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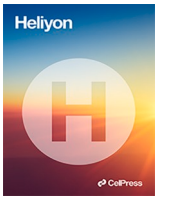

Research article

Leptin protects chondrocytes by inhibiting autophagy via phosphoinositide 3 kinase/protein kinase B/mammalian target of rapamycin signaling pathway

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A B S T R A C T

Leptin has been widely studied and found to have a significant impact on the development of osteoarthritis (OA). However, there are conflicting findings regarding the impact of leptin on chondrocytes. The study aimed to examine the impact of leptin on human chondrocytes and rats with OA. In the in vitro experiment, cartilage tissue obtained from patients hospitalized for knee replacement due to OA was collected for primary culture of chondrocytes. The proliferation and apoptosis of chondrocytes were assessed using cell counting kit-8 and flow cytometry. Autophagy levels were evaluated through monodansylcadaverine staining, mRFP-GFP-LC3 fluorescence, and transmission electron microscopy. Additionally, the expression of autophagy-related genes and proteins was analyzed using qRT-PCR and western blotting. In the in vivo experiment, an OA rat model was established. Following treatment with leptin and leptin antagonists, the cartilage tissues were examined using histology analysis (hematoxylin-eosin and Safranin O/fast green staining) and immunohistochemical. Mankin's score was utilized to assess the severity of OA, while qRT-PCR and western blotting were employed to detect the expression of autophagy-related genes and proteins in the cartilage. The ability of leptin to protect chondrocytes is achieved through the inhibition of autophagy via phosphoinositide 3 kinase/protein kinase B/mammalian target of rapamycin signaling pathway.

1. Background

The worldwide prevalence of osteoarthritis (OA) makes it the most prevalent joint disease. Cartilage degradation is the primay characteristic [1]. The degeneration of cartilage can be triggered by various risk factors, including genetics, age, gender, injury, and obesity. Among these factors, the degeneration of cartilage associated with obesity has garnered significant attention. Excessive weight can potentially contribute to the degeneration of cartilage by augmenting the physical loads exerted on joints [2]. The etiology of obesity-related OA is not solely attributed to joint load, the manifestation of this condition can also occur in joints that do not bear weight, such as the fingers and elbows, among individuals with obesity. Additionally, obesity has the potential to induce inflammatory mediators such as TNF-α and IL-1β, which subsequently facilitate cartilage degradation [3]. The secretion of adipokines, including adiponectin and leptin, has been demonstrated to play a significant role in the process of cartilage degradation according to recent studies [4–6]. The presence of elevated leptin levels in the serum and articular cavities of individuals with obesity-related OA suggests a significant role for leptin in the pathophysiology of this condition [4,5].

The first adipocyte-derived hormone identified is leptin [7]. It plays an essential role in regulating metabolism and maintaining

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body weight [8]. Leptin is also implicated in the regulation of cartilage metabolism and the pathogenesis of OA [9]. The concentrations of leptin in serum and articular cavities are elevated in overweight patients, indicating a robust correlation between leptin levels and obesity-related OA [7]. The leptin in the articular cavities triggers activation of the Janus kinase 2/signal transducer and activator of the transcription 3 (JAK2-STAT3) signaling pathway, thereby expediting the progression of OA [10]. Moreover, leptin also stimulates the generation of pro-inflammatory substances and facilitates the formation of matrix metalloproteinases (MMPs), thereby promoting the deterioration of the extracellular matrix (ECM) [5]. However, the impact of leptin on chondrocytes has been a subject of controversy due to conflicting evidence. The research from Lee SW et al. showed that leptin demonstrated a protective impact against the toxicity induced by TNF-α in rat articular joint fluid, thereby safeguarding cartilage from mechanical load and delaying the degeneration of chondrocytes [11]. Dumond H et al. also found leptin significantly enhanced the anabolic functions of chondrocytes in animal studies [12]. Based on these findings, further investigations are necessary to elucidate the impact of leptin on chondrocytes.

The mammalian target of rapamycin (mTOR) is a crucial kinase that regulates cellular growth, metabolism, survival, and apoptosis [13]. mTOR and leptin are partners in metabolism and inflammation [14]. Additionally, the activation of mTOR induced by leptin may potentially contribute to the pathogenesis of obesity-related diseases [15]. The inhibition of autophagy is an additional role performed by mTOR [16]. Autophagy is a crucial process to cellular energy regulation and chondrocyte metabolic function [17]. The dysregulation of chondrocyte autophagy and apoptosis is implicated in the pathogenesis of OA [18,19]. The process of autophagy serves as a protective mechanism against the progression of OA by preventing premature apoptosis in mature chondrocytes [20,21], and the upregulation of autophagy serves as an adaptive mechanism to safeguard cartilage against mechanical loading [22].

The roles of leptin and mTOR signaling pathways have been extensively investigated. However, there is a lack of research specifically focusing on their interaction within the context of articular cartilage. The objective of this study is to explore the impact of leptin on human chondrocytes and its influence on OA in rats, specifically focusing on autophagy regulation through the mTOR signaling pathway.

2. Materials and methods

2.1. Ethical statement

The human clinical samples were collected from patients who provided informed consent and underwent total knee arthroplasties due to OA. The Ethics Committee of the First Affiliated Hospital of Chongqing Medical University approved the protocol (2022-045). The Animal Research Committee of Chongqing Medical University approved animal experiments (IACUC-CQMU-2024-0274).

2.2. Patients

Criteria for inclusion: Patients over 60 years old. Preoperative radiographs were classified as III and IV according to the Kelglen-Genesis (K-L) grading standard.

Criteria for exclusion: Previous history of knee injury, surgery, and knee infection. Patients with diabetes, rheumatoid arthritis, rheumatic diseases, coagulation dysfunction, and tumors. Patients with long-term use of anticoagulant drugs, and hormone drugs. Patients with serious internal diseases. The other changes during the procedure.

2.3. Primary culture of human chondrocytes

Valid cartilage tissue specimens were obtained from 12 patients' tibial plateau. The cartilage samples were collected from patients and immediately transferred to the laboratory for initial isolation. Briefly, the excision of the articular cartilage surface was performed under aseptic conditions, and subsequently subjected to three rinses with phosphate-buffered saline (PBS; Sangon, China). After that, it was cut into 5-mm^3 fragments using ophthalmic scissors, and then transferred to a tube and treated with 0.25 % trypsin (Beyotime, China). Following centrifugation of the sample at 1000 r/m for 10 min, overnight digestion was performed by adding 0.2 % type II collagenase (Sigma, USA). The precipitated sample was passed through a 200-mesh strainer and subjected to centrifugation at 1500 r/min for 5 min. The precipitated substrate was collected and resuspended in DMEM/F12 (Gibco, American) containing a 10 % solution of fetal bovine serum (Gibco, American), followed by incubation at 37 °C for 48 h in a cell incubator with 5 % CO_2. The medium was refreshed every 72 h, and cell subculture was performed once cell fusion reached 80 %. Second-generation human chondrocytes were used in this experiment.

