated protein 3; ER: Estrogen receptor; MMP13: Matrix metalloproteinase 13; ATG16L1: Autophagy-related 16 like 1; TNF-α: Tumor necrosis factor alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

TABLE II
List of primers used in this experiment

Primer name	Sequence (5'-3')	Primer
NLRP3	GATCTTCGCTGCGATCAACAG	Forward primer
	CGTGCATTATCTGAACCCCAC	Reverse primer
ATG16L1	AACGCTGTGCAGTTCAGTCC	Forward primer
	AGCTGCTAAGAGGTAAGATCCA	Reverse primer
GAPDH	GGAGCGAGATCCCTCCAAAAT	Forward primer
	GGCTGTTGTCATACTTCTCATGG	Reverse primer

NLRP3: NOD-like receptor thermal protein domain associated protein; ATG16L1: Autophagy-related 16 like 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

with 0.5% Triton X-100 for 5 min, and washed three times with PBS. The cells were closed with immunofluorescence blocking solution for 30 min at room temperature, incubated overnight with anti-ER primary antibody and anti-NLRP3 primary antibody (1:100), washed three times with PBS and incubated for 60 min with secondary antibody (1:100), washed three times with PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, and washed three times with PBS.

Statistical analysis

Statistical significance was calculated with a one-way ANOVA corrected for multiple comparisons. Statistical analyses were performed on GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA). The Student t-test was used to compare variables between the groups. A p value of <0.05 was considered statistically significant.

RESULTS

ER-ATG16L1-NLRP3 signaling pathway and interaction analysis with bioinformatics software

To detect the effect of ATG16L1 gene on NLRP3 in the pathogenesis of OA, bioinformatics methods were employed for the interaction and pathway analysis, and interactions among ATG16L1, NLRP3, and IL-1β were discovered. These genes constitute a complete functional pathway and possible extracellular and extracellular signaling pathways were speculated: Nucleotide-binding domain leucine-rich receptor signaling pathway and NF-κB signaling pathway, which significantly induced the expression of inflammatory cytokines; i.e., IL-1β and IL-1 (Figure 1).

Effects of ER for autophagy and inflammation proteins

The increased expression of inflammatory protein was detected after the IL-1β treatment in C28I2 cells (Figure 2). The expression differences of ER were exhibited between normal and inflammatory cell groups. After treatment with selective ER-α (A) and β (B) agonist, the decreased expression of related autophagy and inflammatory proteins were detected. Besides, the estrogen α and β receptor agitator agonist treatment induced the different decreased degree of related autophagy and inflammatory protein expression, which indicated the effect of ER on OA process.

Effects of siRNA-mediated knockdown of ATG16L1 for autophagy and inflammation proteins

As showed in Figure 3a, the fluorescence intensities of ATG16L1 decreased after transfection,

