



Elevated levels of 15-lipoxygenase-1 contribute to the abnormal phenotypes of osteoblasts in human osteoarthritis

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ABSTRACT

Aims: 15-lipoxygenase-1 (15-LOX-1) plays a vital role in aggravating the inflammatory response in various pathological processes, including osteoarthritis (OA). Abnormal osteoblast phenotypes including elevated runt-related transcription factor 2 (RUNX2), collagen type 1 alpha 1 (COL1), and osteocalcin (OCN) lead to osteosclerosis of the subchondral bone, which eventually causes OA. However, the pathogenesis of OA is poorly defined, and it is unclear if 15-LOX-1 induces osteoblast abnormal phenotypes in OA. Therefore, this study aimed to determine the roles of 15-LOX-1 on the abnormal phenotypes present in osteoblasts of the subchondral bone in OA.

Main methods: The expression levels of 15-LOX-1 were measured by Immunohistochemistry, qRT-PCR and western blotting from the OA subchondral bone osteoblasts. To further investigate the roles of 15-LOX-1 in abnormal phenotypes of osteoblasts and its mechanisms in OA, 15-LOX-1 siRNA or overexpressing lv-15-lox-1 were transfected into osteoblasts, respectively. The effects of 15-LOX-1 on abnormal phenotypes of osteoblasts in OA were assessed by qRT-PCR, and western blotting. We also examined the role of 15-LOX-1-inhibited autophagy in OA osteoblasts by qRT-PCR, and western blotting, transmission electron microscopy.

Key findings: The expression levels of 15-LOX-1 along with osteoblast phenotype markers such as RUNX2, COL1, and OCN were significantly increased in OA subchondral bone. Furthermore, 15-LOX-1 inhibited autophagy significantly upregulated the expression levels of RUNX2, COL1 and OCN through activated mTORC1. Similarly, treatment with autophagy inhibitors alleviated osteoblast abnormal phenotypes of osteoblasts in OA.

Significance: In conclusion, our results suggested that the expression of 15-LOX-1 on osteoblasts from the subchondral bone increased in OA. 15-LOX-1 inhibited autophagy by activated mTORC1, which in turn upregulated the markers of abnormal osteoblast phenotypes RUNX2, COL1, and OCN.

1. Introduction

The lesions of subchondral bone, mainly manifested as the increase of bone remodeling and osteosclerosis of subchondral bone, is commonly pathological characteristic in the early progression of osteoarthritis (OA) [1,2]. As a mechanical support structure, the subchondral bone sclerosis results in cartilage phenotypes change, cartilage degeneration, and eventually leads to OA [1,4,5]. Abnormal osteoblast phenotypes play a vital role in OA subchondral bone sclerosis including increased release of runt-related transcription factor 2 (RUNX2), collagen type 1 alpha 1 (COL1), and osteocalcin (OCN) [6]. Dysfunction of osteoblasts in subchondral bone lead to the remodeling and sclerosis of subchondral bone in OA [7–10]. Moreover, osteogenic markers genes

including RUNX2, COL-1, and OCN could induce excessive differentiation of osteoblast through the elevated expression of osteogenic markers, and eventually aggravated the sclerosis of subchondral bone [1,7,8,11,12]. Therefore, elucidation of the mechanism of abnormal osteoblast phenotypes might be great significance to explore the possible pathological mechanism of subchondral bone sclerosis and a new treatment target for OA.

15-lipoxygenase-1 (15-LOX-1) is a key enzyme that plays important roles in regulating the inflammatory responses through catalyzing the formation of lipid peroxides in various diseases [13–15]. Progression of OA could be aggravated by overexpression of 15-LOX-1 which inhibited chondrocytes proliferation and accelerated chondrocytes apoptosis. Furthermore, reduced 15-LOX-1 expression alleviated the clinical

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Table 1
Sequence of the primers used in this study.

organism	targets	Forward primer/reverse prime (5'-3')	Genbank accession no.
human	ACTIN	Forward CACCCAGCACAATGAAGATCAAGAT Reverse CCAGTTTTTAAATCCTGAGTCAAGC	NM_001101
human	15-LOX-1	Forward TTCTGTCCCCCTGATGACTT Reverse ACGATTCTTCCACATACCG	NM_001140.3
human	OCN	Forward TCACACTCTCGCCCTATTG Reverse CTCCTGAAAGCCGATGTGGT	NM_199173.5
human	COL1	Forward CCAAGACGAAGACATCCCACCA Reverse CCGTTGTGCGAGCGCAGAT	NM_000088.3
human	RUNX2	Forward GGAGTGGACGAGGCAAGAGTT Reverse GGTCCCGAGGTCCATCTACT	NM_001015051.3

Abbreviations: 15-LOX-1: 15-lipoxygenase-1; OCN: Osteocalcin; COL1: collagen type 1 alpha 1; RUNX2: runt related transcription factor 2.

symptoms of OA [16]. However, it remains unclear whether 15-LOX-1 exacerbates subchondral bone lesions in OA.

Cellular autophagy is an essential homeostatic process to achieve self-renewal and regulate immunity and inflammation by which cells remove damaged cellular structures and break down their own components [17]. This mechanism is of great significance for survival, differentiation, development and homeostasis [18]. Autophagy played an important role in regulating osteoblast phenotypes [19,20]. Moreover, the activation of autophagy could alleviate OA severity via inhibited degeneration of chondrocytes [21,22]. However, whether autophagy could mediate the progression of OA by affecting the expression of osteoblasts abnormal phenotypes in subchondral bone remains unclear.

