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Osteopontin inhibits autophagy via CD44 and avβ3 integrin and promotes cell proliferation in osteoarthritic fibroblast-like synoviocytes

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Abstract

Objective Osteoarthritis (OA) is closely related to aging, and autophagy is implicated in the retardation of aging. Activated synoviocytes play important roles in OA; the synoviocytes could produce osteopontin (OPN) and its main receptors CD44 and integrin, which are all involved in OA. The purpose of this study is to investigate whether OPN has an effect on autophagy in osteoarthritic synoviocytes.

Methods We cultured human OA fibroblast-like synoviocytes (FLS) and treated them with rhOPN and antibodies against CD44 and CD51/61 (αvβ3 integrin) or isotype IgG to block the interaction between receptors and ligands. Infection with lentivirus mRFP-GFP-LC3, laser confocal imaging and Western blotting were used to determine changes in the expression of autophagy markers, and cell proliferation of FLS was assessed with a CCK-8 assay.

Results Our results showed the expression level of autophagy marker protein LC3 II and the mRFP-GFP-LC3 puncta were significantly decreased after treatment with rhOPN when compared with the control group, when the FLS were incubated with antibodies against CD44 or CD51/61 (αvβ3 integrin) or with control isotype IgG for 1 h, followed by rhOPN treatment for 48 h, rhOPN could suppress the relative expression of LC3 II and Beclin1 via integrin and CD44 in the FLS, CCK-8 assay also showed that rhOPN significantly increased the cell proliferation and viability of FLS.

Conclusions OPN could inhibit autophagy via CD44 and αvβ3 integrin and promote the proliferation of FLS, playing an important role in OA synovitis.

Keywords Autophagy, CD44, Integrin, Osteoarthritis, Osteopontin, Synoviocyte

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Introduction

Osteoarthritis (OA), an age-related degenerative disorder, is predominantly characterized by pain and functional impairment in the elderly population [1]. The pathogenesis of OA involves significant contributions from activated synoviocytes, which play crucial roles in disease progression in humans [2]. Radiographic examinations have demonstrated that OA primarily affects individuals aged 65 years and above, with patients over 75 years old constituting 75% of the cases [3]. The synovial tissue and fibroblast-like synoviocytes (FLS), which line the inner joint cavity, play a critical role in secreting synovial fluid to facilitate cartilage matrix synthesis and chondrocyte proliferation [4, 5]. However, during the progression of osteoarthritis (OA), these cells often undergo a series of structural and functional alterations, which can significantly influence the severity and progression of the disease [6, 7]. Therefore, maintaining fibroblast-like synoviocytes (FLS) in a healthy state is considered a crucial factor in promoting chondrocyte proliferation and cartilage matrix synthesis, which ultimately helps prevent the onset and progression of osteoarthritis (OA) [8–10]. Despite its significant impact on global health, the etiology and pathogenesis of OA remain incompletely understood. Consequently, there is an urgent need to further explore the pathophysiology of OA and identify novel therapeutic targets for effective intervention.

Osteopontin (OPN) is a multifunctional phosphorylated protein with a molecular weight ranging from 44 to 75 kDa. It is synthesized and secreted into body fluids by a diverse array of tissues and cells, including osteoclasts, chondrocytes, synoviocytes, macrophages, and epithelial cells [11]. OA patients have higher OPN concentrations in the synovial fluid, articular cartilage, and plasma; the elevated OPN correlates with progressive joint damage and holds potential as a valuable biomarker for assessing disease severity and monitoring OA progression [12, 13]. Osteopontin (OPN) primarily exerts its biological functions by binding to cell surface receptors, notably CD44 and integrins [14, 15]. Both integrins and CD44 have been demonstrated to play pivotal roles in promoting the progression of osteoarthritis (OA). Importantly, the interaction between OPN and these receptors is strongly correlated with the severity of OA, highlighting its significance in the disease's pathogenesis [14, 16, 17]. However, the specific role of OPN in the pathological changes associated with knee osteoarthritis (OA) remains poorly understood and requires further investigation.