2.4. Identification of human chondrocytes

The chondrocytes were resuspended in a complete medium and then inoculated into 12-well plates with a sterile cap at 2×10^4/mL. After the completion of cell adherence, the cells underwent a PBS rinse, and then fixed in 4 % paraformaldehyde (Sangon, China) for 20 min and underwent wash with PBS. Following this, the cells were subjected to a 10 min staining procedure utilizing toluidine blue dye (Leagene, China) for a period of 10 min. After the drying process, the plates were examined under a microscope.

Following fixation of the chondrocytes with paraformaldehyde, the primary antibody against Collagen II (1:100; Beyotime, China) was introduced and incubated overnight at a temperature of 4 °C. Subsequently, it was subjected to three washes with PBS. The secondary antibody (1:50; Beyotime, China) was introduced and incubated at 23 °C for 60 min. Microscopic observations were performed using the DAB (Jiancheng, China) solution. The cells underwent a 5 min counterstaining process with hematoxylin (Jiancheng,

China). Finally, the cells were sealed using neutral gum (Jiancheng, China) and preserved for microscopic examination.

2.5. Animal experiments

A modified Hulth's modeling method (medial meniscectomy was resected, and the anterior cruciate ligament was cut off) was performed to mimic OA. 24 male Sprague Dawley rats (195–215 g) of 8 weeks old were used in the experiment. Firstly, a 1 % pentobarbital solution (40 mg/kg) was administered to induce anesthesia in the rats. Subsequently, the medial joint capsule was exposed and dissected by making a 1.0 cm incision on the medial aspect of the left knee. Careful attention was given to precisely excise the medial menisci and anterior cruciate ligament while avoiding any damage to the cartilage. A sham surgery was performed by incising the cutaneous and articular capsules.

All rats were allocated randomly into four groups: sham group (no surgery; 1 mL PBS treatment from the second day for four weeks, three times a week; left knee joints from six rats, n = 6); OA group (surgery; 1 mL PBS treatment from the second day for four weeks, three times a week; left knee joints from six rats, n = 6); (3) leptin + OA group (surgery; 0.5 mL leptin (200 ng/mL, Abcam, British) and 0.5 mL PBS treatment from the second day for four weeks, three times a week; left knee joints from six rats, n = 6); (4) leptin + leptin antagonist + OA group (surgery; 0.5 mL leptin and 0.5 mL leptin antagonist (200 ng/mL, ProSpec, Israeli) treatment from the second day for four weeks, three times a week; left knee joints from six rats, n = 6).

2.6. Histological and immunohistochemistry

After a four-week period, all the rats were euthanized under anesthesia. Some knee joints were fixed by immersing them in a 4 % paraformaldehyde solution and then transferred into 20 % formic acid for decalcification, and subsequently embedded in paraffin. From these paraffin-embedded specimens, continuous sagittal slices measuring 4-μm were collected. The tissue sections underwent histological examination using staining with hematoxylin-eosin, Safranin O-fast green, and immunohistochemistry. The staining procedures were conducted following the manufacturer's provided instructions (Beyotime, China). The specimens were subjected to immunohistochemistry analysis, wherein they were treated with 3 % H_2O_2 for 15 min, followed by a subsequent wash period of 2 min. Subsequently, 5 % BSA was added for 2 h for blocking. Primary antibodies against IL-1β (1:200; Beyotime, China), MMP-3 (1:100; Beyotime, China), and mTOR (1:150; Beyotime, China) were introduced and incubated overnight at 4 °C. The secondary antibody (1:100; Beyotime, China) was introduced and allowed to incubate at ambient temperature for 60 min. Subsequently, PBS was used for rinsing. DAB was added for chromogenic detection. Tissues were observed using a microscope.

2.7. Histopathological analysis

Histopathological analysis of articular cartilage was evaluated using Mankin scoring systems and double-blind observations. The Mankin scoring system is presented as follows: Structure; normal surface (0 scores), slightly irregular surface (1 score), surface irregularity and pannus formation (2 scores), clefts extending into the transitional layer (3 scores), clefts extending into the radial layer (4 scores), clefts extending into the calcified layer (5 scores), total disorganization (6 scores). Cells; normal cellularity (0 scores), diffuse cell proliferation (1 score), cell clustering (2 scores), cell loss/depletion (3 scores). Tidemark; intact tidemark structure (0 scores), presence of vascularity (1 score). The cumulative sum represents the severity of cartilage destruction.

2.8. Cell counting Kit-8 (CCK-8)

The single-cell suspensions of human chondrocytes were obtained by enzymatic dissociation using 0.25 % trypsin and subsequently seeded in 96-well plates with six replicates per group at a density of 2×10^4/well. The cells were cultured at 37 °C with 5 % CO_2. Once the adherent cell population reached approximately 60 %, samples were added according to their respective groups, and the cultures were further incubated for either 24 or 48 h. After incubation with CCK-8 (Yeasen, China) for an additional 4 h, the optical density (OD) values were quantified at a wavelength of 450 nm.

2.9. Flow cytometry

The human chondrocytes were trypsinized at 37 °C after 48 h of culture, followed by centrifugation at 1000 r/min for a duration of 5 min. Subsequently, the precipitated substrate was resuspended in PBS and then centrifuged again. The precipitated substrate was collected and resuspended in DMEM/F12 supplemented with 195 μL of Annexin V-FITC (Yeasen, China). An additional Annexin V-FITC (5 μL) was introduced and incubated at 23 °C in a light-free environment for 10 min. After centrifugation and resuspension as described above, propidium iodide (10 μL) (Yeasen, China) was introduced to assess the cell viability rate by flow cytometer.

2.10. Monodansylcadaverine (MDC) staining

Each group was incubated with 250 μL of MDC (Beyotime, China) at 37 °C in a light-free environment for 30 min. The cells were thoroughly rinsed with the Assay Buffer. Subsequently, the observation of green fluorescence was conducted by a fluorescence microscope.

2.11. Transfection and fluorescence microscopy

The transfection procedure was performed during the logarithmic growth phase of the cells. Subsequently, 250 μL of mRFP- GFP-LC3 adenovirus (Hanbio, China) was introduced [23]. The following day, cells in each group underwent the aforementioned treatment and were further incubated for 48 h. Afterward, fixed cells in 4 % paraformaldehyde for 30 min, and DAPI was added to observe using a fluorescence microscope.

2.12. Quantitative RT-PCR analysis

The RNAiso Plus kit (Takara, Japan) was employed to extract RNA from human chondrocytes and rat knee cartilage, following the manufacturer's instructions. The qRT-PCR primers were designed based on the nucleotide sequences of autophagy-related gene 5 (*ATG5)* and *ATG7* from NCBI, and synthesized by Thermo Fisher Scientific. Table 1 presents the study results.

The Hifair™ II 1st Strand cDNA Synthesis Kit (gDNA Digester Plus) (Yeasen, China) was utilized for RNA quantification. RT-PCR was conducted on the ABI StepOne Plus™ Real-Time PCR System (Applied Biosystems, American) using Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (Yeasen, China). The β-actin gene served as a reference gene, and the method of $2^{-\Delta\Delta Ct}$ was used to calculate the fold increase in PCR.