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase that belongs to the phosphoinositide 3kinase (PI3K)-related kinase family. mTOR could inhibit autophagy and acted as a catalytic subunit for two distinct protein complexes: mTORC1 and mTORC2, mTORC1 is inhibited by rapamycin [23,24]. Multiple studies showed that activation of the mTORC1 signaling pathway in osteoblasts led to an increased bone mass and resulted in excessive proliferation and differentiation of osteoblast. Furthermore, activated mTORC1 significantly increased subchondral bone ossification, osteogenesis markers, and subchondral bone mass in OA [25–27,43]. Recently, upregulation levels of 15-LOX-1 could reduce the expression of LC3-II and beclin1 in RAW 264.7 cells, which confirmed the regulated role of 15-LOX-1 in cellular autophagy [28]. However, whether 15-LOX-1 could mediate the abnormal phenotypes of osteoblasts through mTORC1 involved autophagy remains unclear in human osteoarthritis. Therefore, this study aimed to determine the role of 15-LOX-1 on the abnormal phenotypes of osteoblast from the subchondral bone of OA patients.

2. Materials and methods

2.1. Human knee subchondral bone procurement

We procured the subchondral bone from the donors (n = 6) with lower limb trauma who required amputation and patients (n = 6) suffering from OA who underwent knee replacement. Relative non-weight bearing/weight-bearing area subchondral bone procurement was performed as previously reported [29,30]. The overlying cartilage were removed and the trabecular bone tissue was dissected from the subchondral bone plate. The ethical review board of the First Affiliated Hospital of Anhui Medical University approved the protocol of human knee subchondral bone sample collection. All patients gave informed consents.

2.2. Subchondral osteoblasts culture

The osteoblasts were isolated from subchondral bone in OA patients as previously reported [29,30]. Briefly, bone biting forceps were used to

cut subchondral bone into small pieces (< 1mm²) which were digested for 4 h using 1 mg/ml of collagenase I in DMEM/F12 medium (Hyclone, USA) without serum at 37°. The digested bone pieces were cultured in T25 flask containing DMEM/F12 medium supplemented with 20% fetal bovine serum (Clark bioscience, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin, and cultured at 37 °C with 5% CO₂ and saturated humidity. When the osteoblasts were observed in the T25 flask, we changed the medium to DMEM/F12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Osteoblasts reach confluence about 20,000–25000 cells/cm², then osteoblasts were digested using Trypsin-EDTA Solution (beyotime, shanghai, china) and plated at 10,000 cells/cm² in T25 culture bottles containing DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37 °C with 5% CO₂ and saturated humidity, then grown for 4–5 days to reach confluence again.

2.3. Cell transfection

The overexpression vector containing human lv-15-LOX-1 was purchased from Genechem (Shanghai, China), The siRNA duplexes against human 15-LOX-1 were synthesized from GenePharma (Shanghai, China) with the sequences: sense 5'- TCACCTTCCTGCTCG CCTAGTG -3', and antisense 5'- GGTGCTGCTGGCTACAGAGAATG -3'. Osteoblasts were inoculated in 6-well tissue culture plates, 2 ml standard growth medium per well, with a cell density of 2 × 10⁵ cells/well, and incubated for 24 h before transfection. Once osteoblasts reached a confluence of about 80%, cells were transfected with 100 nM siRNA using Lipofectamine 3000 reagent (Invitrogen, US) or 10 µl LV-15-LOX-1 (MOI = 100) using 40 µl HitransG P (Genechem, Shanghai, China). According to the manufacturer's protocols.

2.4. Western blotting analysis

Different proteins were extracted from osteoblasts, and 10 µg of each were loaded and separated by SDS-PAGE; 12% for LC3 and Osteocalcin (OCN); 10% for 15-LOX-1, RUXN2, COL1, and beclin1; and 8% for mTORC1 and pmTORC1. 15-LOX-1, RUXN2, COL1, beclin1, mTORC1, pmTORC1 were electroblotted onto a polyvinylidene difluoride membrane (0.45 mm; biosharp, china), LC3, OCN were electroblotted onto a polyvinylidene difluoride membrane (0.22 mm; biosharp, china), then blocking with 5% non-fat dry milk in TBST for 2 h. Then the membranes with anti-15-LOX-1 antibodies were incubated under 4 °C overnight (antibody to TBST, 1 : 1000) (from Santa Cruz Biotechnology, CA, USA), antiCOL1 antibodies (1 : 1500) (from abcam, UK, England), anti- RUXN2 antibodies (1 : 1500) (from abcam, UK, England), anti-beclin1 antibodies (1 : 1500) (from abcam, UK, England) and anti- LC3B antibodies (1 : 1500) (from abcam, UK, England), anti-mTORC1 antibodies (1 : 1500) (from Cell Signaling Technology, Beverly, MA, USA), anti-pmTORC1 antibodies (1 : 1500) (from Cell Signaling Technology, Beverly, MA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies

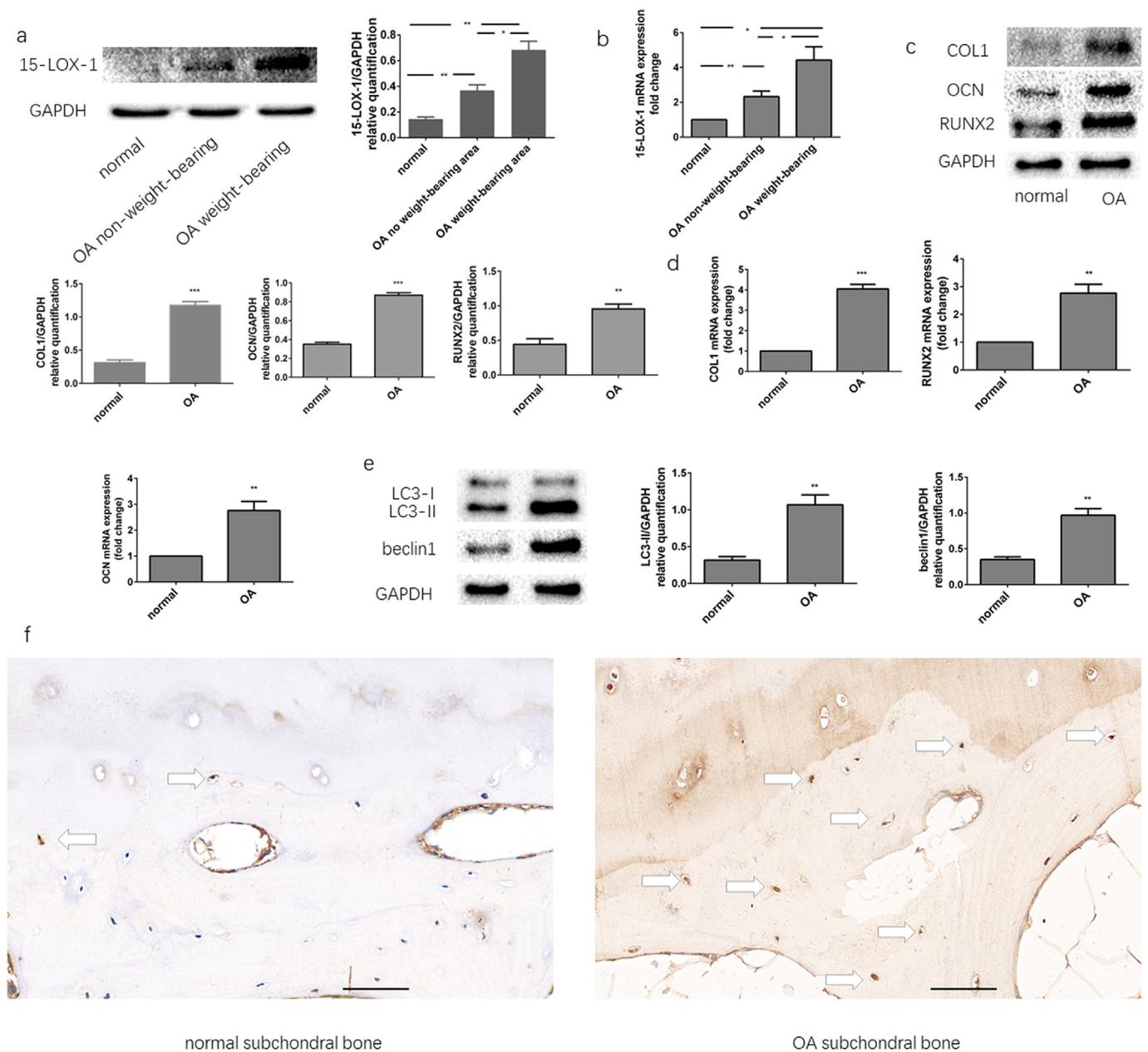


Fig. 1. The expression level of 15-LOX-1, RUNX2, COL1 and OCN in OA subchondral bone was increased. (1a) 15-LOX-1 expression in the OA weight-bearing area, OA non-weight-bearing area and normal subchondral bone by western blot analysis. Normal specimens were obtained from amputees patients without OA ($n = 6$), OA specimens were obtained from OA patients suffering from total knee replacement ($n = 6$), $*P < 0.05$, $**P < 0.01$. The protein concentration was determined by densitometry using ImageJ software. (1b) 15-LOX-1 expression in the weight-bearing area, non-weight-bearing area and normal subchondral bone by qRT-PCR analysis. Normal specimens were obtained from amputees patients without OA ($n = 6$), OA specimens were obtained from OA patients suffering from total knee replacement ($n = 6$), $**P < 0.01$, $*P < 0.05$. (1c) RUNX2, COL1 and OCN expression in the weight-bearing area and the normal subchondral bone by western blot analysis, $**P < 0.01$, $***P < 0.001$. The protein concentration was determined by densitometry using ImageJ software. (1d) RUNX2, COL1 and OCN expression in the weight-bearing area and the normal subchondral bone by qRT-PCR analysis. $**P < 0.01$, $***P < 0.001$. (1e) LC3-II and beclin1 expression in the weight-bearing area and the normal subchondral bone by western blot analysis, $**P < 0.01$. The protein concentration was determined by densitometry using ImageJ software. (1f) 15-LOX-1 expression were observed by immunohistochemistry staining in normal group and OA group. The arrows point to expression of 15-LOX-1. Scale bar = 75 μ m.

(1 : 4000) (sungen biotech, tianjing, China) under room temperature for 1 h. The protein concentration was determined by densitometry using ImageJ software.

2.5. Quantification of mRNA and qRT-PCR

Total RNA was isolated from subchondral bone osteoblasts with TRIzol reagent (Invitrogen, US) as previously described (see online supplementary methods), cDNA was synthesized using 1 μ g of RNA and

a RevertAid First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). cDNA was amplified using the SYBR Premix Ex Tag Kit (TaKaRa) and an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA) by the qRT-PCR. The primer sequences used in this study are shown in Table 1. The fold change of relative mRNA expression levels was evaluated using the $2^{-\Delta\Delta Ct}$ method.

