Original Article

Induction of osteoclast formation by LOX mutant (LOXG473A) through regulation of autophagy

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Background: Lysyl oxidase (LOX) has been identified to modulate osteoclast activity, so we explored the role of LOX_{G473A}, the highest frequency single nucleotide polymorphism in LOX, in osteoclast formation and its potential relationship to autophagy.

Methods: The ability of the LOX mutant, LOX_{G473A}, to promote autophagy and osteoclast formation was evaluated in the pre-osteoclast cell line RAW264.7. Furthermore, autophagy-related protein expression and autophagosomes were detected by western blot and electron microscopy, respectively. Simultaneously, osteoclast formation and resorption ability were also detected using TRAP staining assay and bone resorption assay. In addition, the osteoclast-related proteins and mRNAs, as well as p-AMPKα and p-mTOR proteins, were further evaluated by western blot and qPCR assays.

Results: Autophagy inhibitor 3-MA suppressed the Beclin-1 and ATG5 protein levels and the ratio of LC3-II to LC3-I, as well as autophagosome formation in RAW264.7 transfected with the MUT plasmid and enhanced p62 protein expression. Simultaneously, 3-MA also reduced osteoclast formation and resorption, as well as the F-actin ring level of osteoclasts. In addition, 3-MA inhibited osteoclast-related protein and mRNA expression, including NFATC1, ACP5, CTSK. And the autophagy-related pathway protein p-AMPKα was increased and p-mTOR was reduced by 3-MA treatment. However, autophagy agonist RAPA reversed the effect of 3-MA on RAW264.7 with LOX_{G473A} mutation, indicating that promoting autophagy could enhance the ability of LOX_{G473A} to induce osteoclast formation.

Conclusions: LOX mutant (LOX_{G473A}) might promote osteoclast formation for RAW264.7 by enhancing autophagy via the AMPK/mTOR pathway, which is a new direction for bone disease research.

Keywords: Autophagy; lysyl oxidase (LOX); LOXG473A; osteoclast formation; RAW264.7

Introduction

Bone is a tissue that needs continuous self-renewal to ensure the integrity of the skeleton (1). Bone resorption and formation is a dynamic balanced process; once the balance is disturbed, it will lead to bone-related diseases such as osteoporosis, osteoarthritis, and osteosarcoma (2,3). Of these, osteoporosis, with its features of low bone thickness and high danger of fracture, is a public health issue (4,5). Osteoporosis-related fracture is a common cause of injury and death in the elderly (6). Osteoclasts, which originate

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from precursor cells of macrophages/monocytes, play a key role in bone resorption (4) and abnormal formation of osteoclasts is considered to be the main cause of osteoporosis. In osteoporosis, the formation of osteoblasts increased (7), which means inhibition the activity of osteoclasts may alleviate osteoporosis.

Osteoclast differentiation and formation is a complicated biological procedure involving a variety of signaling pathways and cytokines, among which, receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) are the foremost cytokines (8). M-CSF is an important factor for the proliferation of osteoclast precursor cells, while RANKL stimulates osteoclast precursor cells to mature into osteoclasts (9). Moreover, early studies have shown that extracellular lysyl oxidase (LOX) can catalyze the oxidative deamination of amino acid residues in collagen (10), and the aldehydes produced can form important cross-links to stabilize functional collagen, such as bone collagen (11). Independent of the RANKL system, LOX can also induce bone metastasis and bone destruction in breast cancer patients (12). Human CD14$^+$ monocytes cultured without RANKL but with LOX form osteoclasts (13), indicating that LOX might be a new regulator of osteoclast activation.

G473A is situated in the highly conserved area of the LOX gene and is also currently the most widely studied mutation site, where arginine at position 158 of the encoded protein can be mutated to glutamine, thereby inhibiting the degradation of the LOX proenzyme to the LOX propeptide and thus increasing the expression of active LOX (14). In addition, a LOX mutant (LOX$_{G473A}$) is also related to abnormalities in various diseases (15,16). Therefore, LOX$_{G473A}$ is an important gene locus that affects human health. Recently, we confirmed for the first time that LOX$_{G473A}$ is closely related to lumbar fractures in postmenopausal women (17). Compared with the GG genotype, the fracture risk of people with GA and AA genotypes increased by 1.28- and 1.74-fold, respectively (17). Nevertheless, the mechanism by which LOX$_{G473A}$ drives osteoclast differentiation remains vague in osteoporosis.

Autophagy can degrade lipids, damaged proteins and organelles to maintain cell homeostasis, and it is also an evolutionary conservative process (18,19). Autophagy is a pleiotropic function, involving cell survival, nutritional supply during hunger, antigen presentation, and resistance to pathogens (20). Research in recent years has shown that autophagy participates in the degradation of osteocytes, osteoclasts, and osteoblasts, which may be a novel pathogenic mechanism for bone homeostasis (21). Research has shown that kaempferol restrains bone resorption and osteoclast differentiation by inhibiting autophagy (20). Based on the previously known involvement of LOX$_{G473A}$ in the regulation of osteoclast formation (17), and the role of autophagy in the regulation of osteoclast formation (20), it is not clear whether they have a reciprocal regulatory relationship. In this study, the pre-osteoclast cell line RAW264.7 were used to explore the role of LOX$_{G473A}$ in osteoclast differentiation and the potential role of autophagy in osteoclast differentiation, giving insight to the treatment of osteoporosis. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-4474).

Methods

Cell culture

The murine monocytic macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (TIB-71, ATCC USA) and were cultured in α-MEM medium (Gibco, USA) with the addition of 60 ng/mL recombinant mouse RANKL (PeproTech, USA), 10 ng/mL M-CSF (R&D Systems, USA) and 10% FBS for 5 days at 37 ℃ in 5% CO$_2$ atmosphere for osteoclast differentiation (22).