Autophagy is a fundamental cellular homeostasis mechanism robustly induced across many eukaryotic organisms and diverse cell types under nutrient starvation conditions [18]. Extensive research has demonstrated that autophagy is critical in maintaining physiological processes by eliminating unnecessary intracellular proteins, pathogens, and damaged organelles, including mitochondria, peroxisomes, and the endoplasmic reticulum [19–21]. Importantly, autophagy is implicated in slowing the aging process and enhancing autophagy activity has been shown to significantly reduce the incidence and severity of age-related diseases, such as osteoarthritis (OA) [22]. Previous studies have highlighted autophagy as a key mechanism for cellular homeostasis, with chondrocytes exhibiting elevated autophagy levels during the early stages of OA [18, 23]. Figueroa et al. [21, 24, 25] demonstrated that increased autophagy activity in human chondrocytes, as indicated by elevated levels of ULK1 (unc-51-like kinase 1), Beclin1, and microtubule-associated protein light chain 3 (LC3), protects chondrocytes from dysfunction and promotes cell proliferation, thereby delaying cartilage degeneration. Animal studies have corroborated that autophagy activity regulates cartilage metabolism and influences OA progression. For instance, Takayama et al. [26, 27] found that intra-articular injection of rapamycin, an autophagy inducer, delayed articular cartilage degradation in an OA murine model. This therapeutic effect was evidenced by increased LC3 levels and reduced expression of matrix metalloprotease-13 (MMP-13) [26, 27]. Additionally, another study confirmed the protective role of autophagy in both meniscus and cartilage, as indicated by elevated expression of LC3 and autophagy-related gene 5 (ATG5) [28]. Both human OA studies and animal models have consistently shown that reduced levels of autophagy-related proteins, including ULK1, Beclin1, and LC3-II, are strongly associated with cartilage damage and OA severity [24]. Conversely, activating autophagy or increasing the expression of autophagy-associated proteins, such as LC3-II, has been shown to mitigate the severity of experimental OA [29].

The relationship between autophagy and osteopontin (OPN) in fibroblast-like synoviocytes (FLS) remains poorly understood. This in vitro study aimed to explore the impact of OPN on the expression of autophagy markers in FLS derived from patients with knee osteoarthritis (OA), thereby elucidating the potential role of OPN in OA pathogenesis.

Materials and methods

Cultures of synovial FLS and immunohistochemistry

The study protocol was approved by the Ethics Committee of Xiangya Hospital, Central South University (Approval No. 201602018). For human participants involved in the research, informed consent for participation in the study and consent for publication were obtained from all participants (or their legal guardians). Specifically, informed consent forms were signed by the patients' relatives or guardians. Synovial tissues were collected from the knees of 10 OA patients who underwent

total knee replacement surgery. The baseline characteristics of the patients are summarized in Table 1. After being washed twice with phosphate-buffered saline (PBS, HyClone), the synovial tissue was minced into 1–5 mm^3 sections using a scalpel blade. The synovial tissue was digested with 5–8 mL of 0.2% collagenase II (Sigma-Aldrich) for 12–16 h at 37 °C in a 5% CO_2 atmosphere. The digestion process was terminated by adding 10 mL of Dulbecco's Modified Eagle's Medium (DMEM, HyClone). After centrifugation at 1000 rpm for 5 min, the cell pellets at the bottom of the centrifuge tubes were carefully aspirated and transferred to a culture flask. The pellets were resuspended in 5 mL of DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin solution (Gibco), followed by incubation for 24 h at 37 °C in a 5% CO_2 environment. Non-adherent cells were subsequently removed by washing. The remaining adherent cells were cultured for an additional 2 weeks, with the growth medium replaced every 3 days. After this period, the cells were trypsinized and transferred to new culture flasks. Cells at passages 4 through 6 were identified as fibroblast-like synoviocytes (FLS) and were utilized for phenotypic analysis and subsequent experiments.

The FLS cultured in 6-well plates were fixed with acetone for 15 min, washed twice with phosphate-buffered saline (PBS), and then incubated for 1 h in a humidified chamber with an anti-vimentin antibody (Abcam). Following this, the cells were washed three times with PBS and incubated for 1 h with isotype-matched horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG, Abcam). After three additional washes, the HRP reaction was developed using diaminobenzidine (DAB) according to the manufacturer's instructions (DAB Enhanced Liquid Substrate System, Sigma D3939).

Cell treatment

For all experiments, fibroblast-like synoviocytes (FLS) were seeded in 6-well culture plates at a density of $5 \times 10\text{^}5$ cells per well and subjected to serum deprivation for 24 h in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% fetal bovine serum (FBS). Initially, to assess the impact of osteopontin (OPN) on autophagy, two groups were established: a recombinant human OPN (rhOPN) group, where FLS were treated with 100 ng/mL of rhOPN (1433-OP; R&D Systems) for 48 h, and a control group comprising unstimulated, untreated FLS. Subsequently, blocking experiments were conducted to investigate the potential involvement of CD44 and αvβ3 integrin. In these experiments, FLS were pre-incubated for 1 h with either a mouse anti-CD44 monoclonal antibody (20 μg/mL; BD Biosciences) or an isotype-matched IgG2 control (20 μg/mL; BD Biosciences), as well as with an anti-CD51/61 monoclonal antibody (20 μg/mL; BD Biosciences) to block the interaction between OPN and αvβ3 integrin, or an isotype-matched IgG1 control (20 μg/mL; BD Biosciences). Following this pre-incubation, the cells were treated with rhOPN for 48 h.

mRFP-GFP-LC3 lentivirus infection and laser confocal imaging

The fibroblast-like synoviocytes (FLS) were plated onto glass-bottomed cell culture dishes (Nest, Catalog No. 801002) at a density of $2 \times 10\text{^}5$ cells per dish. On a subsequent day, the cells were transduced with an mRFP-GFP-LC3 lentivirus (provided by Hanbio) at a multiplicity of infection (MOI) 100. After 8-hour incubation, the virus-laden medium was aspirated and replaced with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Following this, the cells were subjected to the treatment protocol detailed in the preceding methodology. After 48 h of incubation, yellow and red punctate structures were observed within the autophagosomes and autolysosomes. These mRFP-GFP-LC3 puncta were visualized using a Leica laser confocal microscope. The number of puncta per cell was quantified using the ImageJ software.