2.13. Western blotting

RIPA buffer (Yeasen, China) was employed to extract proteins from human chondrocytes and rat knee cartilage. The mixture was centrifuged at 12,000 r/min for 20 min at 4 °C after being vigorously oscillated. The bicinchoninic acid method was used to quantify the supernatant. Protein samples were mixed with a ratio of 4:1 with 5 × sodium dodecyl-sulfate (SDS) loading buffer and boiled in water for SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were subjected to SDS-PAGE in equal quantities, which was run at a voltage range between 80 V and 120 V. Afterward, the polyvinylidene fluoride membranes were incubated with skimmed milk at 23 °C for 1 h, followed by treatment with primary antibodies against mTOR (1:1000, Huabio, China), microtubule-associated protein 1 light chain 3B (LC3B; 1:1000, CST, American), signal transducer and activator of transcription 3 (STAT3; 1:2000, Proteintech, China), phospho-STAT3 (1:1000, Proteintech, China), protein kinase B (Akt; 1:2000, Proteintech, China), phospho-Akt (1:2000, Proteintech, China), phospho-ribosomal protein S6 kinase (phospho-S6K; 1:1000, Proteintech, China), phospho-eukaryotic initiation factor 4 (phospho-eIF4B; 1:20000, Proteintech, China), beclin 1 (1:1000, CST, American), p62 (1:1000, CST, American), β-actin (1:1000, Huabio, China) at 4 °C for 8 h. After undergoing a wash with Tris-buffered saline tween (TBST), the membranes were subjected to treatment using the secondary antibody (1:1000, Beyotime, China) at 23 °C for 1 h. Finally, a Tanon-4200 gel imaging system (Tanon, China) was used.

2.14. Transmission electron microscope (TEM)

TEM was applied to observe autophagosomes and autophagolysosomes in chondrocytes. Briefly, the chondrocytes were treated and fixed using a solution specifically designed for electron microscope fixation (Servicebio, China), and TEM (JEOL) was performed after dehydration, infiltration, embedding, and ultrathin sectioning.

2.15. Statistical analysis

The Shapiro-Wilk test was utilized to evaluate the normality of the data. If $p < 0.05$, the data were described using median values, the Mann-Whitney *U* test was employed to compare the differences between the two groups. The Kruskal-Wallis test was employed for conducting comparisons among groups. Conversely, if $p \geq 0.05$, the description involved the utilization of standard deviation and mean, with a T-test applied for comparing two different groups and a one-way analysis of variance (ANOVA) used for comparing differences among multiple groups. All statistical analyses were conducted utilizing SPSS (version 26.0, USA). TEM images were manually counted, and Image J (version 1.8.0, USA) was utilized for the analysis of the remaining images. All results represent three repeated experiments conducted in triplicate. A significance level of $P < 0.05$ indicates statistical significance.

Table 1
Sequences of the primers. According to the nucleotide sequence of *ATG5* and *ATG7* in NCBI.

Gene	Primer sequences of forward (5′-3′)	Primer sequences of reverse (5′-3′)
ATG5	5′-ACCTTCTGCACTGTCCATCT-3′	5′-GAGTTTCCGATTGATGGCCC-3′
ATG7	5′-TGTTTGCTTCCGTGACCGTA-3′	5′-TTGGTCCCATGCCTCCTTTC-3′
β-actin	5′-TGACGTGGACATCCGCAAAG-3′	5′-CTGGAAGGTGGACAGCGAGG-3′

3. Results

3.1. Morphological characteristics of human chondrocytes

The morphological characteristics of human chondrocytes were examined utilizing an inverted phase contrast microscope. The adherent cells exhibited slow growth for approximately 24 h, displaying a triangular or polygonal shape. However, rapid cell proliferation was observed after 3–5 days (Fig. 1A). Toluidine blue staining and examination under a light microscope revealed dark blue nuclei and pale blue cytoplasm and extracellular matrix in the chondrocytes (Fig. 1B). Immunocytochemical staining for Col II confirmed the chondrocytes, as evidenced by specific brown staining of Col II produced and secreted by these cells (Fig. 1C).

3.2. Leptin protected chondrocytes from the damage of IL-1β

The results of CCK-8 demonstrated the OD value in the IL-1β (10 ng/mL, MCE, American) [24] group exhibited a noticeable decline compared to the control group after 24 and 48 h of cultivation. Compared to the IL-1β group, the OD value in the leptin (200 ng/mL, Abcam, British) [25]+IL-1β group exhibited a substantial increase. Moreover, when compared to the leptin + IL-1β group, the OD value in the leptin + leptin antagonist (200 ng/mL, ProSpec, Israeli) + IL-1β group exhibited a decline. Our study observed no significant disparity between the control and leptin groups. Nevertheless, leptin mitigated IL-1β′s inhibitory effect on chondrocytes (Fig. 2A). The same trend was observed by employing flow cytometry following a 48 h period. Compared to the control group, the apoptosis rate increased in the IL-1β group. Conversely, the apoptosis rate significantly decreased in the leptin + IL-1β group when compared to just IL-1β treatment alone. Furthermore, the rate of apoptosis in the group of leptin + leptin antagonist + IL-1β was significantly elevated compared to leptin + IL-1β. Leptin alone had no impact on chondrocytes' behavior (Fig. 2B and C).

3.3. Leptin exerts an inhibitory effect on autophagy in chondrocytes

In comparison to the control group, an increased number of MDC-positive cells were observed in the IL-1β group, indicating enhanced autophagy. Furthermore, there was a reduction in autophagy in the leptin group. Autophagy was also decreased in the leptin + IL-1β group as opposed to the IL-1β group. Conversely, cell autophagy showed an increase in the group treated with both leptin and its antagonist along with IL-1β when compared with the leptin + IL-1β group (Fig. 3A and B). The results obtained from Fig. 3C and D demonstrate that mRFP-GFP-LC3 fluorescence microscopy corroborated the aforementioned findings. Specifically, the IL-1β group exhibited a heightened level of autophagy, while the leptin group displayed a diminished level of autophagy. Furthermore, there was a decrease in autophagy observed in the leptin + IL-1β group when compared to the IL-1β group. Conversely, an increase in autophagy was observed in leptin and its antagonist along with the IL-1β group as compared to the leptin + IL-1β group.

3.4. Leptin inhibited chondrocyte autophagy via mTOR

In comparison with the control group, an increase in mRNA expression of *ATG5* and *ATG7* was observed in the IL-1β group, while the leptin group showed a downregulation. Moreover, the group treated with leptin + IL-1β exhibited a reduction in *ATG*5 and *ATG7* levels when compared to the IL-1β group (Fig. 4A and B). Western blotting was employed to detect LC3B and mTOR. In the IL-1β group, the expression of LC3B was increased in comparison to the control group, whereas there was a decrease observed in mTOR protein levels. Conversely, the group treated with leptin exhibited a decrease in the expression of LC3B and an increase in the levels of mTOR. Compared to the IL-1β group, the group of leptin + IL-1β showed a reduction in the expression of LC3B and an elevation in the levels of mTOR. Furthermore, when compared with the leptin + IL-1β group, the leptin + leptin antagonist + IL-1β group exhibited an increase in the expression of LC3B and a decrease in the levels of mTOR (Fig. 4C–E).