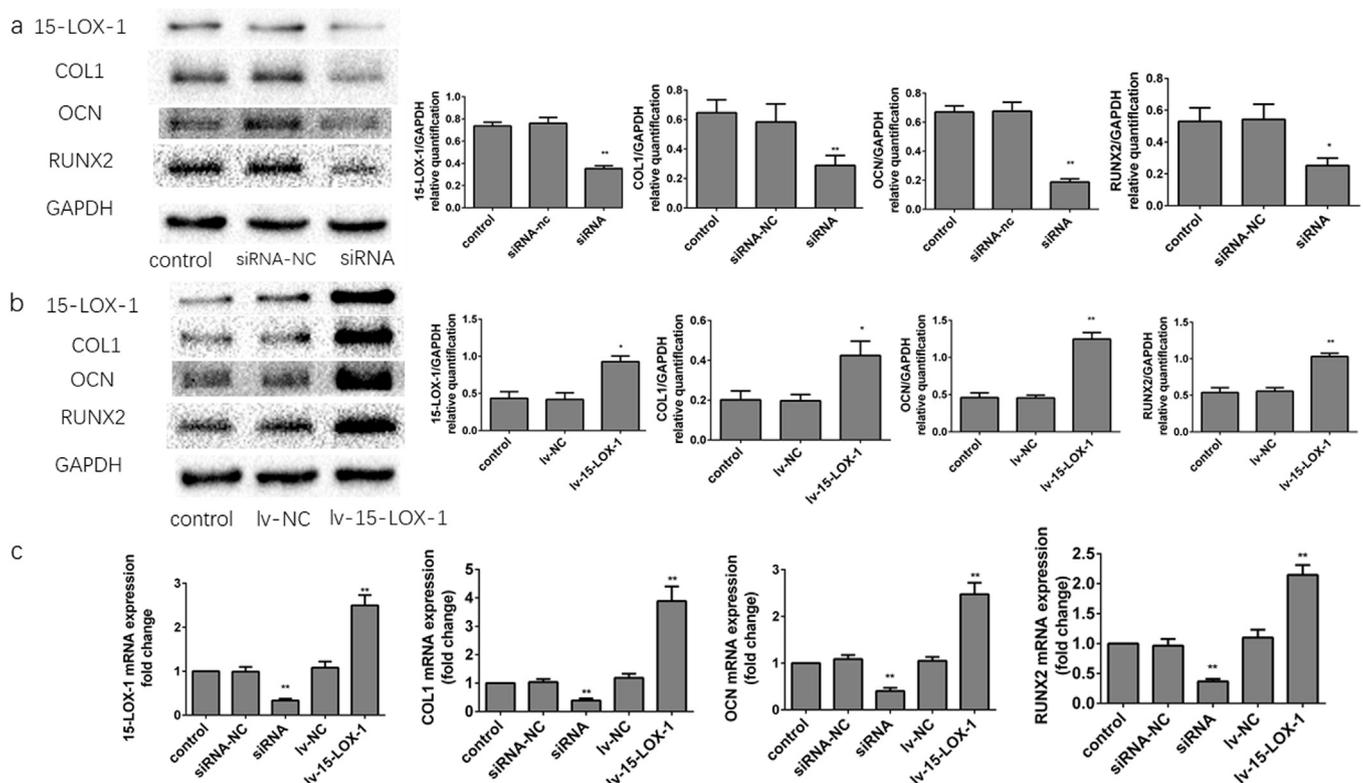


Fig. 2. 15-LOX-1 is involved in the secretion of abnormal phenotype of human osteoarthritic osteoblasts. (2a) 15-LOX-1, RUNX2, COL1 and OCN expression of osteoblasts transfected with siRNA 15-LOX-1, siRNA -NC in the weight-bearing area subchondral bone from OA patients by western blot analysis, ** $P < 0.01$, * $P < 0.05$. The protein concentration was determined by densitometry using ImageJ software. (2b) 15-LOX-1, RUNX2, COL1 and OCN expression of osteoblasts transfected with lv-15-LOX-1, lv-NC in the weight-bearing area subchondral bone from OA patients by western blot analysis, ** $P < 0.01$, * $P < 0.05$. The protein concentration was determined by densitometry using ImageJ software. (2c) 15-LOX-1, RUNX2, COL1 and OCN expression of osteoblasts transfected with lv-15-LOX-1, lv-NC, siRNA 15-LOX-1, siRNA -NC in the weight-bearing area subchondral bone from OA patients by qRT-PCR analysis, ** $P < 0.01$.

2.6. Immunohistochemistry

The expression of 15-LOX-1 was measured by Immunohistochemistry in OA subchondral bone. The specimens were performed by immunohistochemical, according to the manufacturer's protocols (Servicebio wuhan, CHINA). The slides were incubated with a BSA (Servicebio wuhan, CHINA) for 30 min under room temperature, they were covered with the antibody against 15-LOX-1 under room temperature for 2 h. Subsequently, secondary antibody Goat anti-Mouse IgG (H + L) (Servicebio, Wuhan, China) were added into the parts, followed by a peroxidase-labeled streptavidin-biotin staining technique (DAB Kit, Servicebio wuhan, CHINA).

2.7. Transmission electron microscopy

Osteoblasts were fixed in 4 °C glutaraldehyde/0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide, washed, dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, and 100%) and 100% acetone, and embedded in 1:1 acetone/embedding resin. The resin blocks were cut with leica ultramicrotome (leica, Germany). Thin (60–80nm) sections were stained with uranyl acetate and lead citrate. The sections were examined with an H-7700 transmission electron microscope (HITACHI, Ibaraki, Japan).

2.8. Statistical analysis

All quantitative data were expressed as mean \pm SEM. The comparison between the two groups of data was analyzed by Student's *t*-test. We used ANOVA and paired *t*-test to compare the three groups; *p* values < 0.05 were considered statistically significant.