Transfection

Wild-type LOX (WT) and mutant LOX$_{G473A}$ (MUT) genes were obtained from GenePharma (China). Sequences are shown in *Table 1*. The WT and MUT genes were cloned into a pcDNA3.1 plasmid. Synthetic plasmids and empty vector (Vector) were transfected into RAW264.7 using the Lipofectamine TM 3000 transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

TRAP staining assay

TRAP a marker enzyme of osteoclasts. Different RAW264.7 transfected plasmids were cultured in α-MEM medium with the addition of 60 ng/mL recombinant mouse RANKL, 10 ng/mL M-CSF, and 10% FBS for osteoclast differentiation for 5 days (23). The cells were then simultaneously treated with RAPA or 3-MA. The cells were fixed and stained for TRAP 5b using a TRAP staining kit (Sigma-Aldrich, USA) and the manufacturer's protocol (24). Cells stained red were

Table 1 Coding sequence (CDS) of LOX gene

ID	Sequence
Wild-type LOX CDS	ATGCGTTTCGCCTGGGCTGTGCTCCTTCTGGGGCCACTGCAGCTTTGTCCCCTTCTCCGCTGCGCCCCGCAG ACCCCGCGCGAGCCGCCCGCCGCCCCTGGTGCCTGGCGCCAGACAATCCAATGGGAGAACAACGGGCAGG TGTTCAGTCTGCTGAGCCTCGGGGCGCAGTACCAGCCTCAGCGACGCCGCGACCCCAGTGCCACTGCCCGG AGACCCGACGGCGACGCAGCCTCGCAGCCGCGCACGCCCATTCTTCTGCTGCGTGACAACCGCACCGCCTC TACCCGTGCGAGGACGCCAAGCCCGTCTGGGGTCGCCGCGGGTCGTCCCCGGCCCGCCGCCCGCCACTGG TTCCAAGCTGGTTTCTCGCCGTCGGGGGCTCGCGATGGAGCCTCACGGCGCGCGGCGAACCGGACTGCATC GCCACAGCCTCCGCAGCTCAGTAATCTGAGGCCACCCAGCCACATAGATCGCATGGTGGGCGACGACCCCTA CAATCCCTACAAGTACTCCGACGACAACCCCTATTATAACTACTATGACACGTATGAGAGGCCCCGGCCCGGGA GCAGGAACCGACCTGGATACGGCACCGGTTACTTCCAGTACGGTCTCCCGGACCTGGTGCCCGACCCCTACT ACATCCAGGCTTCCACGTACGTCCAGAAGATGTCTATGTACAACCTGAGATGCGCTGCGGAAGAAAACTGCCTG GCCAGTTCAGCATATAGGGCGGATGTCAGAGACTATGACCACAGGGTACTGCTACGATTTCCGCAAAGAGTGAA GAACCAAGGGACATCGGACTTCTTACCAAGCCGCCCTCGGTACTCCTGGGAGTGGCACAGCTGTCACCAACAT TACCACAGCATGGACGAATTCAGCCACTATGACCTGCTTGATGCCAACACACAGAGGAGAGTGGCTGAAGGCC ACAAAGCAAGCTTCTGTCTGGAGGACACGTCCTGTGACTATGGGTACCACAGGCGCTTTGCGTGCACTGCACA CACACAGGGATTGAGTCCTGGATGTTATGACACCTATGCGGCAGACATAGACTGCCAGTGGATTGATATTACAGA TGTACAACCTGGAAACTACATTCTAAAGGTCAGTGTAAACCCCAGCTACCTGGTGCCTGAATCAGACTACACTAA CAATGTTGTACGCTGTGACATTCGCTACACAGGACATCATGCCTATGCCTCAGGCTGCACAATTTCACCGTATTAG
Mutant LOX$_{G473A}$ CDS	ATGCGTTTCGCCTGGGCTGTGCTCCTTCTGGGGCCACTGCAGCTTTGTCCCCTTCTCCGCTGCGCCCCGCAGA CCCCGCGCGAGCCGCCCGCCACCCCTGGTGCCTGGCGCCAGACAATCCAATGGGAGAACAACGGGCAGGTG TTCAGTCTGCTGAGCCTCGGGGCGCAGTACCAGCCTCAGCGACGCCGCGACCCCAGTGCCACTGCCCGGAG ACCCGACGGCGACGCAGCCTCGCAGCCGCGCACGCCCATTCTTCTGCTGCGTGACAACCGCACCGCCTCTAC CCGTGCGAGGACGCCAAGCCCGTCTGGGGTCGCCGCGGGTCGTCCCCGGCCCGCCGCCCGCCACTGGTTC CAAGCTGGTTTCTCGCCGTCGGGGGGCTCGCGATGGAGCCTCACGGCGCGCGGCGAACCGGACTGCATCGCC ACAGCCTCCGCAGCTCAGTAATCTGAGGCCACCCAGCCACATAGATCGCATGGTGGGCGACGACCCCTACAAT CCCTACAAGTACTCCGACGACAACCCCTATTATAACTACTATGACACGTATGAGAGGCCCCGGCCCGGGAGCAG GAACCGACCTGGATACGGCACCGGTTACTTCCAGTACGGTCTCCCGGACCTGGTGCCCGACCCCTACTACATC CAGGCTTCCACGTACGTCCAGAAGATGTCTATGTACAACCTGAGATGCGCTGCGGAAGAAAACTGCCTGGCCA GTTCAGCATATAGGGCGGATGTCAGAGACTATGACCACAGGGTACTGCTACGATTTCCGCAAAGAGTGAAGAAC CAAGGGACATCGGACTTCTTACCAAGCCGCCCTCGGTACTCCTGGGAGTGGCACAGCTGTCACCAACATTACC ACAGCATGGACGAATTCAGCCACTATGACCTGCTTGATGCCAACACACAGAGGAGAGTGGCTGAAGGCCACAA AGCAAGCTTCTGTCTGGAGGACACGTCCTGTGACTATGGGTACCACAGGCGCTTTGCGTGCACTGCACACACA CAGGGATTGAGTCCTGGATGTTATGACACCTATGCGGCAGACATAGACTGCCAGTGGATTGATATTACAGATGTAC AACCTGGAAACTACATTCTAAAGGTCAGTGTAAACCCCAGCTACCTGGTGCCTGAATCAGACTACACTAACAATGT TGTACGCTGTGACATTCGCTACACAGGACATCATGCCTATGCCTCAGGCTGCACAATTTCACCGTATTAG

defined as differentiated osteoclast-like cells. Cells with more than 3 nuclei were considered multinucleated cells. The number of TRACP+ cells was counted using Image-Pro Plus software.

Bone resorption assay

Different RAW264.7 transfected plasmids (1.5×10^3 cells/well) were seeded onto a bone biomimetic synthetic surface (Osteo Assay Surface 24-Well Multiple Well Plates; Corning, USA) (24). The medium was changed every 2 days and after 5 days, the plates were washed with phosphate-buffered saline (PBS) and air-dried for 4 h. The area of osteoclastic absorption was observed under a light microscope and quantified by Image-Pro Plus software.