The rescue experiment was supplemented with Rapamycin, an mTOR inhibitor. In comparison to the control group, the rapamycin (20 nmol/L, MCE, USA) group exhibited a higher number of MDC-positive cells, and there was a further increase in MDC-positive cells in the rapamycin + leptin group compared to the leptin group (Fig. 4F and G). TEM results demonstrated a decrease in the quantity of autophagosomes (red arrow) and autophagolysosomes (blue arrow) in the leptin group, which was subsequently restored through rapamycin treatment (Fig. 4H and I). Additionally, it was observed that LC3B protein expression increased while mTOR protein

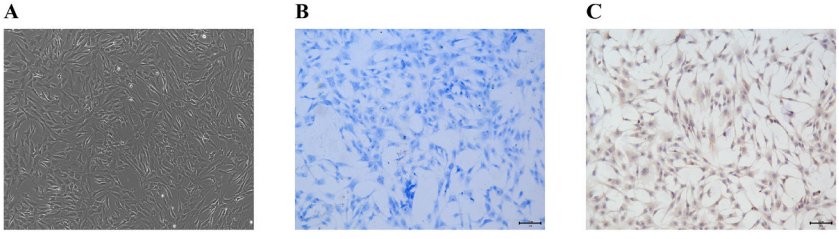

Fig. 1. Identification of human chondrocytes. (A) The morphology of human chondrocytes was observed by the inverted phase contrast microscope (X100). (B) Chondrocytes were stained with toluidine blue. (C) Chondrocytes were stained with Col II immunochemical. Scale bar = 100 μm.

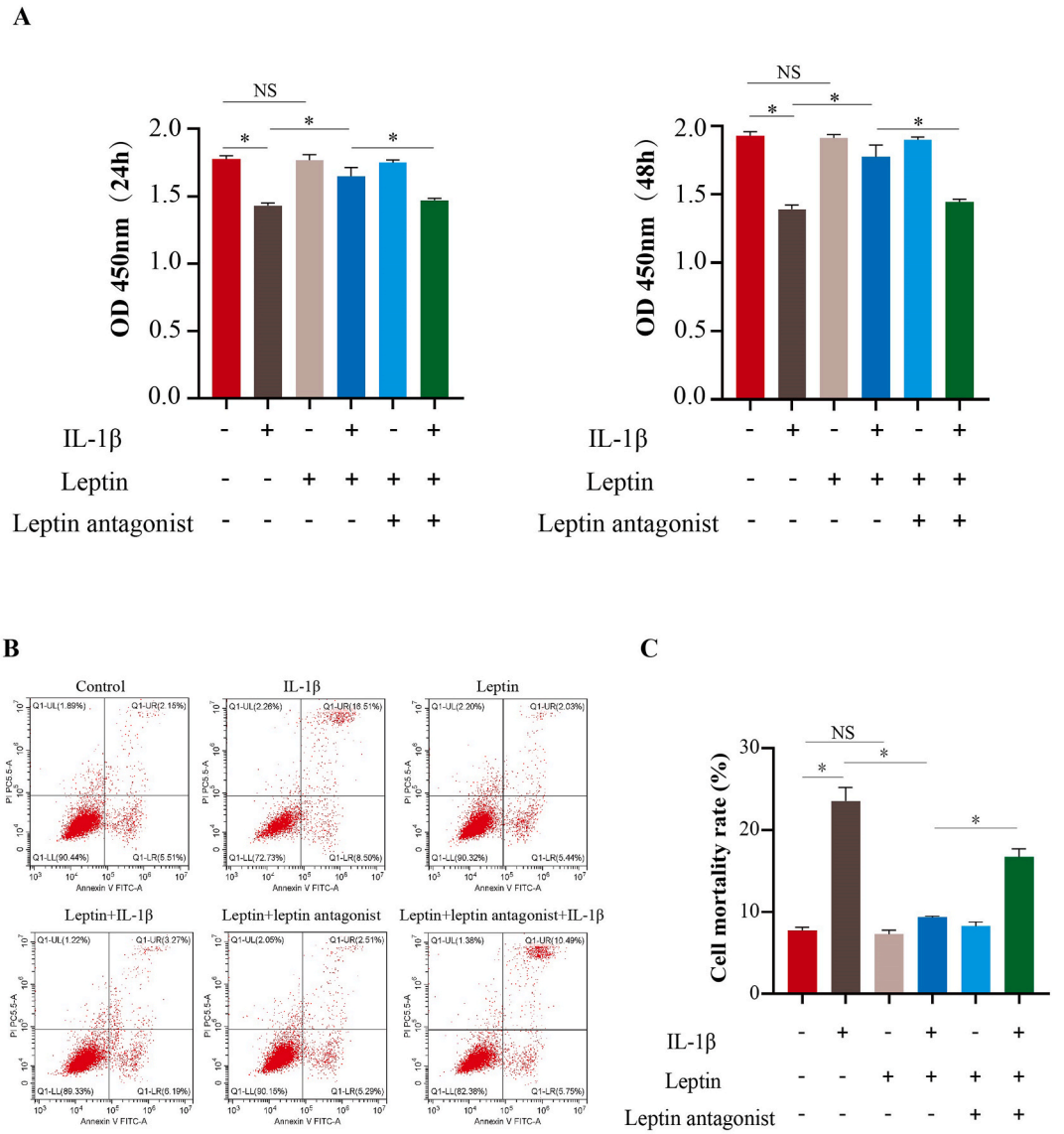

Fig. 2. Leptin protects chondrocytes from the damage of IL-1β. (A) CCK-8 was used to verify the proliferation of cells, and OD value (450 nm) in each group was detected after 24 h and 48 h. (B) The cell mortality rate was determined using a flow cytometer. (C) Comparison of mortality rate in each group. All experiments were performed in triplicate. Data were presented as means ± SD (n = 3) and were analyzed by one-way ANOVA. *, $p < 0.05$.

expression decreased upon exposure to rapamycin (Fig. 4J–L). These findings suggest that leptin inhibited chondrocyte autophagy through mTOR.

3.5. Leptin inhibited chondrocyte autophagy via phosphoinositide 3 kinase/protein kinase B (PI3K/Akt)/mTOR signaling pathway

To further investigate the possible signaling mechanism, we found that both phospho-STAT3 and phospho-Akt levels increased in the presence of leptin (Fig. 5A and B). Next, we examined S6K and eIF4B (both downstream targets of mTOR signaling). Western blotting analysis revealed that phospho-S6K and phospho-eIF4B were decreased after LY294002 (inhibitor of PI3K/Akt, 20 μmol/L, MCE, American) treatment. In contrast, there was no noticeable change after AG490 (inhibitor of JAK/STAT3, 50 μmol/mL, MCE, American) treatment (Fig. 5C–E). The findings suggest that the PI3K/Akt/mTOR signaling pathway is involved in mediating leptin-induced inhibition of autophagy.