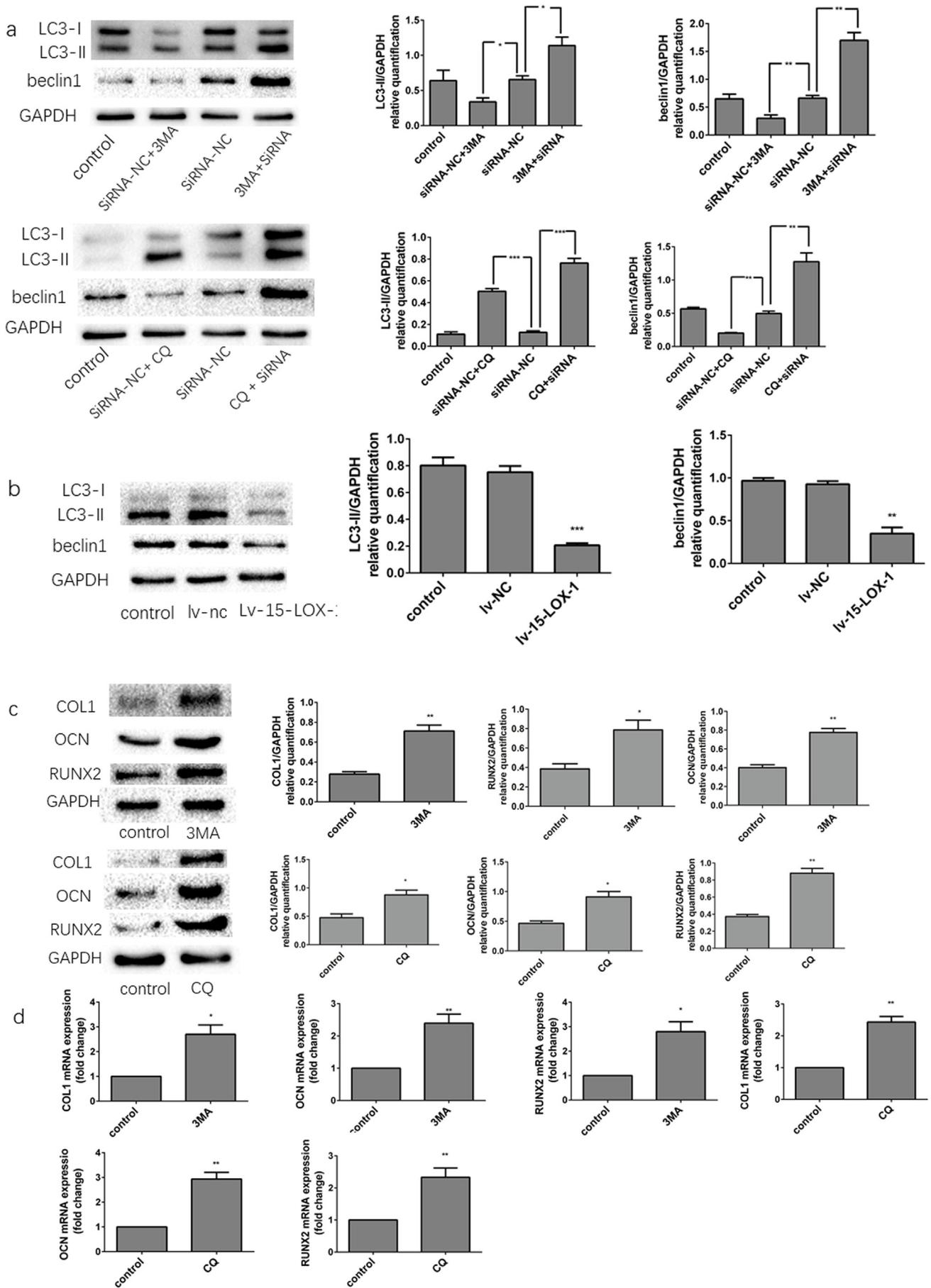
3. Results

3.1. The expression levels of 15-LOX-1, RUNX2, COL1 and OCN in subchondral bone were increased in OA

The differences in the expression of 15-LOX-1 of osteoblasts in the OA subchondral bone and the normal subchondral bone was measured. Normal specimens were obtained from amputees, OA specimens were obtained from OA patients suffering from total knee replacement. Western blot and qRT-PCR were used to detect the expression levels of 15-LOX-1. When compared to normal specimen, the expression levels of 15-LOX-1 in OA specimen were increased. Whereas the expression levels of 15-LOX-1 were more obviously increased in OA weight-bearing area specimens from in non-weight-bearing area specimens (Fig. 1a and b). Immunohistochemistry staining revealed that expression of 15-LOX-1 in the OA group was significantly higher than the normal group (Fig. 1f). The expression levels of RUNX2, COL1 and OCN in normal and OA osteoblasts were measured by western blot and qRT-PCR, the expression levels of RUNX2, COL1 and OCN was significantly increased in osteoblasts of patients with OA (Fig. 1c,d), which showed that samples from OA patients were reliable. The expression of beclin-1 and LC3-II in the OA area and the normal subchondral bone were measured by western blot, the expression levels of beclin-1 and LC3-II was significantly increased in OA osteoblasts (1e).

3.2. 15-LOX-1 induce expression of RUNX2, COL1 and OCN

15-LOX-1 siRNA or overexpressing lv-15-LOX-1 was transfected into osteoblasts extracted from the subchondral bone of OA. Western blotting results showed that the expression of 15-LOX-1, RUNX2, COL1 and



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Fig. 3. 15-LOX-1 is involved in autophagy in human osteoarthritic osteoblasts. (3a) LC3-II, beclin1 expression of osteoblasts transfected with siRNA 15-LOX-1, siRNA-NC in the weight-bearing area subchondral bone from OA patients by western blot analysis, **P < 0.01. The protein concentration was determined by densitometry using ImageJ software. (3b) LC3-II, beclin1 expression of osteoblasts transfected with lv-15-LOX-1, lv-NC in the weight-bearing area subchondral bone from OA patients by western blot analysis, **P < 0.01. (3c) RUNX2, COL1 and OCN expression of osteoblasts pretreated with 3MA and CQ in the weight-bearing area subchondral bone from OA patients by western blot analysis, **P < 0.01. The protein concentration was determined by densitometry using ImageJ software (3d) RUNX2, COL1 and OCN expression of osteoblasts pretreated with 3MA and CQ in the weight-bearing area subchondral bone from OA patients by qRT-PCR analysis, **P < 0.01.

OCN were upregulated in transfected with overexpressing lv-15-LOX-1 osteoblasts (Fig. 2a). Moreover, the expression of 15-LOX-1, RUNX2, COL1 and OCN were significantly reduced in transfected with 15-LOX-1 siRNA osteoblasts (Fig. 2b), and the similar results were further confirmed by qRT-PCR (Fig. 2c). These results suggested that 15-LOX-1 could induce increased expression of RUNX2, COL1 and OCN.

3.3. 15-LOX-1 inhibits autophagy in osteoblasts

15-LOX-1 was involved in the regulation of autophagy in multiple previous reports. Therefore, we further applied 15-LOX-1 siRNA or lv-15-LOX-1 in osteoblasts to test the relationship between 15-LOX-1 and autophagy. The osteoblasts were treated with autophagy inhibitors CQ (10 μ M) or 3-MA (5mM) for 2 h. 3MA blocks Class III PI3K and prevents early autophagy, while CQ is a lysosomal lumen alkaliizer that prevents late autophagy. In the present study, we found that the expression of LC3-II was significantly increased in the CQ group. However, the expression of LC3-II was significantly decreased in the 3-MA group. Moreover, the expression levels of beclin1 were both significantly downregulated in the CQ group or 3-MA group. In the next experiment, osteoblasts were pretreated with CQ or 3-MA after 15-LOX-1 siRNA were transfected into osteoblasts. We showed that the expression levels of LC3-II and beclin1 were significantly increased after 15-LOX-1 siRNA were transfected into osteoblasts (Fig. 3a). Meanwhile, the expression levels of LC3-II and beclin1 level were significantly reduced after lv-15-LOX-1 were transfected into osteoblasts (Fig. 3b). These results suggested that 15-LOX-1 could inhibit autophagy. Western blotting results suggested that the expression of RUNX2, COL1 and OCN were upregulated after pretreated with 3-MA and CQ (Fig. 3c), the similar results were obtained by qRT-PCR (Fig. 3d), the results suggest that inhibition of autophagy could induce abnormal phenotypes of osteoblasts in OA.