F-actin ring-formation assay

The F-actin ring is a precondition for osteoclast bone resorption and the most significant feature of mature osteoclasts. The culture method of RAW264.7 for osteoclastic differentiation in the TRAP staining experiment was used in the ring-formation experiment. RAW264.7 cells were cultured for osteoclast differentiation for 5 days and then fixed with 4% paraformaldehyde at 4 ℃ for 10 min and stained with 0.1% phalloidin after the osteoclasts formed (25). The images were observed by confocal microscopy (Leica, Germany).

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Table 2 Primer sequences for quantitative real-time PCR (qPCR) detection

ID	Sequence (5'-3')	Product length (bp)
β-actin. Forward	CATTGCTGACAGGATGCAGA	139
β-actin. Reverse	CTGCTGGAAGGTGGACAGTGA	
LOX. Forward	TCTTCTGCTGCGTGACAACC	117
LOX. Reverse	GAGAAACCAGCTTGGAACCAG	
NFATC1. Forward	GACCCGGAGTTCGACTTCG	97
NFATC1. Reverse	TGACACTAGGGGACACATAACTG	
ACP5. Forward	CACTCCCACCCTGAGATTTGT	118
ACP5. Reverse	CATCGTCTGCACGGTTCTG	
CTSK. Forward	GAAGAAGACTCACCAGAAGCAG	102
CTSK. Reverse	TCCAGGTTATGGGCAGAGATT	

Electron microscopy

RAW264.7 transfected with MUT plasmid after 3-MA or RAPA treatment were fixed with 4% paraformaldehyde for 30 min. Cells were incubated using 1.5% osmium tetroxide, followed by dehydration with acetone and then they were embedded in Durcupan resin. The prepared sections were stained with lead citrate and observed under TECNAI 10 electron microscope (Philips, Holland) at 60 kV to observe the formation of autophagy bodies.

qPCR

Total RNA from plasmids transfected RAW264.7 cells or 3-MA or RAPA treated cells was separated using the Trizol reagent and cDNA was obtained from mRNA using the PrimeScipt MMLV RT reagent Kit (TakaRa, Japan) based on the manufacturer's protocol. The quantitative real-time PCR (qPCR) reaction was executed using SYBR green (Bio-Rad, USA) as the fluorescent reporter and an ABI-7500. A total of 40 cycles (95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 2 min) were run for each primer. β-actin was used as a standardized internal control. Primers are shown in *Table 2*.

Western blot

Treated cells were dissolved in RIPA buffer including protease inhibitors, and then 20 μg protein samples were added to SDS-PAGE gel electrophoresis followed by a transfer of protein to a PVDF membrane. Membranes were sealed using 5% fat-free milk for 1 h and then cultured with antibodies against LOX (Abcam, ab174316), Beclin-1 (Abcam, ab207612), LC3B (Abcam, ab192890), p62 (Abcam, ab109012), ATG5 (Abcam, ab108327), mTOR (Abcam, ab134903), p-mTOR (Abcam, ab109268), AMPKα (Abcam, ab32047), p-AMPKα (Abcam, ab133448), NFATC1 (Abcam, ab25916), ACP5 (Abcam, ab238913), CTSK (Abcam, ab239506), and GAPDH (Cell Signaling Technology, #5174) overnight at 4 ℃. After a 30-min washing step in TBS containing 0.1% Tween20, the membranes were cultured with HRP (horseradish peroxidase)-conjugated secondary antibodies (Abcam, ab205718 and ab6728). Visualization was carried out using Pierce™ ECL western blotting substrate (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Analysis was performed with Image-Pro Plus software.

Statistical analysis

All experiments were performed thrice. All data were statistically analyzed with Graphpad Prism 8.0.2, using one-way analysis of variation, followed by Tukey's multiple comparisons test. $P<0.05$ were considered statistically significant.

Results

Effect of LOX$_{G473A}$ on level of autophagy in RAW264.7

We constructed WT and MUT LOX plasmids. Simultaneously, the MUT and Vector were transfected into RAW264.7. The sequencing results showed that the

G in *LOX* was successfully mutated to A (*Figure 1A*). In addition, the mRNA and protein of LOX in the WT and MUT transfected groups were significantly higher than in the Vector transfected group, suggesting that the WT and MUT plasmids were successfully transfected into RAW264.7 (*Figure 1B-1D*). The western blot analysis results also implied that LOX and LOX_{G473A} promoted the levels of Beclin-1, ATG5, and LC3-II protein, as well as the conversion of LC3-II to LC3-I, and suppression of LC3-I and p62 protein expression, suggesting that both WT and MUT induced autophagy (*Figure 1B,1E-1J*). Moreover, the Beclin-1, ATG5, and LC3-II protein expressions in the MUT group were higher than in the WT group (*Figure 1B, 1E,1G,1J*). The LC3-I and p62 protein expressions in the MUT group were lower than those in the WT group, showing that LOX_{G473A} was better able to induce cell autophagy than LOX (*Figure 1B,1F,1I*).

Effect of LOX_{G473A} on osteoclast formation in RAW264.7

We detected the role of LOX_{G473A} in the bone formation and bone resorption of RAW264.7 cells differentiated into osteoclasts. TRAP staining showed significantly increased osteoclast formation in the WT group compared with the Vector group, and significantly increased osteoclast formation in the MUT group compared with the WT group (*Figure 2A*). In addition, bone resorption experiments indicated that the bone resorption area was much greater in the WT group compared with the Vector group, and remarkably higher in the MUT group compared with the WT group (*Figure 2B*), suggesting that MUT promoted bone resorption by osteoclasts.

Effect on LOX_{G473A} promotion of autophagy by 3-MA or RAPA

Western blot analysis indicated that in the MUT + 3-MA group, the p62 protein expression was remarkably higher than in the MUT group, but the Beclin-1 and ATG5 protein levels, as well as the conversion of LC3-I to LC3-II, were remarkably lower than in the MUT group (*Figure 3A,3B*). RAPA showed the opposite effects on the expression levels of the above proteins, indicating that RAPA enhanced the ability of LOX_{G473A} to promote autophagy and 3-MA reduced the ability of LOX_{G473A} to promote autophagy (*Figure 3A,3B*). Furthermore, the electron microscopy results also showed that 3-MA suppressed the production of autophagosomes in RAW264.7 cells transfected with

MUT plasmids, and RAPA promoted the production of autophagosomes inRAW264.7 transfected with MUT plasmids (*Figure 3C*).

