P2X7 receptor knockdown suppresses osteoclast differentiation by inhibiting autophagy and Ca^{2+}/calcineurin signaling

YONGGANG MA[1-3*], RAN DI[1-3*], HONGYAN ZHAO[1-3], RUILONG SONG[1-3], HUI ZOU[1-3] and ZONGPING LIU[1-3]

[1]College of Veterinary Medicine, Yangzhou University; [2]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses; [3]Joint International Research Laboratory of Agriculture and Agri-Product Safety of The Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu 225009, P.R. China

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Abstract. Bone is continuously remodeled in a dynamic process maintained by osteoclasts and osteoblasts, and imbalances in the relative activities of these cell types can cause various pathological conditions, including rheumatoid arthritis and osteoporosis. Osteoclasts are multinucleated cells that serve an important role in regulating the development of osteoporosis. Furthermore, P2X7 receptor activation has a vital role in physiological and pathological reactions in bone, including bone disease. Therefore, the present study aimed to investigate the effect of P2X7 receptor on osteoclast differentiation and to explore the underlying molecular mechanism by western blotting and tartrate-resistant acid phosphatase staining. The results indicated that the expression levels of P2X7 receptor and intracellular Ca^{2+} concentration levels were very high in mature osteoclasts. Furthermore, P2X7 receptor overexpression increased the number of multinucleated osteoclasts and the expression of osteoclastogenesis-related proteins. P2X7 receptor overexpression was also associated with downstream activation of Ca^{2+}/calcineurin/nuclear factor of activated T cells c1 (NFATc1) signaling and increased expression of autophagy-related proteins during osteoclast differentiation. By contrast, knockdown of P2X7 receptor exerted the opposite effects. Notably, FK506 (a Ca^{2+}/calcineurin/NFATc1 signaling inhibitor) abrogated P2X7 receptor overexpression-induced osteoclast differentiation and activation of autophagy. Moreover, 3-MA (an autophagy inhibitor) significantly suppressed P2X7 receptor overexpression-induced osteoclast differentiation. In conclusion, P2X7 receptor knockdown may suppress osteoclast differentiation by modulating autophagy and the Ca^{2+}/calcineurin/NFATc1 signaling pathway.

Correspondence to: Dr Zongping Liu, College of Veterinary Medicine, Yangzhou University, 88 South University Avenue, Yangzhou, Jiangsu 225009, P.R. China
E-mail: liuzongping@yzu.edu.cn

Abbreviations: Atg5, autophagy-related 5; CK, cathepsin K; CAII, carbonic anhydrase II; MMP-9, matrix metalloproteinase-9; NC, negative control; OPG, osteoprotegerin; OE, overexpression; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T cells c1

[*]Contributed equally

Key words: P2X7, Ca^{2+}/calcineurin/NFATc1, osteoclast, differentiation

Introduction

Bone is made up of an organic protein matrix, and is continuously remodeled in a dynamic process maintained by osteoblasts and osteoclasts. During this process, osteoblasts and osteoclasts are important for normal bone metabolism, and imbalances in the relative activities of these cell types can cause various pathological conditions, including rheumatoid arthritis and osteoporosis (1,2). Osteoclasts are multinucleated cells that are formed by the fusion of mononuclear macrophages derived from hematopoietic stem cells (3). Macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL) are important factors that induce the differentiation of osteoclast precursors into mature osteoclasts (4). A previous study evaluated regulation of various genes during osteoclastogenesis (5); however, the mechanism underlying osteoclast differentiation has not been clearly elucidated.

Vega *et al* (6) proposed that the RANK/RANKL/osteoprotegerin (OPG) system is a vital signal during osteoclast differentiation. In addition, a previous study (7) demonstrated that the RANK/RANKL/OPG system may trigger a series of signaling molecules during osteoclast differentiation, and the calcium signaling pathway has been identified as essential during osteoclast differentiation. The activation of calcium signaling can induce osteoclast differentiation through Ca^{2+} oscillations (8). The P2X7 receptor (P2X7R), an ion channel receptor, constitutes a calcium-permeable cationic channel (9). It has previously been reported that P2X7 receptor activation has a vital role in physiological and pathological reactions, including autophagy and metabolic responses (10). A previous study (11) focused on the role of the P2X7 receptor in skeletal diseases, and loss of function polymorphisms in the P2X7 receptor have been shown to be associated with bone loss and increased fracture risk. In addition, the activation of

the P2X7 receptor is vital in the formation and apoptosis of osteoclasts (12). These studies suggested that P2X7 may have an important role in bone disease, and could act as a potential therapeutic target for medicinal development. Therefore, elucidating its role during osteoclast differentiation may have critical implications for therapeutic strategies in bone disease.