3.4. 15-LOX-1 can inhibit autophagy through mTORC1

Osteoblasts were pretreated with mTORC1 inhibitor rapamycin (5mM) for 2 h, Western blotting analysis showed that rapamycin effectively blocked phosphorylation of mTORC1 and induced the expression of LC3-II and beclin1. The expression of LC3-II and beclin1 were significantly upregulated by combination of rapamycin and siRNA 15-LOX-1, compared with rapamycin alone (Fig. 4a). While the expression of LC3-II and beclin1 were significantly reduced by combination of rapamycin and transfected with lv-15-LOX-1, compared with rapamycin alone (Fig. 4b), suggesting that 15-LOX-1 can inhibit autophagy through mTORC1 activation. Osteoblasts were pretreated with rapamycin, COL1, RUNX2 and OCN levels were significantly decreased (Fig. 4c,d). These results suggested that autophagy could inhibit abnormal phenotypes of osteoblasts in OA.

3.5. The inhibition of autophagy caused by 15-LOX-1 was confirmed by transmission electron microscopy

After 15-LOX-1 siRNA or lv-15-LOX-1 was transfected into osteoblasts, we found that the number of autophagosomes and autolysosomes in osteoblasts transfected with 15-LOX-1 siRNA significantly increased, whereas pretreatment with lv-15-LOX-1 obviously reduced the number of autophagosomes and autolysosomes. The results suggested that 15-LOX-1 can inhibit autophagy (Fig. 5a,b,5c,5d).

4. Discussion

In previous reports, 15-LOX-1 was involved in various pathological processes and exacerbated the inflammatory response [13,15]. Moreover, 15-LOX-1 resulted in cartilage degeneration in OA [16], but whether 15-LOX-1 can affect OA subchondral bone is not clear. Sclerosis of the subchondral bone can directly lead to the occurrence of OA and participate in the development of the condition. The appearance of abnormal phenotypes in the subchondral bone osteoblasts plays the most critical role in subchondral bone sclerosis. Therefore, it is particularly important to study the mechanisms that induce abnormal phenotypes in these osteoblasts during OA.

Increasing the expression of mice 15-LOX-1 enhanced the abnormal phenotypes of mouse osteoblasts and increased bone regeneration [31–35]. However, the expression of 15-LOX-1 in human subchondral bone had not been reported. Thus, we first determined the expression of 15-LOX-1 in human subchondral bone osteoblasts. Our results showed that the levels of 15-LOX-1 were higher in the subchondral bone of OA than that in normal subchondral bone. Furthermore, the expression of 15-LOX-1 was higher in the weight-bearing than that in the non-weight-bearing area of the subchondral bone of OA. To the best of our knowledge, this is the first study reporting the expression levels of 15-LOX-1 in the human subchondral bone.

The abnormal phenotype-related genes RUNX2, COL1, and OCN were also highly expressed in the osteoblasts of the subchondral bone of OA patients. RUNX2 is a specific transcription factor of osteogenic differentiation which plays a key role in regulating bone metabolism and bone formation, and increasing the abnormal phenotypes of osteoblasts inducing the generation of COL1 and OCN [1,7,8,11,12]. COL1 is a primary extracellular matrix of osteoblast synthesis [36]. OCN plays an important role in promoting mineral deposit in bone tissues [37]. The results of the present study demonstrated that the expression of RUNX2, COL1, and OCN were upregulated in accordance with previous reports [7,38]. We further observed that the expression of RUNX2, COL1, and OCN significantly decreased after 15-LOX-1 silencing, and significantly increased after 15-LOX-1 overexpression, suggesting that 15-LOX-1 could regulate the abnormal phenotypes of osteoblasts, and further induce the sclerosis of subchondral bone, and eventually accelerate the progression of OA.

Autophagy plays an important role in regulating the expression of osteoblast osteogenic phenotypes and could promote normal osteoblast differentiation [20,39,40]. However, the role of autophagy in OA osteoblasts had not been clarified. On the one hand, autophagy can maintain cell structure and function balance. On the other hand, previous studies [7,38] had indicated that OA osteoblasts overexpress osteogenic phenotypes. Therefore, we speculated that autophagy activity was increased in OA osteoblast and could inhibit the overexpression of abnormal osteoblast phenotypes. Our results showed that the expression of autophagy markers beclin-1 and LC3-II were significantly upregulated. Meanwhile, our data also showed that autophagy inhibitors 3MA and CQ reduced the expression of RUNX2, COL1, and OCN in OA osteoblasts. Therefore, autophagy inhibited the overexpression of genes involved with osteogenic phenotypes in osteoblasts during OA and could potentially alleviate OA symptoms. To the best of our knowledge, this is the first report of the relationship between autophagy and OA in subchondral bone osteoblasts.