### Effect on LOX_{G473A} induction of osteoclast formation by 3-MA or RAPA

Our results showed that RAPA enhanced the ability of LOX_{G473A} to promote autophagy and 3-MA reduced the ability of LOX_{G473A} to promote autophagy. Furthermore, the effect of 3-MA or RAPA on the ability ofLOX_{G473A} to induce osteoclastogenesis was further explored. The TRAP staining results showed that in the MUT + 3-MA group, the number of red cells was remarkably lower in contrast to the MUT group, while in the MUT + RAPA group, the number was much higher in contrast to the MUT group (*Figure 4A*). The result of semiquantitative analysis also indicated that the number of osteoclasts in the MUT + 3-MA group was much fewer than in the MUT group and the number of osteoclasts in the MUT + RAPA group was much greater than in the MUT group (*Figure 4A*). The results indicated that 3-MA reduced osteoclast differentiation of RAW267.4 and RAPA enhanced osteoclast differentiation of RAW267.4 (*Figure 4A*). In addition, bone resorption experiments showed that in the MUT + 3-MA group, the area of bone resorption was markedly less than in the MUT group, while in the MUT + RAPA group, the area of the cells was markedly higher in contrast of the MUT group (*Figure 4B*), suggesting that 3-MA inhibited osteoclast resorption and RAPA promoted osteoclast resorption.

### Effect of blocking autophagy on F-actin expression in osteoclasts

We also tested whether 3-MA and RAPA could affect the production of the F-actin rings of osteoclasts (26). Our results showed that 3-MA inhibited the area of F-actin rings forming in RAW267.4 cells induced by M-CSF (10 ng/mL) and recombinant mouse RANKL (60 ng/mL) (*Figure 5A,5B*). RAPA reversed this phenomenon (*Figure 5A, 5B*). These results indicated that 3-MA might promote osteoclast differentiation by blocking autophagy, while RAPA might induce osteoclast differentiation by promoting autophagy.

Effect of AMPK/p-mTOR in osteoclast differentiation

In order to explore if 3-MA and RAPA affect the
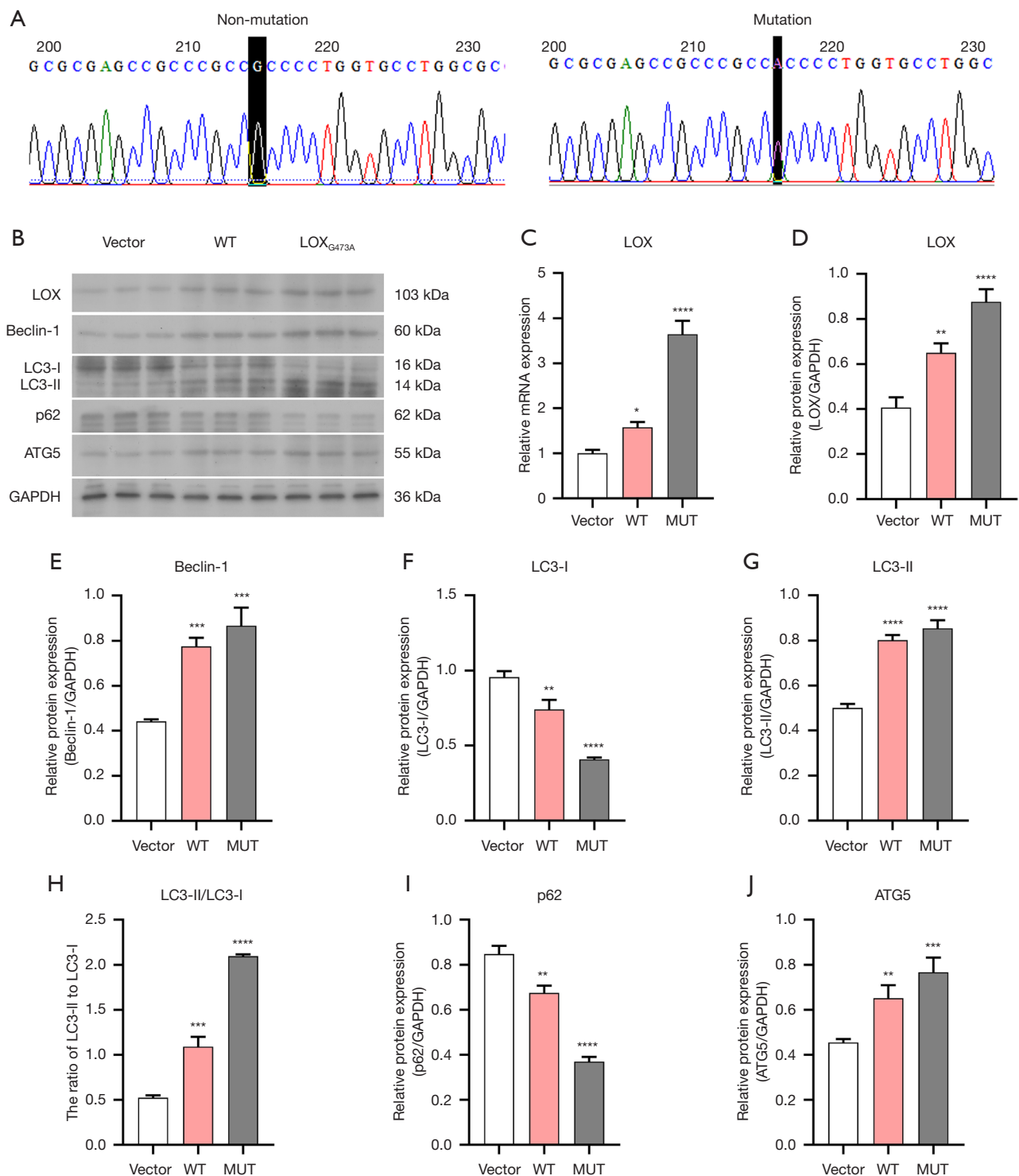
Figure 1 Effect of LOX mutant (LOX_{G473A}) on autophagy level in RAW264.7 cells. (A) Mutation site of LOX gene. (B) The LOX protein and autophagy-related protein expressions in RAW264.7 transfected with wild-type LOX (WT) and mutant LOX_{G473A} (MUT) plasmids tested by western blot. (C) LOX mRNA expression in RAW264.7 transfected with WT and MUT plasmids tested by qPCR. (D-J) Protein expression level in (B) quantified using Image-Pro Plus software. *, P<0.05; **, P<0.01; ***, P<0.001; and ****, P<0.0001 *vs*. Vector represent significant differences.
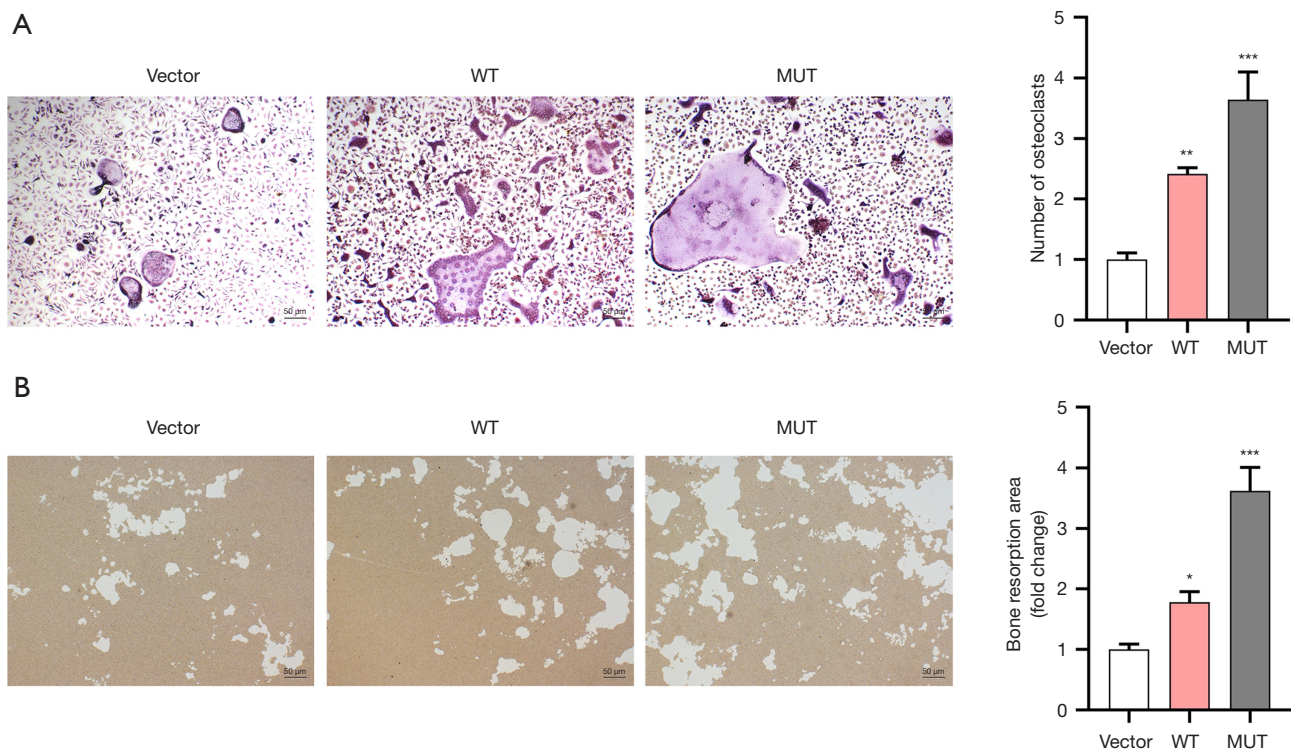
Figure 2 Effect of LOX mutant (LOX_{G473A}) on the differentiation of RAW264.7 into osteoclasts. (A) Osteoclast expression in RAW264.7 transfected with wild-type LOX (WT) and mutant LOX_{G473A} (MUT) plasmids detected by TRAP staining. Magnification ×100. (B) Level of osteoclast resorption level in RAW264.7 transfected with WT and MUT plasmids analyzed by Image-Pro Plus software. *, P<0.05; **, P<0.01 *vs.* Vector and ***, P<0.001 *vs.* WT represent significant differences.