Table 1 Characteristics of the patients

Patient	Sex	Age	BMI	Disease	KL degree	Hypertension	Diabetes
1	Male	66	28.04	OA	4	+	-
2	Female	63	32.09	OA	4	+	-
3	Female	69	23.44	OA	4	-	-
4	Male	71	28.10	OA	4	+	-
5	Male	69	30.12	OA	4	-	-
6	Female	72	30.48	OA	4	+	-
7	Female	71	23.28	OA	4	-	-
8	Female	61	24.49	OA	4	+	+
9	Female	68	29.38	OA	4		-
10	Female	73	26.84	OA	4	+	-

OA: osteoarthritis

Western blotting

The fibroblast-like synoviocytes (FLS) were lysed with 100 µL/well of sodium dodecyl sulfate (SDS) buffer supplemented with protease inhibitor at a ratio of 100:1. The protein concentration of the resulting lysate was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific). For subsequent experiments, 40 µg of protein was loaded per sample. Protein separation was performed using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Genescript), with an initial run at 80 V for 30 min followed by 120 V for 60 min. Subsequently, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at a constant current of 300 mA for 75 min. Following transfer, the membranes were blocked with a nonfat dry milk solution for 1 h at room temperature. They were then incubated overnight at 4 °C with primary antibodies targeting Beclin1 (1:1000 dilution, Cell Signaling Technology) or LC3 (1:1000 dilution, Cell Signaling Technology), diluted in primary antibody dilution buffer. After primary antibody incubation, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:2000 dilution) for 1 h at room temperature. β-actin was used as an internal loading control to ensure consistent protein quantification. Protein bands were visualized using enhanced chemiluminescence (ECL) (NCM Biotech), and the chemiluminescent signals were captured and quantified using the Bio-Rad ChemiDoc-XRS imaging system with Image Lab Software (Bio-Rad, Richmond).

Cell proliferation assay

The fibroblast-like synoviocytes (FLS) were planted in a 96-well plate at a density of 6,000 cells per well, with five replicate wells for each treatment group. To measure cell proliferation 48 h after treatment, we used the Cell Counting Kit-8 Assay (CCK-8, from Beyotime). At this point, 90 µl of DMEM culture medium mixed with 10 µl of CCK-8 solution was added to each well. The plate was then incubated for an additional 2 h. Finally, the absorbance of each well was read at a wavelength of 450 nm using a microplate reader (Bio-Rad, Richmond).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software). Data are presented as the mean ± standard error of the mean (SEM). Differences between the two experimental groups were assessed using Student's t-test. For comparisons involving multiple groups, one-way analysis of variance (ANOVA) was employed, followed by the Bonferroni post hoc test to evaluate differences between individual groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

Phenotypic features of synovial fluid-derived FLS

The fibroblast-like synoviocytes (FLS) from passages 4 through 6 displayed a consistent spindle-shaped and fibroblast-like morphology, as illustrated in Fig. 1A. Immunocytochemical staining demonstrated a positive reaction for the anti-vimentin antibody in the majority of the FLS, as depicted in Fig. 1B.

RhOPN inhibits LC3 expression

Our results showed that, in comparison to the control group, the expression level of the autophagy marker protein LC3 II was markedly reduced in fibroblast-like synoviocytes (FLS) treated with recombinant human osteopontin (rhOPN), decreasing to 0.62 ± 0.08-fold of the control level. This difference was statistically significant ($P = 0.029$, $P < 0.05$), as illustrated in Fig. 2A and B. Additionally, visualization of mRFP-GFP-LC3 puncta at a multiplicity of infection (MOI) of 100 using a Leica laser confocal microscope further confirmed that rhOPN