Autophagy is a process associated with the degradation of long-lived proteins and organelles, which has an important role in maintaining cell homeostasis. Notably, autophagy has been shown to be activated during osteoclastogenesis (13). A series of autophagy-related proteins are involved in polarization of osteoclasts, including autophagy-related 5 (Atg5), Atg7 and LC3 (14). In addition, Beclin-1 serves a crucial role in osteoclastogenesis. Li and Bai (15) reported that the level of autophagy was associated with the activation state of P2X7R and P2X7R activation could affect a series of signals to regulate autophagy.

Based on previous findings, it was hypothesized that P2X7 may regulate autophagy and calcium signaling, and thereby modulate osteoclast differentiation. In addition, it was hypothesized that RANKL may affect P2X7R activation to modify osteoclast function by regulating calcium signaling. Therefore, the present study aimed to investigate the effects of P2X7 on osteoclast differentiation.

Materials and methods

Animals. In the present study, all mice were provided by Yangzhou University. BALB/c mice (age, 4 weeks; weight, 10 g) were given free access to food and water and were sacrificed by cervical dislocation. A total of 20 mice were used to isolate primary cells. The health and behavior of mice were monitored every day; no mice died prior to sacrifice and all mice were maintained in specific pathogen-free animal housing for 1 month at 20-25˚C and 40-70% humidity under a 12/12-h light/dark cycle.

Cell culture. The 4-week-old BALB/c mice were euthanized and primary bone marrow cells were isolated from mice femurs; the marrow was aspirated from the marrow cavity using a syringe to collect the cells. Cells were maintained in an α-MEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) for 2 days in a humidified atmosphere containing 5% CO_2 at 37˚C. After 2 days, the suspended cells were collected and cultured in the presence of M-CSF (30 ng/ml; R&D Systems, Inc.) and RANKL (60 ng/ml; R&D Systems, Inc.) for 4 days. When the percentage of osteoclast confluence reached ~60%, they were used for subsequent experiments. The medium was replaced every 2 days and the cells used for osteoclastogenesis were from passage one. In addition, BMMs overexpressing P2X7 were cultured in the presence of M-CSF and RANKL for 12 h; after 12 h, cells were treated treatment with 2 μM FK506 (cat. no. HY-13756; MedChemExpress) or 5 mM 3-MA (cat. no. 19312; MedChemExpress) for 12 h in a humidified atmosphere containing 5% CO_2 at 37˚C. BMMs were cultured in the presence of M-CSF as a control.

Tartrate-resistant acid phosphatase (TRAP) staining. To identify osteoclasts, the cells were washed with 1X PBS, and then fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were stained using the TRAP staining kit (MilliporeSigma) according to the manufacturer's instructions. The TRAP-positive cells were observed under a light microscope (Leica Microsystems GmbH), and multinucleated (≥3 nuclei) cells were counted as osteoclasts. The number of mature osteoclasts was counted in three randomly selected fields of view.