differentiation of osteoclasts by blocking autophagy and the underlying mechanism of their effects, western blot and qPCR assays were carried out to detect the levels of related proteins and mRNAs. The results implied that 3-MA inhibited the NFATC1, ACP5 and CTSK protein expressions in the MUT group and that RAPA promoted the NFATC1, ACP5 and CTSK protein expressions in MUT group (*Figure 6A,6B*). Simultaneously, 3-MA also inhibited the NFATC1, CTSK and ACP5 mRNA levels in the MUT group, and RAPA also promoted the NFATC1, CTSK and ACP5 mRNA levels in the MUT group (*Figure 6C*). These results further illustrated that blocking autophagy could inhibit the osteoclast differentiation ability of RAW264.7 transfected with MUT plasmids, and that promoting autophagy could reverse this phenomenon. Further analysis showed that 3-MA could inhibit the protein levels of p-AMPKα and promote the protein levels of p-mTOR (*Figure 6A,6B*), that RAPA had the opposite effect, suggesting that AMPKα/p-mTOR is involved in autophagy-affected osteoclast differentiation.

Discussion

Osteoporosis is a common bone metabolism disease characterized by bone loss, bone microstructural changes and increased bone fragility (27). Excessive activation of osteoclasts leads to bone metabolism illness such as rheumatoid arthritis and osteoporosis (28,29). Studies have shown that inhibiting the differentiation of osteoclasts and reducing the excessive activation of osteoclasts is an effective therapy for bone loss diseases (30). In addition, osteoporosis is also a polygenic disease, although most of the genes related to osteoporosis are still unknown (31). In this study, the pre-osteoclast cell line RAW264.7 was used to study the role of the LOX mutant, LOX_{G473A}, in osteoclast differentiation. LOX_{G473A} showed a significant increase in LOX expression, as well as osteoclast formation and autophagy in RAW264.7. Further studies showed that this phenomenon could be inhibited by 3-MA and enhanced by RAPA, suggesting that LOX_{G473A} can induce excessive activation of osteoclasts via regulation of autophagy.