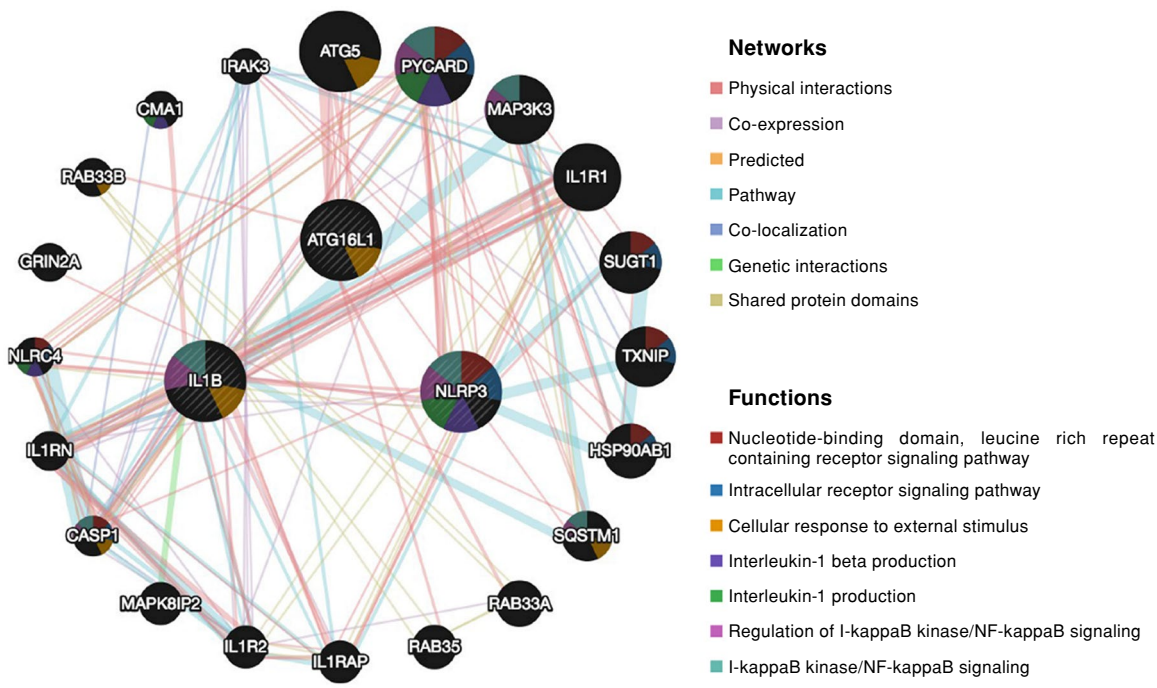


FIGURE 1. Analysis of the relationship between ATG16L1 and NLRP3 using bioinformatics software.
ATG16L1: Autophagy-related 16 like 1; NLRP3: NOD-like receptor thermal protein domain associated protein.

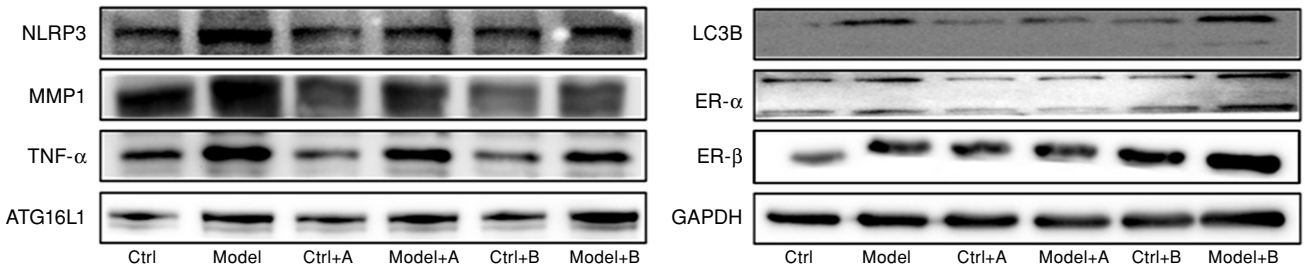


FIGURE 2. The expression of autophagy and inflammatory related proteins before and after ER intervention. NLRP3, MMP1, TNF-α, Atgl6L1, LC3B, ER-α and ER-β were involved in western blot analysis.

ER: Estrogen receptor; NLRP3: NOD-like receptor thermal protein domain associated protein; MMP1: Matrix metalloproteinase 1; TNF-α: Tumor necrosis factor alpha; Atgl6L1: Autophagy-related 16 like 1; LC3B: Light chain 3 beta; ER-α: Estrogen receptor-alpha; ER-β:Estrogen receptor-beta.