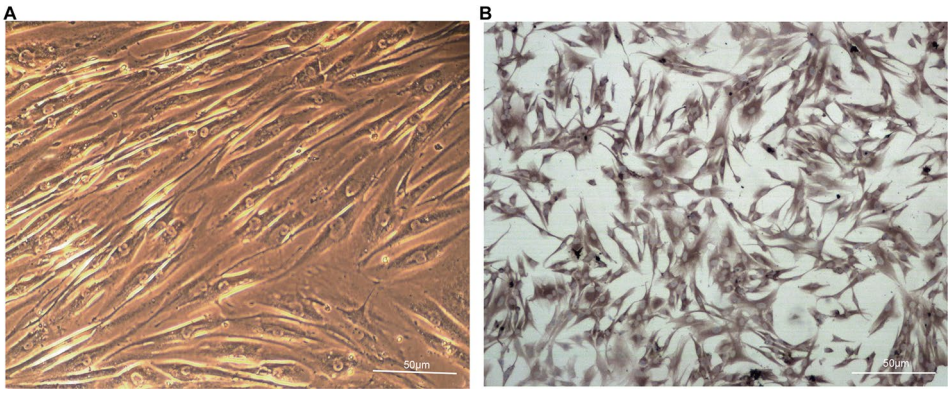

Fig. 1 Phenotypic features of the FLS passages 4 through 6. (**A**) Light microscopy showed that FLS were homogeneous and spindle-shaped in morphology. (**B**) Immunocytochemical staining revealed that the majority of FLS stained positive with anti-vimentin antibody

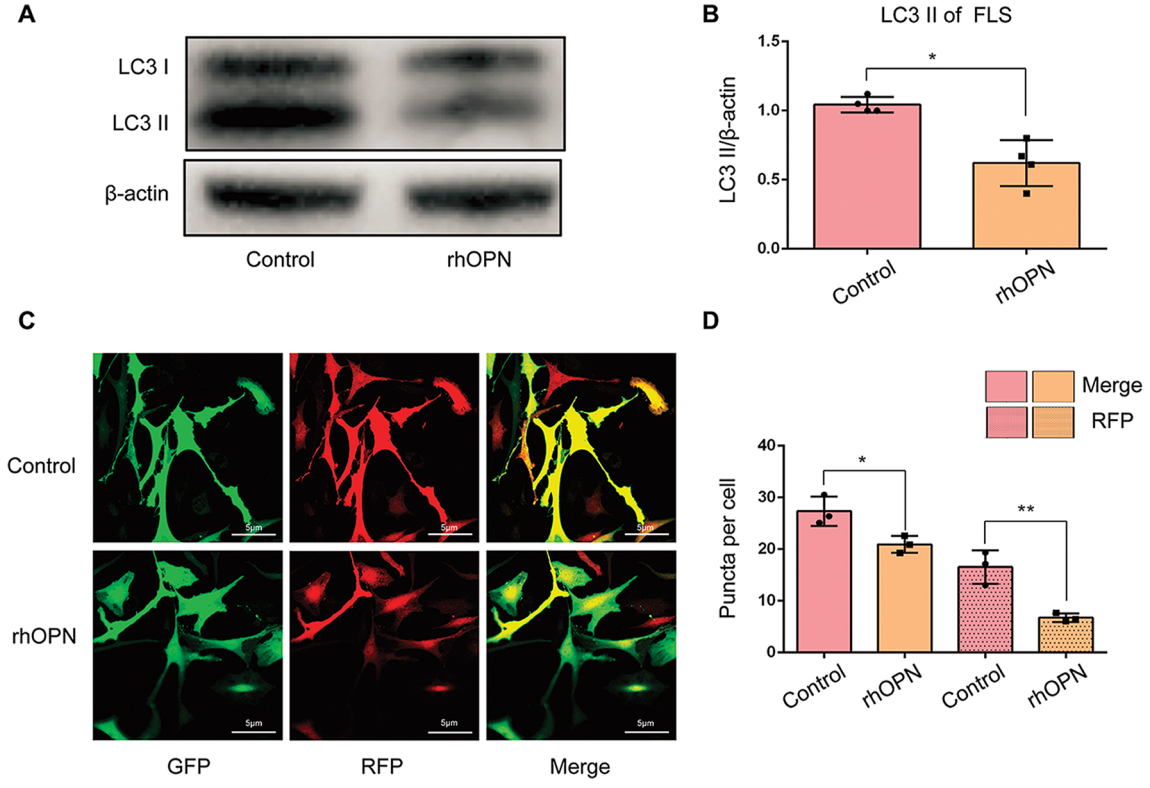

Fig. 2 rhOPN inhibits autophagy activity via suppressing the expression of LC3. (**A**) The LC3 II of FLS was significantly decreased in rhOPN group compared to the control group. (**B**) The expression of LC3 II between the two groups were statistically significant. (**C**) The mRFP-GFP-LC3 puncta confirmed the inhibitory effects of LC3 II in rhOPN group compared to the control group. (**D**) The expression of the mRFP-GFP-LC3 puncta between these two groups were statistically significant; * means $P < 0.05$ when compared between the two groups, ** means $P < 0.01$ when compared between the two groups

decreased LC3 II expression, as depicted in Fig. 2C and D.