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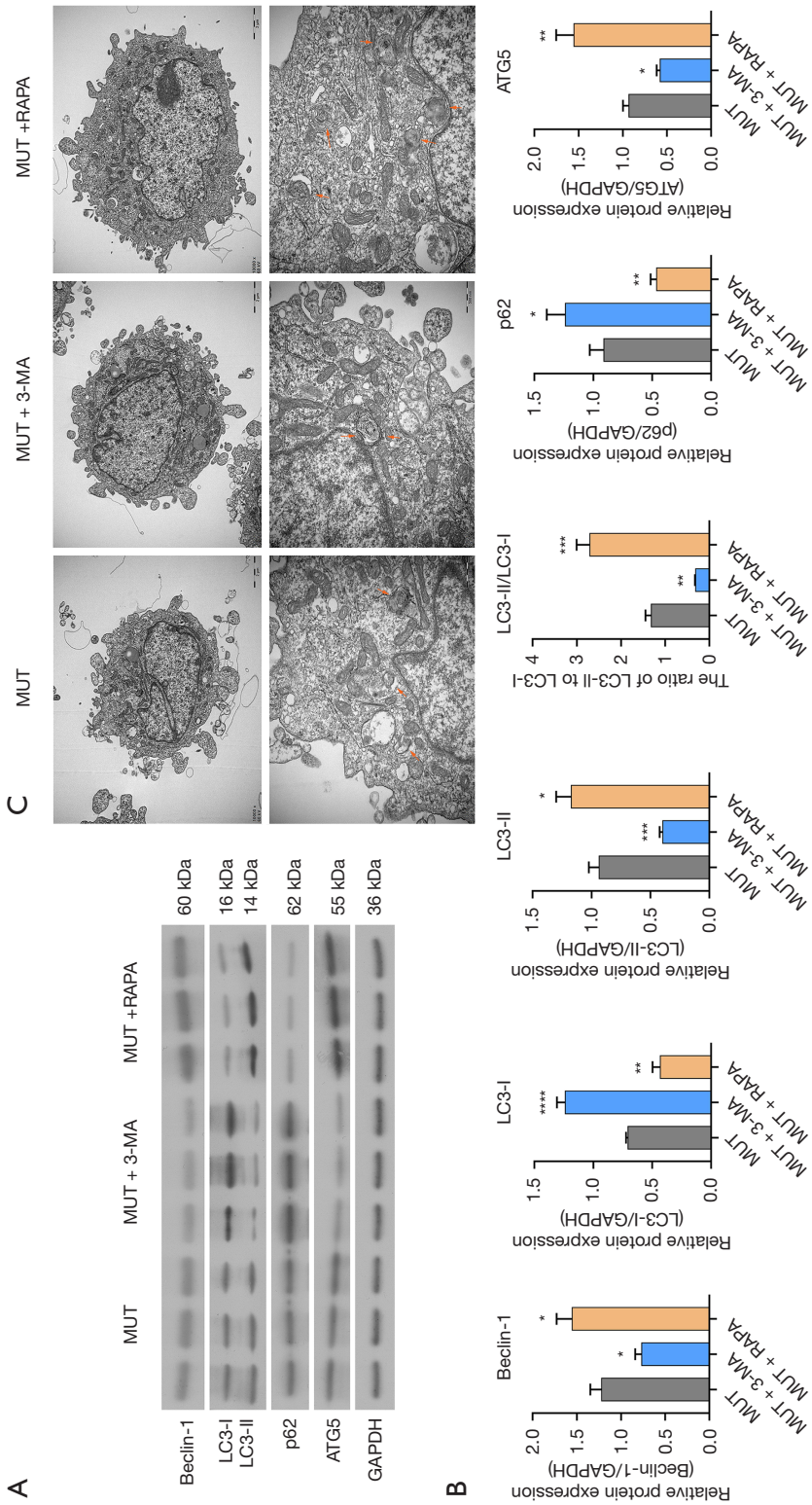
Figure 3 Effects of autophagy inhibitors and agonists on autophagy levels in RAW264.7 with LOX mutant (LOX$_{G473A}$). (A) Expression of autophagy protein in RAW264.7 transfected with mutant LOX$_{G473A}$ (MUT) plasmid and treated with 3-MA and RAPA detected by western blot. (B) Protein expression level quantified through Image-Pro Plus software in (A). (C) Autophagosomes in RAW264.7 transfected with MUT plasmid and incubated with 3-MA and RAPA detected by transmission electron microscopy. Magnification ×10,000 up and ×40,000 down. Red arrows indicate Autophagosomes. *, P<0.05; **, P<0.01; ***, P<0.001; and ****, P<0.0001 *vs.* MUT represent significant differences.
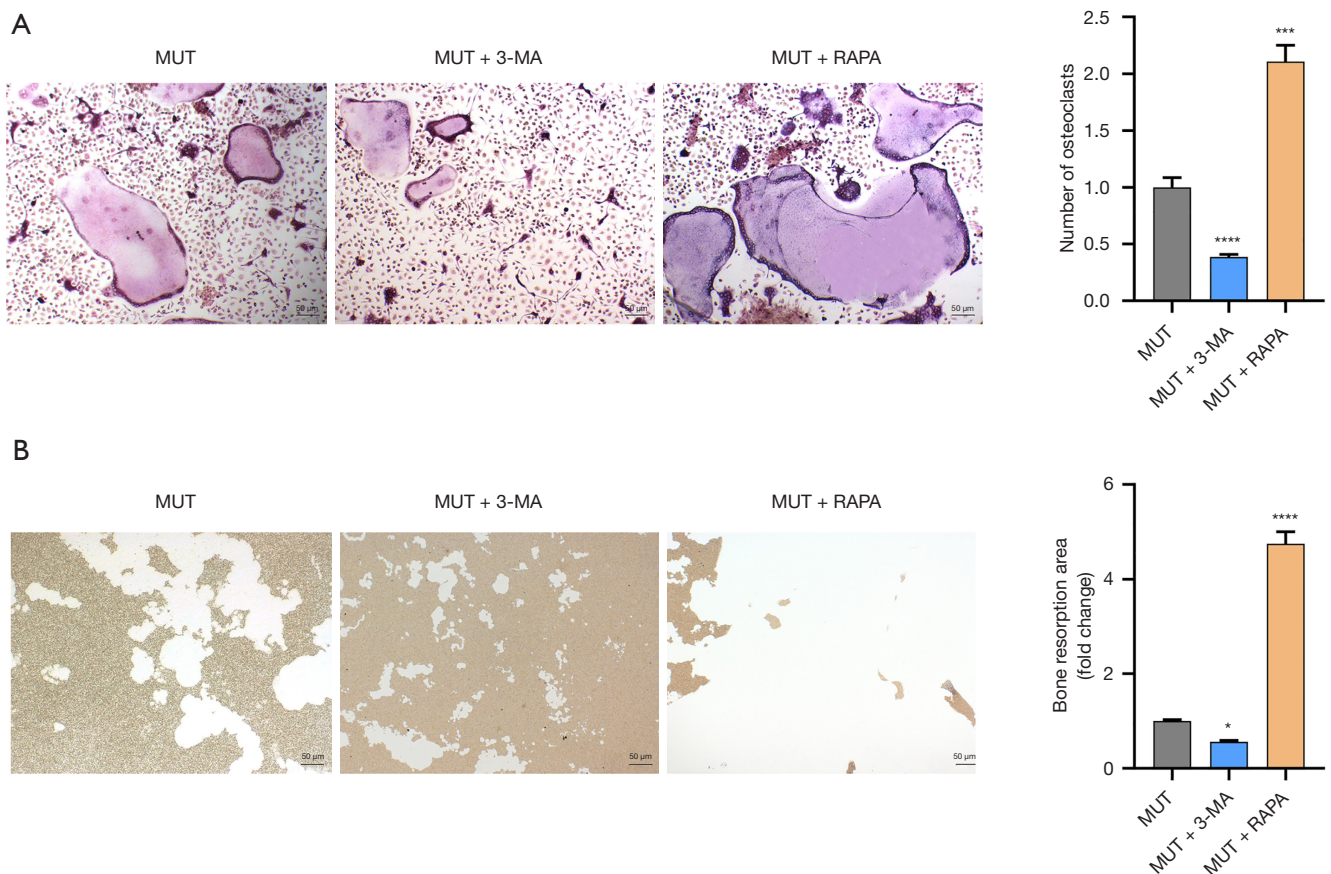
Figure 4 Effects of autophagy inhibitors and agonists on osteoclast differentiation and resorption. Magnification ×100. (A) Osteoclast expression in RAW264.7 transfected with mutant LOX_{G473A} (MUT) plasmid and treated with 3-MA and RAPA detected by TRAP staining. Semiquantitative analysis of the number of osteoclasts by Image-Pro Plus software. (B) Level of osteoclast resorption in RAW264.7 transfected with MUT plasmid and treated with 3-MA and RAPA analyzed by Image-Pro Plus software. *, P<0.05; ***, P<0.001; and ****, P<0.0001 *vs.* MUT represents significances.