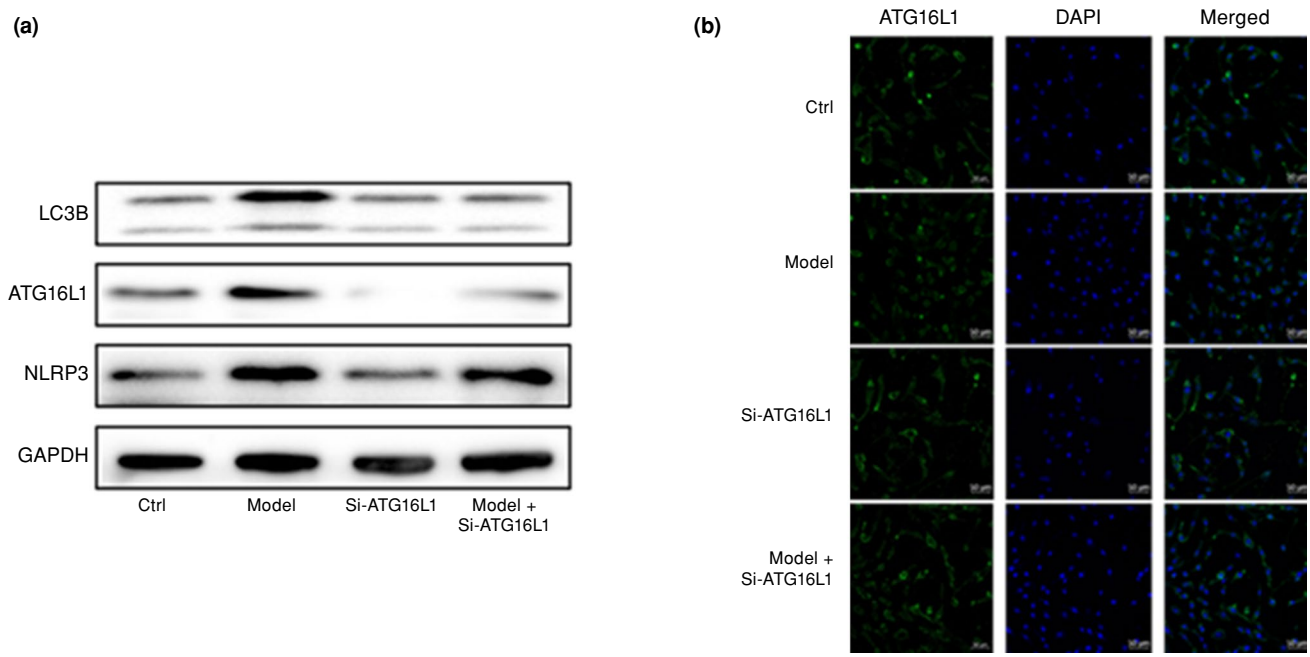


FIGURE 3. The expression of autophagy and inflammatory related proteins before and after ATG16L1 protein silencing treatment. **(a)** LC3B, ATG16L1 and NLRP3 were involved in WB analysis; **(b)** The expression ATG16L1 was detected by Confocal laser analysis.

LC3B: Light chain 3 beta; ATG16L1: Autophagy-related 16 like 1; NLRP3: NOD-like receptor thermal protein domain associated protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: Diamidino-2-phenylindole; Ctrl: Control; Si-ATG16L1: SiRNA-Autophagy-related 16 like 1.

which was consistent with results of Western blot. Besides, compared to the model group, both NLRP3 and LC3B expressions were reduced (Figure 3b), indicating the regulatory role of ATG16L1 on autophagy and inflammation process in C28I2 cells.

Direct protein-protein interaction by ER and NLRP3

The RT-qPCR analysis exhibited the increased gene expression of ATG16L1 and NLRP3 after treated with IL-1β in C28I2 cells. However, in the IL-1β and ER-β agonist combined treatment group, the expression of ATG16L1 was upregulated with a downregulated expression change for NLRP3 (Figure 4a). Further CO-IP analysis confirmed the direct interaction between ER and NLRP3 protein (Figure 4b), which indicated the effect of ER β on autophagy and inflammation process.

DISCUSSION

Autophagy is the central mechanism that modulates homeostasis, participating in the progress of multiple diseases including OA with increasingly