Resorption activity. BMMs ($1x10^6$/ml) were seeded in 48-well plates with 120-140-μm bovine cortical bone slices (IDS Nordic) in the presence of M-CSF. BMMs were transduced with shRNA-P2X7 adenovirus and HBLV-m-P2Rx7-3xfag-2s Green-Puro for 12 h. RANKL was then added. After 3 days, the cells were washed with 1X PBS, fixed in 2.5% glutaraldehyde solution overnight at 4˚C, dehydrated using an alcohol gradient and dried. To observe their morphology, the specimens were coated with gold using an SCD 500 sputter-coater (Leica Microsystems GmbH) and examined using a GeminiSEM 300 field-emission environmental scanning electron microscope (Carl Zeiss). Bone resorption area was calculated by ImageJ 1.48 (National Institutes of Health).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from mature osteoclasts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, RNA was reverse transcribed into complementary (c)DNA using the HiScript qRT SuperMix kit according to the manufacturer's protocol. (Vazyme Biotech Co., Ltd.). cDNA templates were then amplified using a ChamQ SYBR q-PCR Master Mix kit according to the manufacturer's protocol. (Vazyme Biotech Co., Ltd.). Thermocycling conditions were as follows: Initial denaturation for 2 min at 95˚C, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min and final extension for 15 sec at 95˚C, 15 sec at 60˚C and 15 sec at 95˚C. The expression levels of target genes [P2X7, c-fos, NFATc1 and TRAP] were normalized to the reference gene GAPDH. The $2^{-\Delta\Delta Cq}$ method was applied to calculate relative gene expression; $2^{-\Delta\Delta Cq} = 2^{-(C_{q,target} - C_{q,GAPDH})}_{experiment\ group} - (C_{q,target} - C_{q,GAPDH})_{control\ group}$ (16). All of the qPCR reactions were performed in triplicate and the primers used for qPCR are shown in Table I. The primers were obtained from Shenzhen Huada Gene Technology Co., Ltd.

Western blot analysis. Western blot analysis was performed to detect protein expression levels. Total protein was isolated from mature osteoclasts using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Equal amounts of protein (25 μg/lane) were separated by SDS-PAGE on 10% gels and transferred onto PVDF membranes. After blocking with 5% non-fat skim milk at room temperature for 2 h, the membranes were incubated at 4˚C overnight with primary antibodies against P2X7 (cat. no. NBP1-20180; 1:1,000; Novus Biologicals, LLC), LC3 (cat. no. L7543; 1:1,000; MilliporeSigma), TRAP (cat. no. ab238033; 1:1,000), CK (cat. no. ab37259; 1:1,000), CAII (cat. no. ab182611; 1:1,000), MMP-9 (cat. no. ab76003; 1:1,000) (all from Abcam), NFATc1 (cat. no. 8032; 1:1,000), c-fos (cat. no. 2250; 1:1,000), calcineurin (cat. no. 2614; 1:1,000), Beclin-1 (cat. no. 3495; 1:1,000) and β-actin (cat. no 4970; 1:1,000; all from Cell

Table I. Nucleotide sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene name	Annealing temperature (˚C)	Forward primer sequence	Reverse primer sequence
GAPDH	60	5'-TCAAGAAGGTGGTGAAGCAG-3'	5'-AGTGGGAGTTGCTGTTGAAGT-3'
NFATc1	58	5'-CTCGAAAGACAGCACTGGAGCAT-3'	5'-CGGCTGCCTTCCGTCTCATAG-3'
TRAP	62	5'-CTGGAGTGCACGATGCCAGCGACA-3'	5'-TCCGTGCTCGGCGATGGACCAGA-3'
c-fos	64	5'-GGAGAATCCGAAGGGAACGG-3'	5'-GCAATCTCAGTCTGCAACGC-3'
CAII	52	5'-GGGGATACAGCAAGCACAAC-3'	5'-GACTGCCGGTCTCCATTG-3'
CK	52	5'-CGAAAAGAGCCTAGCGAACA-3'	5'-TGGGTAGCAGCAGAAACTTG-3'
MMP-9	52	5'-ACGACATAGACGGCATCCA-3'	5'-GCTGTGGTTCAGTTGTGGTG-3'
P2X7	60	5'-AGATCGTGGAGAATGGAGTG-3'	5'-TTCTCGTGGTGTAGTTGTGG-3'

Primer amplicon size, 50-150 bp; primer concentration, 10 μmol/l; CAII, carbonic anhydrase II; CK, cathepsin K; MMP-9, matrix metalloproteinase-9; NFATc1, nuclear factor of activated T cells c1; TRAP, tartrate-resistant acid phosphatase.

Signaling Technology, Inc.). Subsequently, membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (cat. nos. 7074 and 7076; 1:1,000; Cell Signaling Technology, Inc.). The proteins were visualized using an ECL kit (cat. no. P2300; New Cell & Molecular Biotech) and the data were analyzed using ImageJ software; the protein expression levels were calculated according to the gray value of the bands.