OPN suppresses autophagy via integrin and CD44

To identify the receptors responsible for the observed effects of osteopontin (OPN), fibroblast-like synoviocytes (FLS) were pre-treated with antibodies against CD44 or CD51/61 (αvβ3 integrin) or with control isotype IgG for 1 h, followed by treatment with recombinant human OPN (rhOPN) for 48 h. The relative expression levels of LC3 II and Beclin1 in each group were analyzed. Compared to the control group, the expression levels of LC3 II and Beclin1 in the rhOPN group were significantly reduced to 0.61 ± 0.07-fold and 0.53 ± 0.09-fold, respectively ($P = 0.0308$ and $P = 0.0353$, $P < 0.05$), confirming that rhOPN inhibits autophagy in FLS. In the rhOPN + CD44 antibody group, the expression levels of LC3 II and Beclin1 were 1.03 ± 0.11-fold and 1.16 ± 0.04-fold, respectively, showing no significant difference compared to the control group ($P = 0.8361$ and $P = 0.0795$, $P > 0.05$). This indicated that the CD44 antibody, at a concentration of 20 μg/ml, effectively blocked OPN's inhibitory effect on LC3 II and Beclin1 expression. In contrast, the rhOPN + IgG2 group exhibited reduced expression levels of LC3 II and Beclin1 (0.53 ± 0.06-fold and 0.51 ± 0.08-fold, respectively), which were significantly lower than those in the control group ($P = 0.0168$ and $P = 0.0241$, $P < 0.05$). Similarly, in the rhOPN + CD51 antibody group, the expression levels of LC3 II and Beclin1 were 1.01 ± 0.15-fold and 0.99 ± 0.27-fold, respectively, showing no significant difference compared to the control group ($P = 0.9521$ and $P = 0.974$, $P > 0.05$). However, the rhOPN + IgG1 group exhibited significantly reduced expression levels of LC3 II and Beclin1 (0.69 ± 0.03-fold and 0.79 ± 0.05-fold, respectively) compared to the control group ($P = 0.0113$ and $P = 0.0445$, $P < 0.05$). These results collectively demonstrate that OPN suppresses the relative expression of LC3 II and Beclin1 in FLS through interactions with both integrin and CD44 receptors. The data are summarized in Fig. 3A and B, and 3C.

OPN promotes cell proliferation in FLS

The proliferation and viability of fibroblast-like synoviocytes (FLS) were assessed using the CCK-8 assay. The results demonstrated that recombinant human osteopontin (rhOPN) significantly enhanced FLS proliferation/viability, with an optical density (OD) value of 1.16 ± 0.07 at 450 nm compared to the control group (OD = 0.85 ± 0.04; $P = 0.0092$). Additionally, all other experimental groups exhibited markedly elevated OD450 values relative to