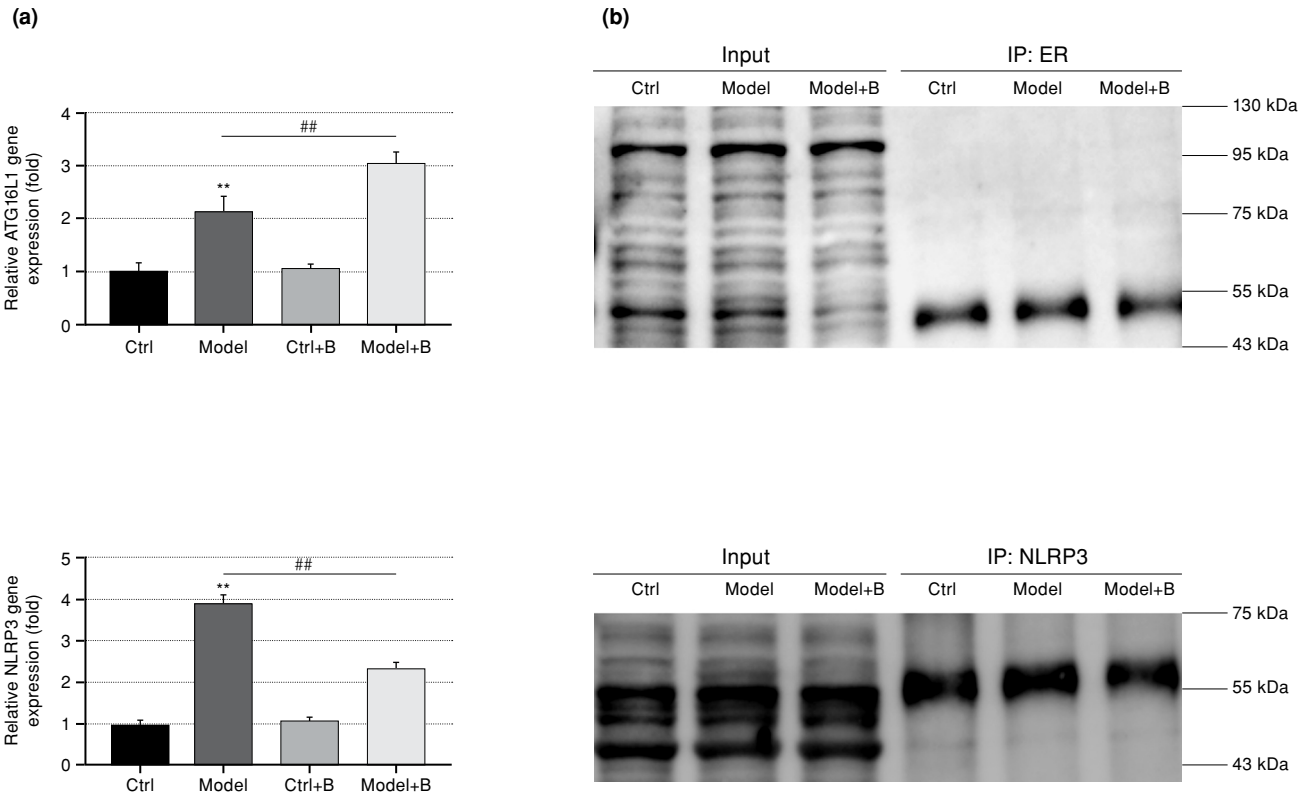


FIGURE 4. The effect of ER on the related gene expression and the direct interaction between ER and NLRP3. **(a)** ATG16L1 and NLRP3 were involved in RT-qPCR analysis; **(b)** The direct interaction between ER and NLRP3 was implied by CO-IP analysis.

ATG16L1: Autophagy-related 16 like 1; Ctrl: Control; NLRP3: NOD-like receptor thermal protein domain associated protein; IP: Immunoprecipitation; ER: Estrogen receptor; RT-qPCR: Real-time quantitative polymerase chain reaction.

attention and is negatively correlated with the NLRP3 inflammasome.[18,19] The enhancement of the autophagy response in Wistar rats not only enhances the expression of autophagy proteins such as LC3B, but also inhibits the expression of NLRP3 and its mediated secretion of IL-1β.[20]

Through the enrichment analysis of biological informatics pathway and cell biology experiments, we revealed that autophagy protein ATG16L1 played a critical role in the onset of OA. The cell autophagy takes part in the metabolic regulation of chondrocytes in the entire process. In the early stage of OA, cell autophagy acts on joint cartilage as a protective effect; however, in the late OA stage, excessive autophagy induces cartilage cell death,[21] which provides new insights into exploring the intervention of OA. Based on the crucial importance of autophagy, improving OA in regulation of cell autophagy could be beneficial. Recently, Vinatier et al.[22] revealed that autophagy, a protective mechanism in normal cartilage to avoid cell death driven by aging and trauma, was engaged in the OA cartilage aging and degeneration. Chen et al.[23] proposed that autophagy was a cellular homeostasis mechanism that clear dysfunctional organelles and macromolecules, which alleviate OA and delay joint aging by activating chondrocyte autophagy. Zuo et al.[24] validated that chondrocyte autophagy modulated the generation of reactive oxygen species (ROS) in chondrocytes and the expression of OA-related proteins: Aggrecan, collagen type 2 alpha 1 (COL2A1), matrix metalloproteinase 13 (MMP13), recombinant A disintegrin and metalloproteinase with thrombospondin 5 (ADAMTS5). Cai et al.[25] confirmed the coexistence of autophagy and apoptosis in the cell death of chondrocytes. Briefly, moderate autophagy is beneficial to the human body with the function of obliterating mitochondria damaged by ROS and maintaining the cell morphology of chondrocytes and other cells. Furthermore, the activation of NLRP3 can be induced with the defective autophagy, which promoted the production of ROS, accumulation of damaged mitochondria and