Studies have shown that LOX and interleukin (IL)-6 synergistically induced osteoclast differentiation of RANKL-dependent mouse primary bone marrow cells (32). Human CD14+ monocytes treated with LOX can differentiate into osteoclasts (13). Interestingly, our research also found that in the WT group, both osteoclast number and bone resorption area of processed RAW264.7 were remarkably higher compared with the Vector group. That result further illustrated that LOX can induce osteoclasts. In addition, studies show that LOX_{G473A} is associated with diabetic foot ulcers, compared with healthy individuals (33). Our results indicated that LOX_{G473A} enhanced osteoclast formation inRAW264.7 according to TRAP staining, compared with the WT group, showing that LOX_{G473A} might be related to the activation of osteoclasts and a correlation between LOX mutation and osteoporosis.

Interestingly, we found the osteoclast formation and resorption, as well as F-actin ring production of osteoclasts, were inhibited in RAW264.7 with the LOX_{G473A} mutation through suppression of the expression of autophagosomes and autophagy-related proteins. Other research has also shown that kaempferol inhibits the expression of NFATC1, and also the levels of autophagy-related proteins, including P62 and Beclin-1, as well as the ratio of LC3-I/LC3-II in RAW264.7 (20). The expression of TRAP+ multinucleated cells and TRAP mRNA were also downregulated in bone marrow macrophages induced by IL-17A via inhibition of autophagy (34). These findings show that inhibiting autophagy could alleviate LOX_{G473A}-induced osteoporosis. However, our study also demonstrated that LOX_{G473A} can

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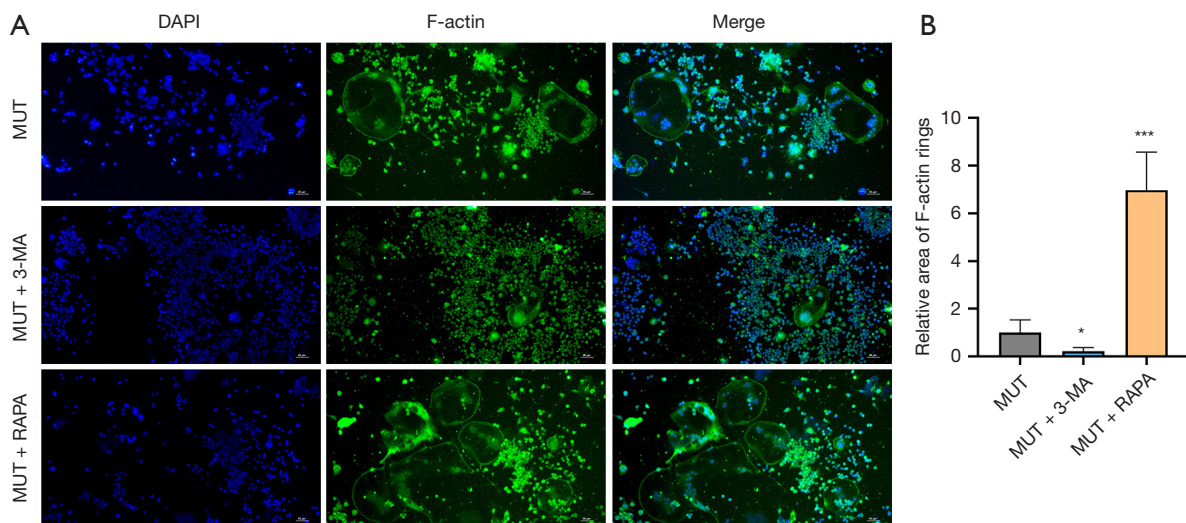
Figure 5 Effects of autophagy inhibitors and agonists on F-actin ring formation of osteoclasts. (A) F-actin ring expression in RAW264.7 transfected with mutant LOX$_{G473A}$ (MUT) plasmid and treated with 3-MA and RAPA detected by phalloidin staining. Magnification ×100. (B) Semiquantitative analysis of the area of F-actin ring by Image-Pro Plus software. *, P<0.05; and ***, P<0.001 *vs*. MUT represents significance.

promote the level of autophagy of osteoclast differentiation of RAW264.7. It could be inferred that LOX$_{G473A}$ interacted with autophagy to regulate the formation and absorption of osteoclasts.