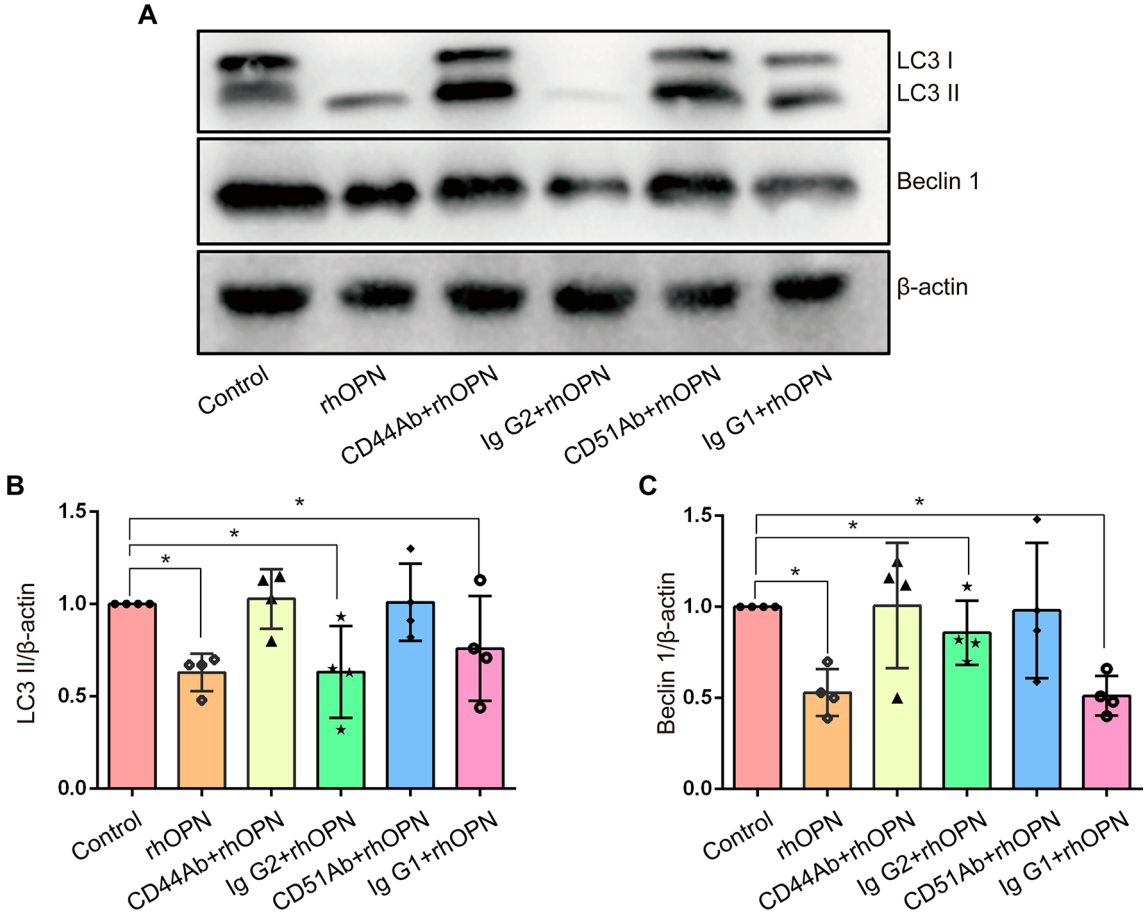

Fig. 3 rhOPN inhibits autophagy activity through the intervene of Integrin in FLS. (**A**) The expression of LC3 II and Beclin1 in FLS treated by rhOPN, CD44 Ab + rhOPN, Ig G2 + rhOPN, CD51 Ab + rhOPN, Ig G1 + rhOPN compared to the control group. (**B**) The analysis showed that expression of LC3 II was statistically significant in groups Control vs. rhOPN, control vs. Ig G2 + rhOPN and Control vs. Ig G1 + rhOPN; (**C**) The analysis showed that expression of Beclin1 was statistically significant in groups Control vs. rhOPN, Control vs. Ig G2 + rhOPN and Control vs. Ig G1 + rhOPN. * means $P < 0.05$ when compared between the two groups

the control group: rhOPN + CD44 antibody (1.26 ± 0.11), rhOPN + IgG2 (1.43 ± 0.13), rhOPN + CD51 antibody (1.17 ± 0.13), and rhOPN + IgG1 (1.22 ± 0.07) (all $P < 0.05$; Fig. 4). These findings collectively indicate that rhOPN promotes the in vitro proliferation of FLS, with CD44 and integrin receptors potentially mediating this effect.

Discussion

OA exhibits intricate and multi-tissue pathologies, encompassing cartilage, subchondral bone, and synovial inflammation [30, 31]. Synovial inflammation stands as a pivotal factor in OA pathogenesis, with osteopontin (OPN) produced by synovial fibroblasts showing a positive correlation with cartilage invasion and articular cartilage degeneration [32]. The concentration of OPN in synovial fluid correlates with the intensity of joint pain following an anterior cruciate ligament rupture [33]. Prior studies have confirmed that the heightened expression of OPN mRNA in OA might be attributed to elevated hyaluronic acid (HA) levels in OA synovitis [2, 34]. OPN not only facilitates the attachment of cells to cartilage but also contributes to matrix degradation in rheumatoid arthritis (RA) by stimulating chondrocyte secretion of collagenase 1 [32]. Recent research has found that OPN regulates chondrocyte hypertrophy and infrapatellar fat pad inflammation in OA progression, suggesting that downregulating OPN levels could be an innovative therapeutic strategy for OA by mitigating inflammatory activity and cartilage erosion [35]. OPN treatment has been shown to increase the secretion of interleukin-6 (IL-6) and IL-8 in human OA chondrocytes, with this upregulation positively correlated with OPN concentration [36]. Slovacek et al. [37] investigated the relationship between OPN and inflammatory factors in OA patients and found that OPN could elevate the expression of IL-1, IL-6, and C-reactive protein (CRP). They also suggested that downregulating OPN levels might promote cartilage matrix metabolism by reducing the expression of a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4). However, another study indicated that intra-articular

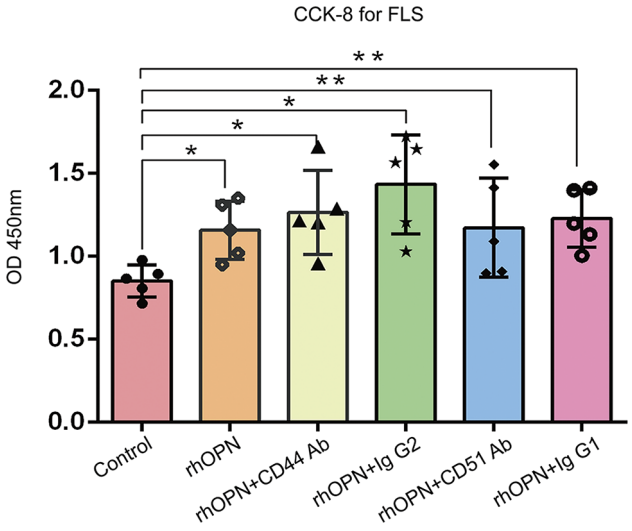

Fig. 4 rhOPN could promote the cell proliferation of FLS in vitro and that even CD44 and CD51 Ab or their isotype Ig G could not block this effect. The CCK8 results showed the FLS proliferation were statistically significant in groups Control vs. rhOPN, Control vs. rhOPN + CD44 Ab, Control vs. Ig G2 + rhOPN, Control vs. rhOPN + CD51 Ab, and Control vs. Ig G1 + rhOPN. * means $P < 0.05$ when compared between the two groups, ** means $P < 0.01$ when compared between the two groups

OPN could inhibit the production and expression of IL-1, nitric oxide (NO), and prostaglandin E2 (PGE2) in chondrocytes [38]. Furthermore, OPN plays a crucial role in the intrinsic regulation of cartilage degradation through its impact on MMP-13 expression and proteoglycan loss in both instability-induced and aging-associated spontaneous OA [39], but the role of OPN in OA has not been absolutely determined.