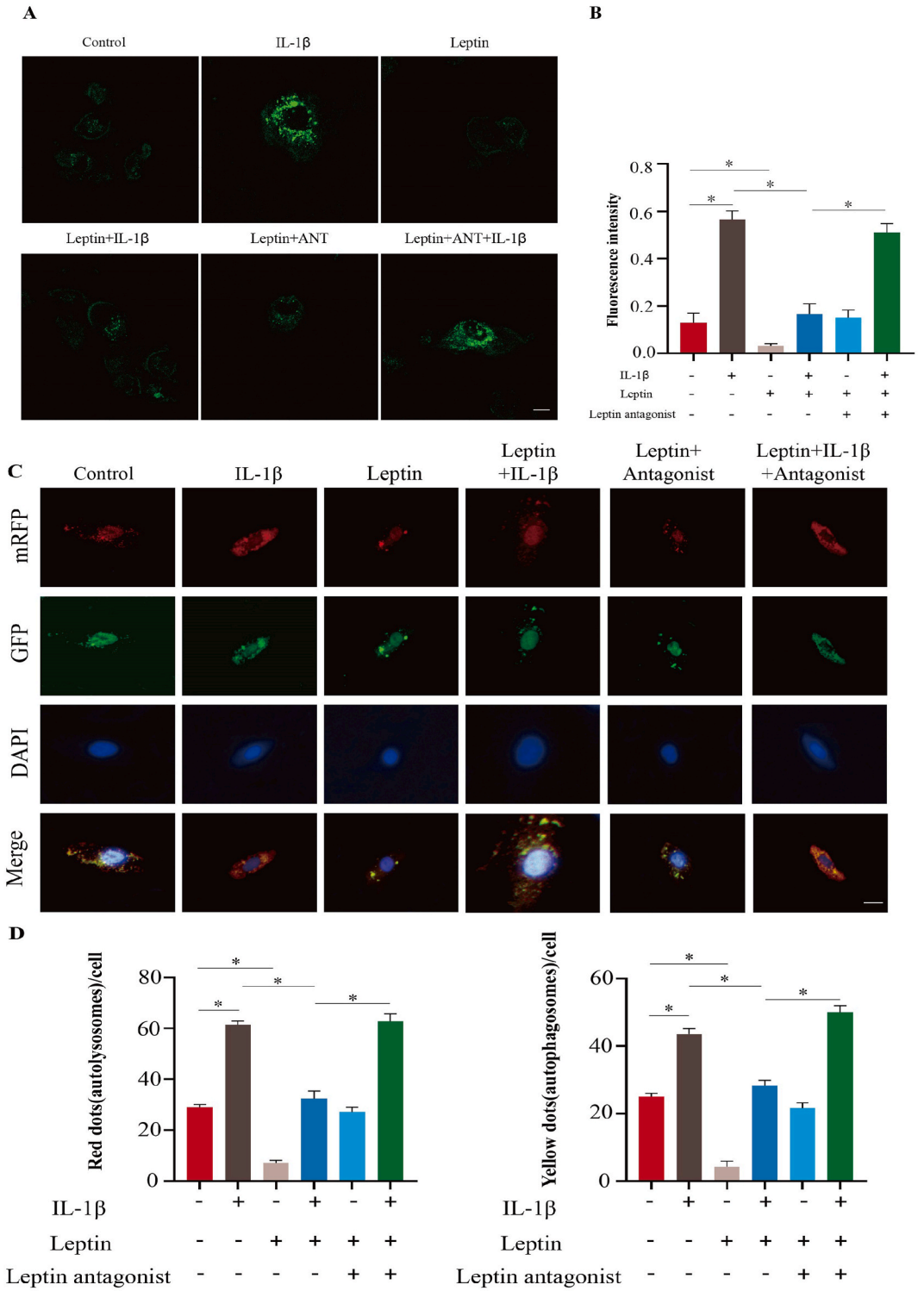

Fig. 3. Leptin inhibited chondrocyte autophagy. (A) The images of autophagosome formation were observed by MDC staining in each group (n = 3 biological replicates/group). Scale bar = 25 μm. (B) Quantitative analysis of fluorescent intensity was performed using Image J software. (C) The images of mRFP-GFP-LC3 adenovirus double label in each group (n = 3 biological replicates/group), mRFP (red dots) indicated autolysosomes, merge (yellow dots) indicated autophagosomes. Scale bar = 25 μm. (D) Quantitative analyses of red dots (autolysosomes) and yellow dots (autophagosomes) were performed using Image J software. ANT, antagonist. Data were presented as means ± SD (n = 3) and were analyzed by one-way ANOVA. *, $p < 0.05$.