release of mitochondrial DNA. From previously published studies, the internal biological clocks in the chondrocytes have dramatic influence on the cartilage repair and remodeling. Knockout of the clock associated genes improve antioxidants enzyme activity, mitigate the generation of ROS to restore autophagy, and attenuate the aging of chondrocytes.[26] Transcription factor EB (TFEB) is the main regulator of the autophagy flux with initiation of autophagy related genes lysosomal biogenesis.[27] However, little is known about its relevance with autophagy protein ATG16L1, which may be a potential target for the treatment of OA.

By exploring the expression of autophagy-like protein ATG16L1 in chondrocytes in vitro, we confirmed that ER acts on ATG16L1 to mediate autophagy. Further CO-IP analysis confirmed the direct interaction between ER and NLRP3 protein, which indicated the effect of ER-β on autophagy and inflammation process. Estrogen is a major steroid hormone that plays a crucial role in regulating many physiological functions, including cell growth, differentiation, metabolism, and death. Since the early 20th century, studies have demonstrated that estrogen deficiency is inseparable from OA, and ER α and β genes are expressed in human chondrocytes, indicating that articular cartilage is one of the target tissues of estrogen.[15] Moreover, estrogen replacement therapy can reduce the occurrence of OA, alleviate its development, and improve its clinical symptoms. Different subtypes of ER inhibitors differentiated on autophagy and inflammation-related protein expression, among which the augment in ER-α expression have significant relevance with the aging and degeneration and negatively correlated with proliferative chondrocytes.[28] In addition to ERs α and β, ER-γ directly induce the expression of MMP3 and MMP13 in articular chondrocytes, which is critical for cartilage destruction. The ER gamma (ER-γ) is a novel catabolic regulator of OA pathogenesis and may also regard as a therapeutic target for OA.[29] In addition, miR-203 combines and negatively regulates ER-α in postmenopausal OA rats, resulting in cellular inflammation and cartilage destruction. After ovariectomy, the expression of miR-140-5p levels is significantly attenuated.[30] Therefore, miRNA can be a therapeutic target for women with OA. The expression of ER protein affects the expression of autophagy and inflammation-related proteins, and different subtypes of receptors can also participate in OA through disparate pathways. Further investigation should focus on the chondrocyte autophagy-like protein ATG16L1 and miRNA as a promising target for OA therapy.

In conclusion, through bioinformatics methods and molecular biology experiments, we demonstrate that through autophagy and inflammasome activation ER modulate the progress of OA, which are connected by the autophagy-like protein ATG16L1 in articular cartilage degeneration. Although several works have been completed, we still need to investigate the role of inflammation in OA autophagy process, The direct interaction between ER and NLRP3 also needs to be confirmed by other methods. In addition, autophagy is closely bound up with OA and there are plentiful potential targets for further exploration.

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Ethics Committee Approval: This project was approved by the ethics committee of Anhui Public Health Clinical Center (North District of The First Affiliated Hospital of Anhui Medical University) in accordance with the principles of the Declaration of Helsinki and Guide for the Care and Use of Laboratory Ani-mals (date: 02.29.2024, no: PJ-YX2024-014). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Writing-original draft, conceptualization, investigation, methodology, validation: F.X.L.; Investigation, methodology, formal analysis, resources, data curation: S.Y.; Software, formal analysis, visualization: Z.H.L.; Methodology, Investigation: K.D.B.; Formal analysis, validation, conceptualization: P.F.X.; Writing-review & editing, supervision, funding acquisition, conceptualization, project administration: J.C.

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