Autophagy is a vital cellular homeostatic mechanism responsible for eliminating dysfunctional cellular organelles and macromolecules, and its activity is enhanced under catabolic and nutritional stresses [18, 40, 41]. In normal cartilage, autophagy serves as a protective mechanism, and its age-related decline is associated with the secretion of inflammatory factors, degradation of cartilage matrix proteins, and chondrocyte death in OA [29]. Pharmacological activation of autophagy presents a promising therapeutic approach for OA [30]. The mammalian target of rapamycin (mTOR) functions as a key suppressor of autophagy. Elevated mTOR expression correlates with increased chondrocyte apoptosis in OA, while mTOR ablation provides significant protection against destabilization of the medial meniscus (DMM)-induced OA, accompanied by a notable reduction in articular cartilage degradation, apoptosis, and synovial fibrosis [42, 43]. Previous research has demonstrated that OPN inhibits the expression of LC3 II and Beclin1 in chondrocytes by binding to CD44 and αvβ3 integrin receptors, ultimately regulating the ERK-mitogen-activated protein kinase (MAPK) signaling pathway to execute its physiological functions [44]. Our current study further reveals that OPN can inhibit autophagy in osteoarthritic fibroblast-like synoviocytes (FLSs). Specifically, we observed that OPN decreased the expression of LC3 II protein, and the mRFP-GFP-LC3 puncta also confirmed the inhibitory effects of recombinant human OPN (rhOPN) on LC3 II expression. Consistent with our findings, a previous study also showed that OPN inhibits apoptosis and autophagy in colorectal cancer, promoting cell migration and invasion [45].

The cell surface receptors integrin and CD44 play pivotal roles in the progression of osteoarthritis (OA). Specifically, the expression of CD44 is significantly elevated in human OA synovial tissue, and its level positively correlates with cartilage damage [16, 17]. Integrins mediate communication primarily through binding to chondrocyte extracellular matrix (ECM) proteins, ultimately regulating cell proliferation, survival, differentiation, and matrix remodeling signals [46].

To investigate the interaction between OPN and CD44 or integrins, our study incubated fibroblast-like synoviocytes (FLSs) with antibodies against CD44 or CD51/61 (integrin) or with control isotype IgG before recombinant human OPN (rhOPN) treatment. The results indicated that both the CD44 antibody and the integrin antibody could block the inhibitory effect of OPN on the expression of LC3 II and Beclin1, suggesting that OPN interacts with both receptors to regulate autophagy. In rheumatoid arthritis (RA), the overexpression of OPN in synovial T cells is associated with the local inflammatory milieu, and OPN acts as a crucial mediator in amplification and perpetuation. The binding of OPN to CD44 and integrin cell surface receptors mediates the functional properties of OPN on the target cells [47]. Another study reported that increased expression of OPN and αvβ3 integrin in synovial tissues facilitates the production of pro-inflammatory factors, ultimately leading to joint destruction with higher levels of IL-1 and MMP-13 [48]. In RA, the OPN level in urine may reflect the severity of active inflammatory arthritis, as higher OPN levels in urine are correlated with inflammation markers such as erythrocyte sedimentation rate (ESR), CRP, rheumatoid factors, and the severity of cartilage erosion [49]. Zheng et al. [50] also observed that elevated OPN concentrations correlated with serum levels of inflammatory markers such as macrophage inflammatory protein-1β (MIP-1β) and monocyte chemoattractant protein 1 (MCP-1) in monocytes. OPN acts as a regulatory factor involving the NF-κB signaling pathway and exerts biological effects by activating protein 38 (p38), MAPK, and c-Jun NH2-terminal kinase (JNK). Take et al. [51] reported increased expression of OPN in the co-culture of FLS cells and B lymphocytes from RA patients. They also found that upregulated OPN levels could enhance the expression of