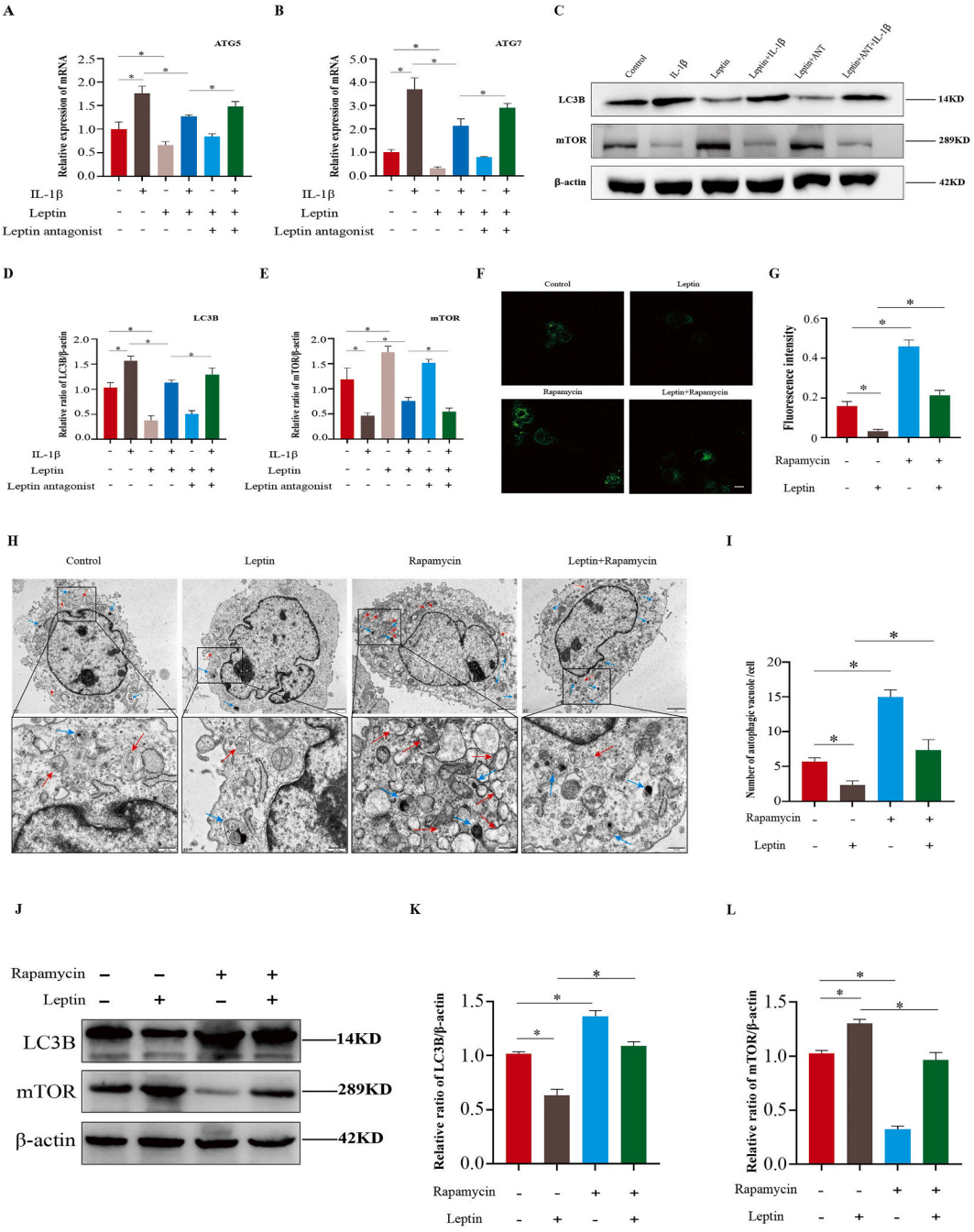

Fig. 4. Leptin inhibited chondrocyte autophagy via mTOR. (A, B) Real-time RT-PCR was performed to determine the gene expression of *ATG5* and *ATG7*. (C) Western blot was used to analyze the proteins of LC3B and mTOR. (D, E) Protein level of LC3B and mTOR normalized to β-actin using Image J software. (F) The images of autophagosome formation were observed by MDC staining in each group (n = 3 biological replicates/group). Scale bar = 25 μm. (G) Quantitative analysis of fluorescent intensity was performed using Image J software. (H) The images of autophagosomes (red arrow) and autophagolysosomes (blue arrow) in the cytoplasm were observed by TEM in each group (n = 3 biological replicates/group). Scale bar = 2 μm, 500 nm. (I) Quantitative analyses of the number of autophagic vacuoles were performed using manual counting. (J) Western blot was used to analyze the proteins of LC3B and mTOR. (K, L) Protein level of LC3B and mTOR normalized to β-actin using Image J software. ANT, antagonist. All experiments were performed in triplicate. Data were presented as means ± SD (n = 3) and were analyzed by one-way ANOVA. *, $p < 0.05$.

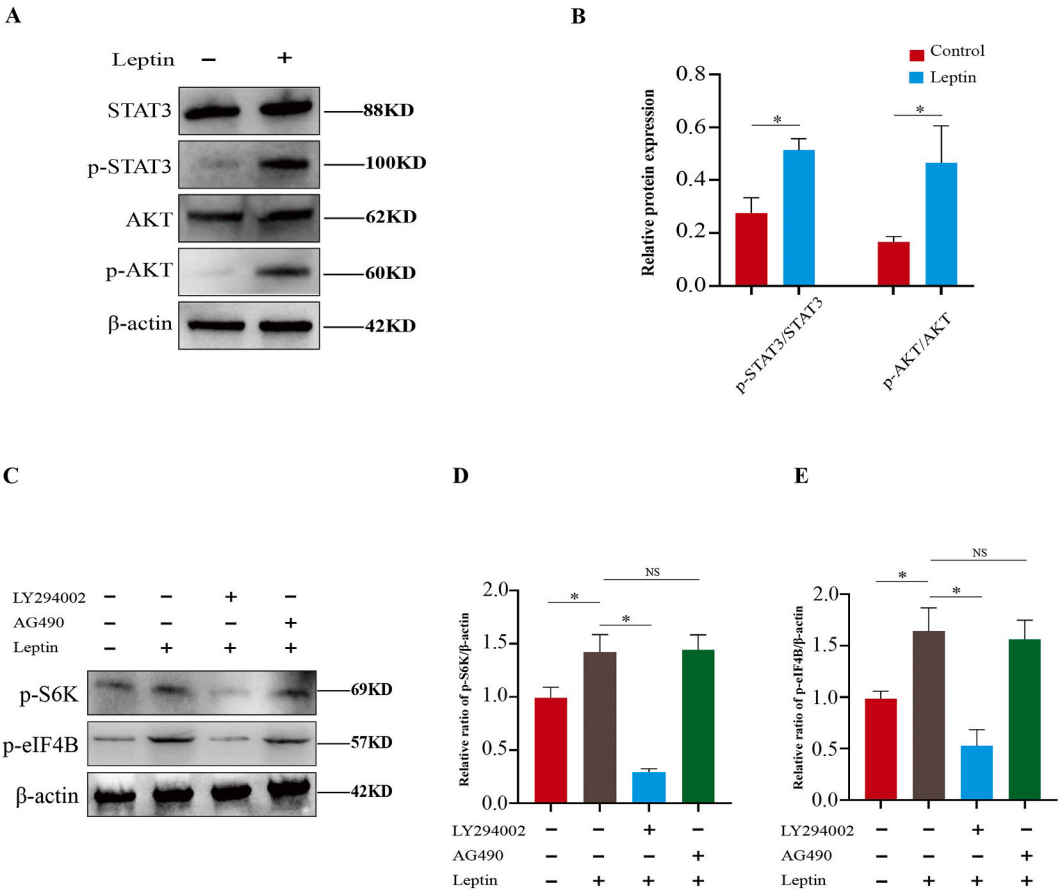

Fig. 5. Leptin inhibited chondrocyte autophagy via the PI3K/Akt/mTOR signaling pathway. (A) Western blot was used to analyze the proteins of STAT3, p-STAT3, Akt, and *p*-Akt. (B) Protein level of STAT3, p-STAT3, Akt, and *p*-Akt normalized to β-actin using Image J software. (C) Western blot was used to analyze the proteins of p-S6K and *p*-eIF4B. (D, E) Protein level of p-S6K and *p*-eIF4B normalized to β-actin using Image J software. All experiments were performed in triplicate. Data were presented as means ± SD (n = 3) and were analyzed by Student's t-test and one-way ANOVA. *, $p < 0.05$.

3.6. The intra-articular administration of leptin protected rat cartilage

In comparison with the OA group, the leptin + OA group showed a reduction in cartilage matrix damage and an increase in cartilage layer thickness. Conversely, in the leptin + leptin antagonist + OA group, there was an increase in cartilage damage (Fig. 6A–C). Similar findings were observed for the Mankin score (Fig. 6D). Additionally, immunohistochemistry results demonstrated a decrease in IL-1β and MMP-3 expression within the leptin + OA group (Fig. 6E and F).

3.7. The intra-articular administration of leptin inhibited the expression of autophagy-related genes and proteins of rat articular cartilage

In comparison with the OA group, a decrease in the expression levels of *ATG5*, *ATG7*, Beclin1 protein, and LC3B protein was observed in the leptin + OA group, meanwhile, there was an elevation in p62 protein and mTOR protein. Conversely, in the leptin + leptin antagonist + OA group, *ATG5*, *ATG7*, and LC3B were upregulated while p62 and mTOR were downregulated (Fig. 7A–G and Fig. 6E and F).