Later study demonstrated that 15-LOX-1 plays an important role in

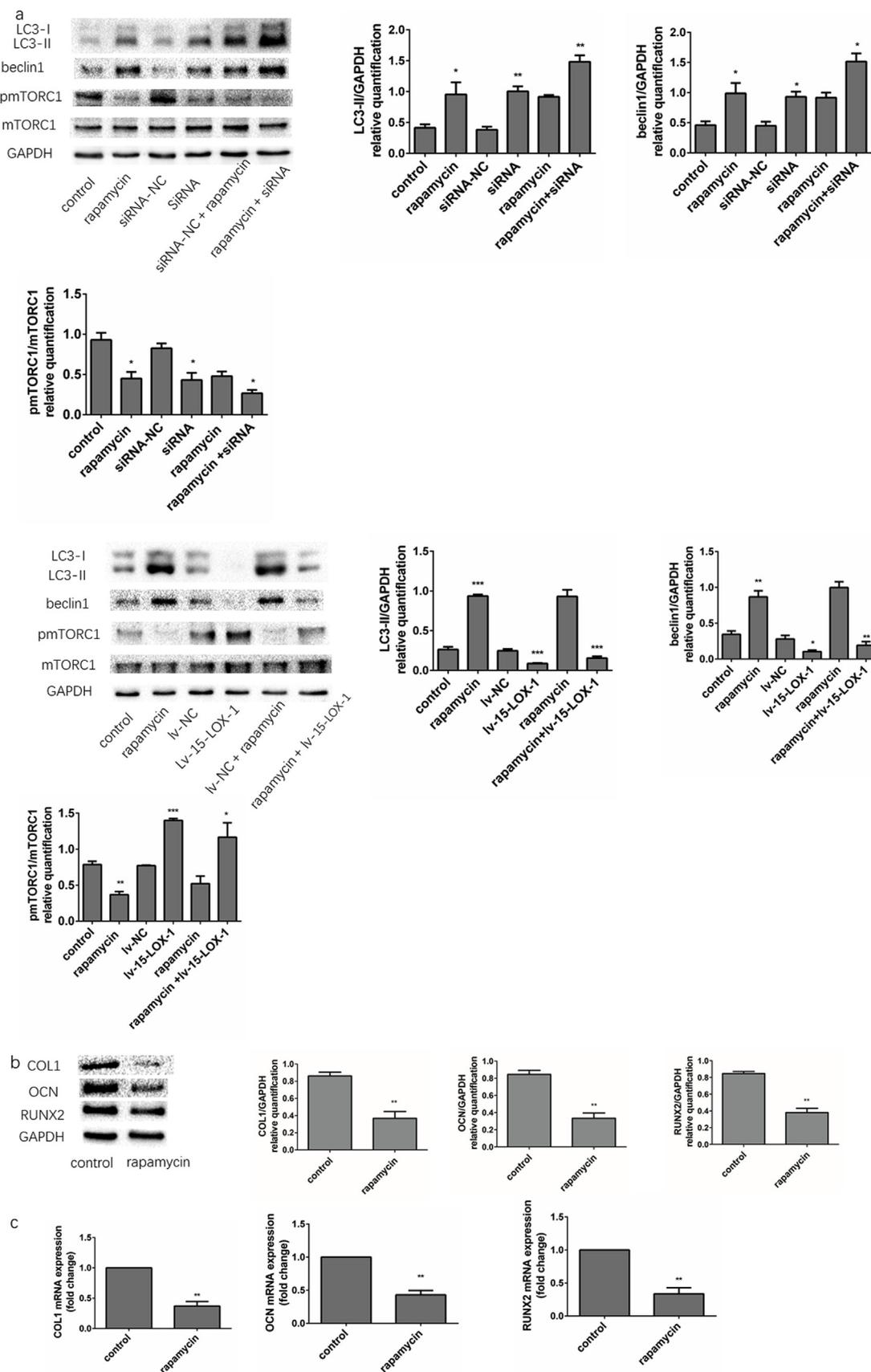
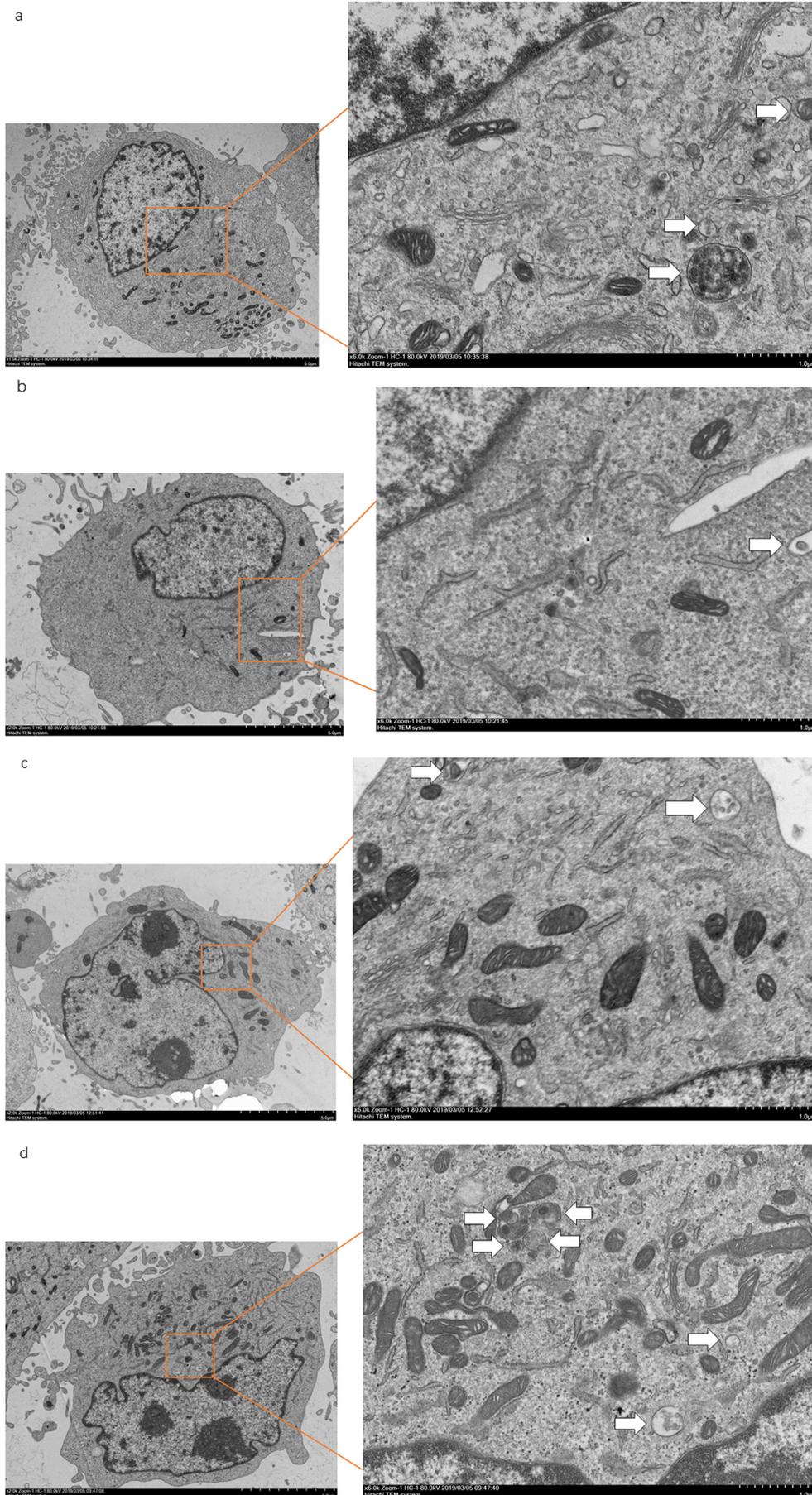