Cell transduction. A P2X7 short hairpin RNA (shRNA) knockdown construct (pHBAd-U6-Scarmble-GFP) and P2X7 overexpression system were constructed by Hanheng Biology. Empty vector lentivirus was used as negative control. Knockdown and overexpression were performed using shRNA-P2X7 adenovirus and HBLV-m-P2Rx7-3xfag-2s Green-Puro, respectively. BMMs were grown in α-MEM supplemented with 10% fetal bovine serum at 37˚C and 5% CO_2. An adenoviral vector containing shRNA-P2X7 and HBLV-m-P2Rx7-3xfag-2s Green-Puro (MOI, 1-3) were introduced into BMMs in the presence of M-CSF (30 ng/ml); after 24 h, transduction efficiency was observed using fluorescence microscopy and silencing and overexpression efficiency were determined by western blotting. The sequences of the shRNA-P2X7 and non-targeting negative control (NC)-shRNA were as follows: NC-shRNA sense, 5'-AATTCGTTCTCCGAACGTGTCACGTAATTCAAGAGATTACGACACGTTCGCAGAATTTTTTG-3' and antisense, 5'-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACACGTTCGGAGAACT-3' and shRNA-P2X7 sense, 5'-AATTCGACGAAGTTAGGACACAGCATCTTTGTTCAAGAGACAAAGATGCTGTGTCCTAACTTCGTTTTTTTG-3' and antisense, 5'-GATCCAAAAAAACGAAGTTAGGACACAGCATCTTTGTctcttgaaCAAAGATGCTGTGTCCTAACTTCGTCG-3'.

Immunofluorescence staining. BMMs on glass coverslips were incubated in the presence of M-CSF and RANKL, and cells were transduced with adenovirus-mediated shRNA-P2X7 and NC-shRNA. For SP2X7 overexpression, cells were transduced with HBLV-m-P2Rx7-3xfag-2s Green-Puro and NC. When the confluence of osteoclasts reached ~60%, they were used for the subsequent experiment. Osteoclasts were fixed with 4% paraformaldehyde for 20 min at room temperature, washed in PBS twice (5 min/wash), permeabilized with 0.5% Triton X-100 for 30 min at room temperature, washed in PBS twice (5 min/wash) and blocked with 5% FBS for 30 min at room temperature. Osteoclasts were then incubated overnight at 4˚C with an anti-LC3 primary antibody (cat. no. L7543; 1:200; Sigma-Aldrich; Merck KGaA). Subsequently, the coverslips were washed and incubated for 2 h at room temperature with Alexa Fluor® 488-conjugated secondary antibody (cat. no. A0428; 1:200; Beyotime Institute of Biotechnology), followed by two further washed with PBS (5 min/wash). Cells were then observed under a confocal microscope. In the present study, mature osteoclasts were chosen for confluence analysis. Fluorescence intensity was analyzed using ImageJ.

Intracellular Ca^{2+} measurement. BMMs were seeded onto confocal microscope-specialized cover glass. When the confluence of osteoclasts reached ~60%, they were used for the subsequent experiment. Osteoclasts were incubated with 1 μM Fluo3-AM (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 5% CO_2 and 37˚C. The medium was then replaced and intracellular Ca^{2+} concentrations were observed using a confocal microscope. Fluorescence images were captured and analyzed using ImageJ.

Statistical analysis. Each experiment was repeated at least three times. All of experimental data were analyzed using SPSS 21.0 (IBM Corp.). An independent samples Student's t-test was used for two-sample comparisons with data exhibiting a normal distribution. Multiple groups were compared using one-way analysis of variance followed by Tukey's or Tamhane's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

P2X7R expression and Ca^{2+} levels increase during osteoclast differentiation. The results of the present study demonstrated that the expression levels of osteoclastogenesis key genes were significantly increased at the mRNA and protein levels