Furthermore, osteoclast differentiation of RAW264.7 with mutant LOX$_{G473A}$ showed downregulated p-AMPKα protein expression and upregulated p-mTOR protein expression after blocking of autophagy. Other research has shown that osteoprotegerin restrains osteoclast differentiation and bone resorption through regulating of autophagy via the AMPK/mTOR/p70S6K signaling pathway (35). AMPK is a key regulator of bone homeostasis by negatively regulating osteoclast formation and bone resorption (36). AMPK activation can also suppress the activation of mTOR, which can affect autophagy (37). It could be inferred that promoting autophagy would increase LOX$_{G473A}$-induced osteoclast differentiation by the p-AMPKα/mTOR signal pathway. In addition, restricted mTOR activation also inhibits expression of CTSK and matrix metalloprotein-9, RANK and NFATC1 in osteoclasts (38), further indicating that the autophagy signaling pathway of p-AMPKα/mTOR might inhibit the production of osteoclasts by restraining the NFATC1, ACP5, and CTSK proteins as shown in our research. Considering the existence of other autophagic signaling pathways, such as PI3K/AKT/mTOR (39) and mTOR-independent (40) signaling pathways, it is unclear whether they play a regulatory role in osteoclast formation, and we will follow up with further studies to explore whether LOXG473A regulates osteoclast formation through cross-talk of different autophagic pathways. Moreover, whether inhibiting osteoclast differentiation by suppressing autophagy brings other potential risks should be taken into consideration in subsequent studies.

Conclusions

In summary, LOX$_{G473A}$ could induce excessive activation of osteoclasts by enhancing activation of the AMPKα/mTOR signaling pathway associated with autophagy. LOX$_{G473A}$ might be a pathogenic gene causing osteoporosis and could be a potential therapeutic target.
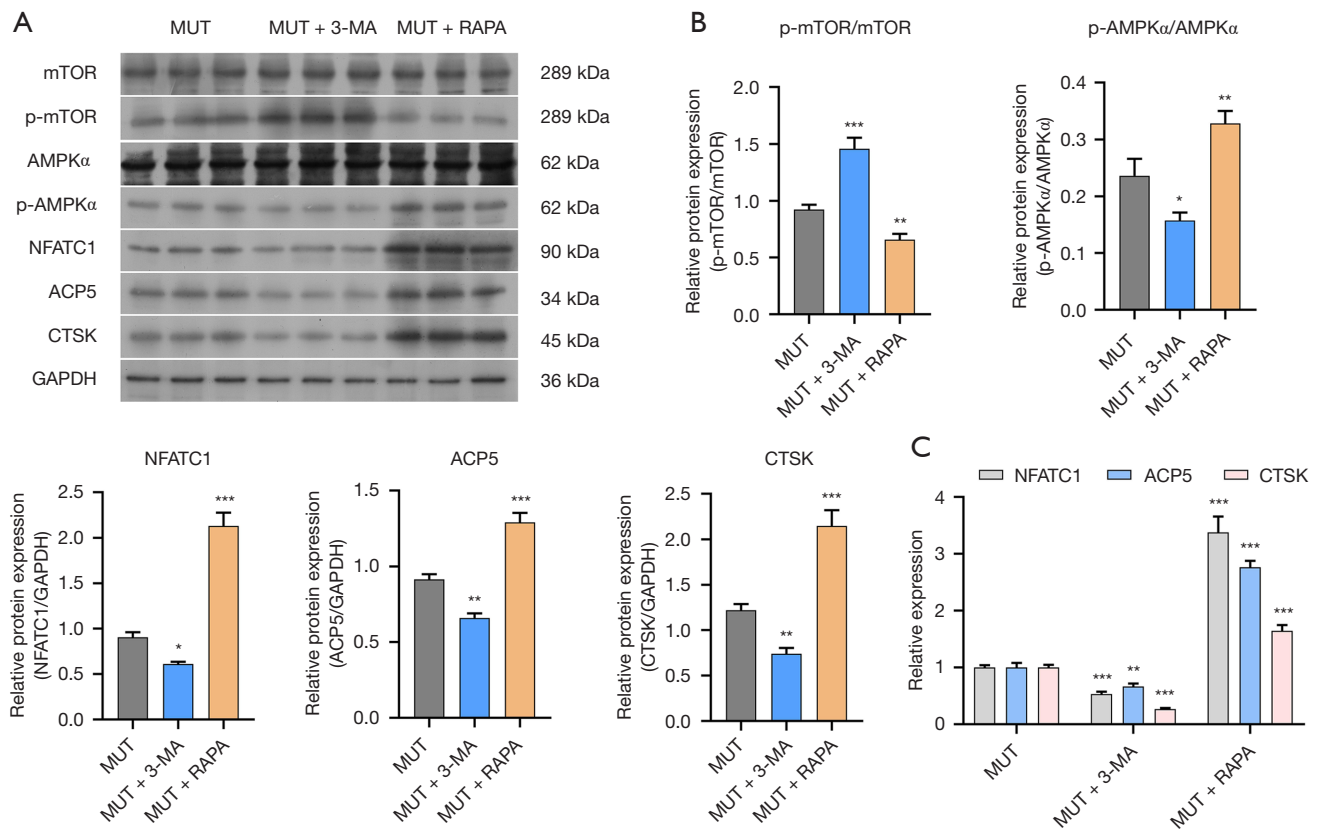
Figure 6 Effects of autophagy inhibitors and agonists on autophagy-related mechanism protein, as well as osteoclast protein and mRNA levels in osteoclasts. (A) Expression of autophagy-related mechanism protein and osteoclast protein in RAW264.7 transfected with mutant LOX_{G473A} (MUT) plasmid and treated with 3-MA and RAPA detected by western blot. (B) Protein expression level in (A) was quantified by Image-Pro Plus software. (C) Osteoclast mRNA level in RAW264.7 transfected with MUT plasmid and treated with 3-MA and RAPA detected by qPCR. *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ vs. MUT represent significant difference.

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