Fig. 4. 15-LOX-1 can inhibit autophagy through mTORC1. (4a) LC3-II, mTORC1, pmTORC1, and beclin1 expression of osteoblasts transfected with siRNA15-LOX-1, siRNA-NC,lv-15-LOX-1, lv-NC and pretreated with rapamycin in the weight-bearing area subchondral bone from OA patients by western blot analysis, **P < 0.01. (4b) COL1,RUNX2,OCN pretreated with rapamycin in the weight-bearing area subchondral bone from OA patients by western blot analysis, **P < 0.01. The protein concentration was determined by densitometry using ImageJ software. (4c) RUNX2, COL1 and OCN expression of osteoblasts pretreated with rapamycin in the weight-bearing area subchondral bone from OA patients by qRT-PCR analysis, **P < 0.01.



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Fig. 5. The inhibition of autophagy caused by 15-LOX-1 was confirmed by transmission electron microscopy. The number of autophagosomes and autolysosomes in OA osteoblasts transfected with lv-NC (5a) is higher than in OA osteoblasts transfected with lv-15-lox-1(5b); and the number is lower in OA osteoblasts transfected with siRNA-NC(5c) compared to OA osteoblasts transfected with siRNA 15-lox-1 (5d). The arrows point to autophagosomes and autolysosomes.

regulating autophagy. Inhibition 15-LOX-1 could increase level of autophagy in macrophages [41], meanwhile, increasing the level of 15-LOX-1 inhibit autophagy in RAW 264.7 cells [28]. However, 15-LOX-1 whether can affect autophagy in human osteoblasts is unclear. Our results showed that the expression levels of 15-LOX-1 was involved in regulating autophagy marker proteins lc3-II and beclin1 in OA osteoblasts and that transfection with 15-LOX-1 siRNA induced the expression of osteoblast autophagy markers. Meanwhile, transfection with lv-15-LOX-1 suppressed osteoblast autophagy markers.

The results of transmission electron microscopy showed that upregulating the expression of 15-LOX-1 decreased the number of autophagosomes and autolysosomes in osteoblasts while reducing the expression of 15-LOX-1 increased the number of autophagosomes in osteoblasts. These results suggest that 15-LOX-1 upregulated the osteogenic phenotypes in OA osteoblasts by inhibiting autophagy, eventually leading to the over-ossification of subchondral bone, which exacerbates the progression of OA.

Activation of the mTORC1 in articular cartilage and subchondral bone plays an important role in aggravating OA progression, mTORC1 could result in chondrocyte apoptosis and degeneration [42–44]. mTORC1 could also promote subchondral bone osteogenesis and osteosclerosis and the expression of RUNX2, COL1, and OCN which are markers of osteoblast osteogenic phenotypes [43,45]. Previous studies have reported that 15-LOX-1 stimulated angiogenesis in adipose tissue by activating the mTORC1 pathway [47]. Meanwhile, the mTORC1 pathway is a classic pathway that can inhibit autophagy [23]. Our study found that 15-LOX-1 inhibited autophagy and that osteoblasts transfected with siRNA 15-LOX-1 blocked the phosphorylation mTORC1 and enhanced the expression of LC3-II.

Meanwhile, osteoblasts transfected with lv-15-LOX-1 increased phosphorylation mTORC1 and inhibited LC3-II expression. Additionally, the treatment with mTORC1 inhibitor rapamycin increased the expression of RUNX2, COL1, and OCN. Therefore, reduced expression of 15-LOX-1 in the osteoblasts of the OA subchondral bone could inhibit phosphorylation mTORC1, increase the levels of autophagy, and inhibit the overexpression of abnormal osteoblast phenotypes, eventually decelerate OA progression.

5. Conclusion

Our results suggested that the expression of 15-LOX-1 on osteoblasts from the subchondral bone increased in OA. 15-LOX-1 inhibited autophagy by activated mTORC1, which in turn upregulated the markers of abnormal osteoblast phenotypes RUNX2, COL1, and OCN.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

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Abbreviations

15-LOX-1	15-lipoxygenase-1
3MA	3-Methyladenine
COL1	collagen type 1 alpha 1
CQ	chloroquine
mTOR	mammalian target of rapamycin

OA	osteoarthritis
OCN	Osteocalcin
PI3K	phosphoinositide 3kinase
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RUNX2	runt-related transcription factor 2

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2019.116980>.

Author contribution

All authors contributed to data interpretation and to the writing of the paper, made final decisions on all parts of the paper, and approved the final version of the submitted paper. Moreover, all authors agreed to any changes to the author list after the initial submission. YP Wan analyzed dates and collected the specimen, constructed, revised and wrote the manuscript. D Li analyzed dates and revised the manuscript, YX Lv, MM Wu, L Li analyzed partly dates. ZS Yin was in charge of designing the experiment.

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