4. Discussion

The pathogenesis of OA involves the progressive degradation of articular cartilage, thereby leading to joint dysfunction [26]. Although OA has been widely studied, the mechanism of OA is still unclear. The viewpoint that adipokines play a crucial role in obesity-related OA has been amply supported [27,28]. Increasing evidence suggests leptin plays a powerful regulatory role in the pathophysiology of OA [29]. The secretion of leptin primarily originates from adipocytes, and its levels are associated with the amount of adipose tissue. This hormone regulates the body's energy balance by inducing appetite suppression and enhancing energy expenditure through targeted modulation of specific hypothalamic regions [30].

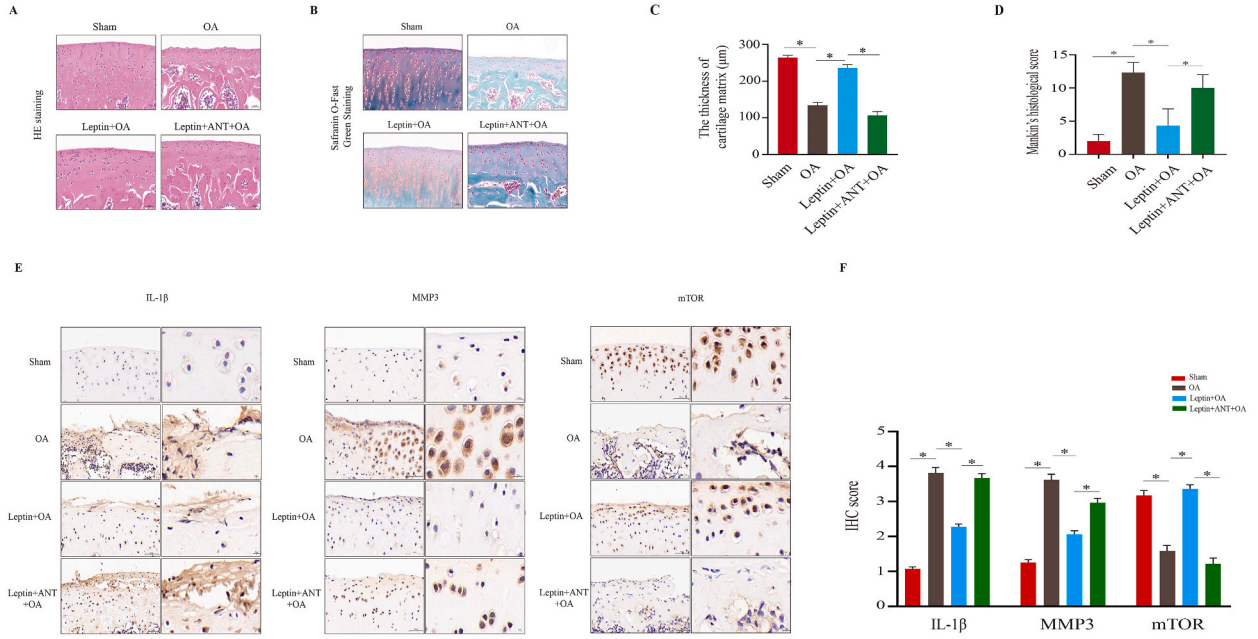

Fig. 6. Intra-articular injection of leptin protected the cartilage of rats. (A) The images of HE staining of rat cartilage tissue in each group (n = 3 biological replicates/group). Scale bar = 50 μm. (B, C) The images of safranin O/fast green staining of rat cartilage tissue in each group (n = 3 biological replicates/group) and quantitative analyses of cartilage thickness were performed. Scale bar = 50 μm. (D) Mankin's histological scores of cartilage tissue in each group (n = 3 biological replicates/group) were performed. (E, F) The images of immunohistochemistry of rat cartilage tissue in each group (n = 3 biological replicates/group) and quantification of IHC score was performed using Image J software. Scale bar = 50 μm, 10 μm. OA, osteoarthritis; ANT, antagonist. Data were presented as means ± SD (n = 3) and were analyzed by one-way ANOVA. *, $p < 0.05$.

The prevailing perspective in previous research suggests that leptin may exert detrimental effects by promoting the secretion of MMPs and synergizing with pro-inflammatory cytokines [4,7]. In addition, leptin also contributes to the regulation of the immune system by promoting the proliferation and activation of immune cells, thereby stimulating inflammatory activity and enhancing cartilage degeneration [9]. However, leptin also can enhance the synthesis of proteoglycan and collagen, its bidirectional effect on cartilage was suggested to be a response at different stages of OA progression [29]. In our study, we noted that leptin demonstrated a safeguarding impact on chondrocytes against the detrimental effects induced by IL-1β, which is consistent with the outcomes reported by Lee SW et al. [11] and Okano T et al. [31]. The same results also be observed in the vivo experiment, the presence of leptin conferred protective effects on cartilage, as demonstrated in Fig. 6. Moreover, the leptin + OA group exhibited a higher Makin score compared to the OA group, and immunohistochemistry results indicated a reduction in IL-1β and MMP-3 levels. These findings suggest that leptin exhibits both anti-inflammatory and pro-inflammatory properties [32]. Furthermore, the concentration of leptin is additionally a pivotal factor. It has been reported that low concentrations of leptin enhance the formation of proteoglycan and type 2 collagen, whereas high concentrations of leptin promote the proliferation of chondrocytes [7]. The study by Bo Yu et al. demonstrated that physiological levels of leptin have the ability to stimulate chondrocyte differentiation and proliferation, as opposed to high levels [33]. Gila Maor et al. also demonstrated that physiological levels of leptin increased the zone of the chondroprogenitor, however, high concentrations of leptin exhibited a suppressive effect [34]. Therefore, we suggest that leptin exerts its influence on cartilage in OA through the modulation of inflammation and the immune system, encompassing both local and systemic levels. The response of leptin to inflammatory stimuli, as well as variations in its concentration and receptor sensitivity, elicit diverse effects on chondrocytes.

mTOR serves as a pivotal regulator governing cellular processes including growth, proliferation, motility, survival, and autophagy [13]. The proteins mTOR and others have the ability to form distinct protein complexes, which are referred to as mammalian target of rapamycin complex (mTORC) 1 and mTORC2 [35]. mTORC1 promotes the production of proteins, lipids, nucleotides, and ATP and inhibits intracellular autophagy. The precise functioning of mTORC2 remains elusive, and indications suggest its potential collaboration with Akt to enhance the phosphorylation layer, thereby influencing its activity [35]. The downregulation of mTOR can trigger autophagy, thereby promoting the synthesis of extracellular matrix and enhancing chondrocyte proliferation [36]. However, the results of our investigation demonstrated that leptin upregulated mTOR and downregulated autophagy. Meanwhile, the downregulation of autophagy by leptin can be reversed by rapamycin. Therefore, the regulation of chondrocyte autophagy by leptin involves the participation of the mTOR signaling pathway.