IL-6, ultimately enhancing the interactions and adhesion of FLS cells to B lymphocytes. Furthermore, the role of OPN in Th17 differentiation and its promoting effect on the secretion of the inflammatory cytokine IL-17 are also mediated by the receptors CD44 and integrin in RA [52]. Over-proliferation of human FLS may contribute to the OA pathological process and amplify synovitis [53, 54]. Our results showed that OPN increased the cell proliferation and viability of the FLS, consistent with previous studies indicating that OPN could promote chondrocyte proliferation in OA [53] and that elevated OPN expression could promote synoviocyte proliferation in OA [54]. In OA synovium tissues, the increased OPN leads to a higher expression of MMP-13, IL-6 and IL-8. Inhibiting OPN expression could result in decreased MMP-13, IL-6, and IL-8 and downregulation of synoviocyte proliferation, which might be an effective strategy for OA treatment [54]. It has been established that inflammatory diseases are accompanied by autophagy dysfunction [55]. Rapamycin treatment in mouse knee joints to induce autophagy can reduce the severity of cartilage degradation and decrease the synovitis levels [56]. Ribeiro et al. [57] also investigated the effect of autophagy activity on cartilage integrity in type-2 diabetes. They induced OA model in *db/db* mice ((B6.BKS (D)-*Leprdb*/J mice), which are prone to obesity and diabetes due to a deficiency of the leptin receptor. They found that rapamycin treatment could greatly alleviate cartilage damage and synovial inflammatory activity.

In summary, our findings demonstrate that OPN suppresses autophagy-related markers LC3 II and Beclin1 in FLS through interactions with integrin (αvβ3) and CD44 receptors while promoting FLS proliferation. These dual effects collectively suggest that OPN may play a key role in driving synovial inflammation and disease progression in OA.

Limitations

There are some potential limitations to this study. Firstly, our investigation was confined to in vitro studies using FLS cells, which may not fully capture the complex physiological interactions and functions of OPN in vivo. This in vitro setting may not accurately reflect the full spectrum of OPN's effects on FLS cells under physiological conditions. Secondly, the FLS cells utilized in our study were sourced from OA patients, and it is conceivable that these cells may exhibit distinct characteristics compared to FLS cells derived from healthy adults or individuals with other arthritic conditions, such as rheumatoid arthritis (RA), hand OA, or hip OA. Recent research has illuminated that FLS cells isolated from different anatomical sites, including the hand, knee, hip, and foot synovial tissue, display variations in their transcriptomic profiles, proliferation rates, inflammatory responses, and metabolic activities [58]. These differences highlight the need for more nuanced analyses when studying the role of OPN in various arthritic contexts. Thirdly, our study was constrained by a relatively small sample size, with synovial tissue collected from only 10 patients. This limitation may have impacted our ability to generalize our findings to a broader OA patient population. To address this, future research endeavors should endeavor to include a larger cohort of OA patients, as well as patients with other types of arthritis and healthy adults, to more comprehensively explore the function of OPN in FLS cells across diverse groups.

Conclusion

Our results demonstrate that OPN suppresses autophagy in FLS through CD44 and αvβ3-integrin signaling while promoting FLS proliferation, thereby contributing critically to the pathogenesis of OA-associated synovitis.

Abbreviations

OA	osteoarthritis
OPN	osteopontin
LC3	microtubule associated protein 1 light chain 3
ULK1	unc-51-like kinase 1
ATG5	autophagy-related gene5
FLS	fibroblast-like synoviocytes
PBS	phosphate-buffered saline
DMEM/F12	Dulbecco's modified Eagle's medium/F12
FBS	fetal bovine serum
HRP	horseradish peroxidase
rhOPN	recombinant human OPN
DAB	Diaminobenzidine
rhOPN	recombinant human OPN
SDS	sodium dodecyl sulfate
SDS	PAGE-SDS-polyacrylamide gel electrophoresis
PVDF	polyvinylidenedifluoride
MOI	multiplicity of infection
ECL	enhanced chemiluminescence
CCK-8	Cell Counting Kit-8
HA	hyaluronic acid
RA	rheumatoid arthritis
MMP	13-matrix metalloproteinase-13
IL	interleukin
NO	nitric oxide
PGE2	prostaglandin E2
ADAMTS-4	a disintegrin and metalloproteinase with thrombospondin motifs-4
ESR	erythrocyte sedimentation rate
CRP	C-reactive protein
JNK	c-Jun NH2-terminal kinase
MIP-1β	macrophage inflammatory protein-1β
MCP-1	monocyte chemoattractant protein1
mTOR	mammalian target of rapamycin
DMM	destabilization of the medial meniscus
ECM	extracellular matrix
db/db mice	B6.BKS (D)-Leprdb/J mice

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions
BRJ and ZFJ had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. BRJ and ZFJ are joint corresponding authors. All authors have read, provided critical feedback on intellectual content and approved the final manuscript. Concept and design: BRJ and ZFJ. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: LM, WCB and LHF. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: LM, WCB and LHF. Obtained funding: BRJ, ZFJ, WCB and LHF.

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Data availability
The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate and publication
The studies that involved human participants were meticulously reviewed and granted approval by the Institutional Review Board of Xiangya Hospital, Central South University. All patients/participants provided their written informed consent prior to their involvement in this study.

Data Sharing Statement
All data generated or analyzed during this study are included in the manuscript.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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