Previous research has provided evidence that autophagy has the potential to repair impaired chondrocytes and effectively hinder the advancement of OA [37–39]. Our study demonstrated a contrary outcome, which could potentially be attributed to the following circumstances. The first, the chondrocytes were sourced from patients with OA, previous studies have reported an upregulation of autophagic activity in individuals with OA [40]. The process of autophagy does not result in the demise of juvenile chondrocytes, it

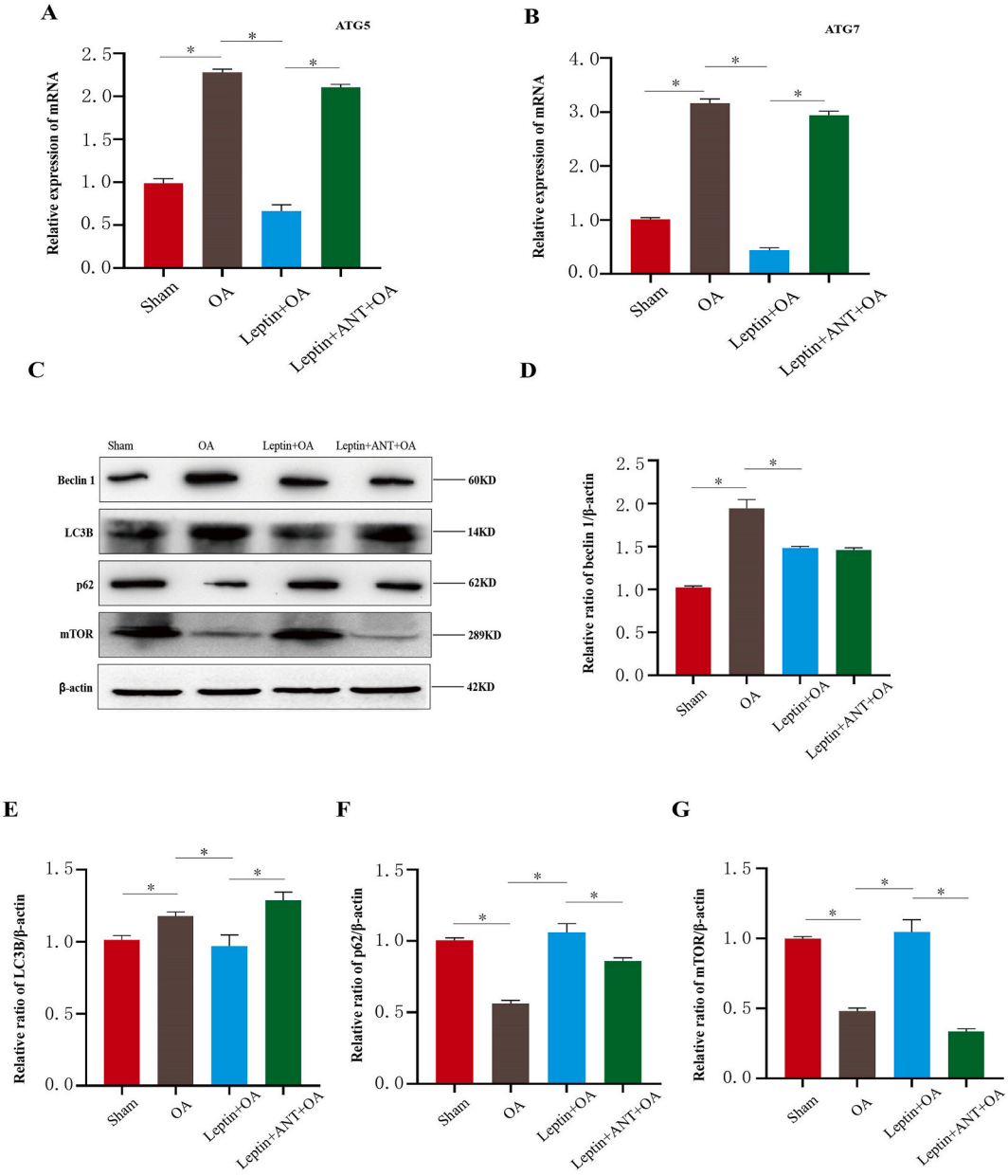

Fig. 7. Intra-articular injection of leptin inhibited the expression of autophagy-related genes and proteins of rat articular cartilage. (A, B) Real-time RT-PCR was performed to determine the gene expression of *ATG5* and *ATG7*. (C) Western blot was used to analyze the proteins of beclin 1, LC3B, p62, and mTOR in the cartilage. (D–G) Protein level of beclin 1, LC3B, p62, and mTOR normalized to β-actin using Image J software. OA, osteoarthritis; ANT, antagonist. All experiments were performed in triplicate. Data were presented as means ± SD (n = 3) and were analyzed by one-way ANOVA. *, $p < 0.05$.

may trigger chondrocyte apoptosis in individuals with OA. This implies that autophagy serves a dual function by both safeguarding and facilitating chondrocyte death during the development of OA [40]. The initiation of autophagy may potentially serve as a defensive mechanism against chondrocyte mortality during the initial phases of OA, while in subsequent stages, it could be simultaneously activated alongside apoptosis to induce cellular demise [41]. The second reason for our results may be an elevated level of autophagy, as prolonged and intensified autophagy can lead to protein and intracellular organelle damage, ultimately resulting in cellular demise [42]. The research conducted by Ma et al. indicated that excessive autophagy is involved in fluoride-induced chondrocyte apoptosis [43]. Therefore, autophagy is a complex process, with its impact on chondrocytes being contingent upon the age of patients, stage of OA progression, and the level of autophagic activity.

Signaling pathway of MAPK/ERK1/2, JAK/STAT3, PI3K/Akt are reported to be downstreams of leptin [44], and PI3K/Akt is also the classical upstream signaling pathway of mTOR [16,39,45]. The mechanistic experiments demonstrated an upregulation of

phospho-STAT3 and phospho-Akt expression in response to leptin. The upregulation of phospho-S6k and *p*-eIF4B (both substrates of mTORC1) induced by leptin was reversed upon inhibition of the PI3K/Akt signaling pathway using LY294002; however, the inhibitor AG490 targeting JAK/STAT3 did not have the same effect. Therefore, the involvement of the PI3K/Akt/mTOR signaling pathway in leptin-induced autophagy was indicated.

In conclusion, our study has demonstrated that leptin exhibits a protective effect on chondrocytes and suppresses cellular autophagy through modulation of the PI3K/Akt/mTOR signaling pathway. To the best of our knowledge, there is a scarcity of reports regarding the protective effects on chondrocytes resulting from autophagy inhibition by leptin. Therefore, our findings establish a fundamental basis for future research in this field. The relationship among leptin, PI3K/Akt/mTOR signaling pathway, and autophagy requires further investigation to provide additional evidence for the application of leptin-related therapy in OA.

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Data availability statement

The data sets generated and analyzed in this study are publicly accessible.

Statement of ethics

This study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Ethical review batch number: 2022-045) and the Animal Research Committee of Chongqing Medical University (Ethical review batch number: IACUC-CQMU-2024-0274).

CRediT authorship contribution statement

Ping Li: Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Weiqian Jiang:** Methodology. **Qiming Yang:** Investigation. **Yang Lu:** Investigation. **Jian Zhang:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jian Zhang reports financial support was provided by The First Affiliated Hospital of Chongqing Medical University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35665.

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