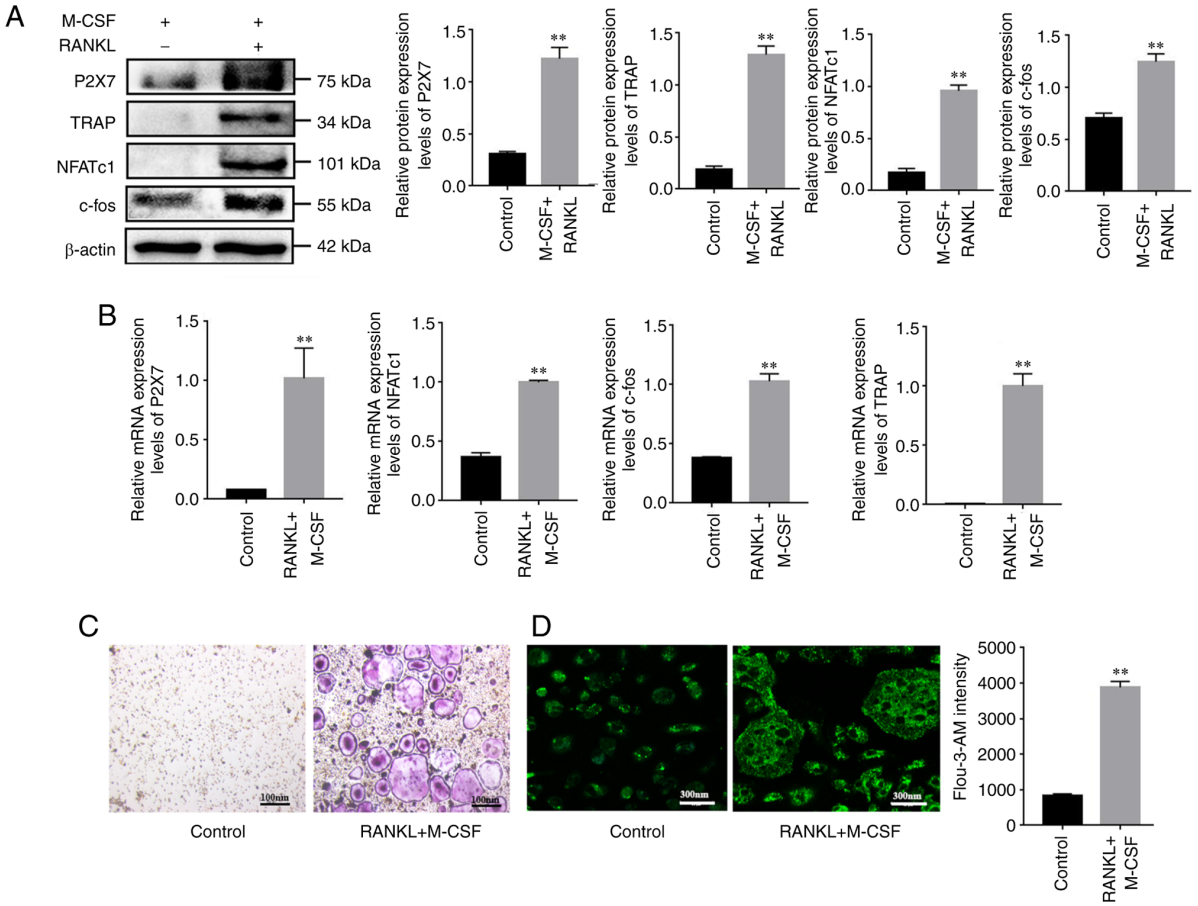

Figure 1. P2X7 expression levels and intracellular Ca^{2+} concentrations are increased during osteoclast differentiation. Bone marrow mononuclear macrophages were cultured in the presence of M-CSF (30 ng/ml) and RANKL (60 ng/ml), and differentiation into mature osteoclasts was evaluated. Control BMMs were cultured in the presence of M-CSF (30 ng/ml). (A) Western blot analysis was performed to examine the protein expression levels of P2X7, TRAP, NFATc1 and c-fos. (B) Reverse transcription-quantitative polymerase chain reaction was performed to detect the mRNA expression levels of P2X7, TRAP, NFATc1 and c-fos. (C) TRAP-positive multinucleated cells were counted as osteoclasts. (D) Fluo3-AM was used to observe intracellular Ca^{2+} fluorescence intensity. Data are presented as the mean ± SD from three independent experiments. $^{**}P<0.01$ vs. control. M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells c1; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase.

following M-CSF and RANKL stimulation compared with in the control cells (M-CSF stimulation; Fig. 1A and B). Furthermore, the number of TRAP-positive osteoclasts was markedly increased after stimulating BMMs with M-CSF and RANKL for 4 days (Fig. 1C). To determine whether P2X7R was involved in regulating osteoclast differentiation by changing intracellular Ca^{2+} influx, the present study measured the mRNA and protein expression levels of P2X7, as well as Ca^{2+} concentration, during osteoclast differentiation. The results suggested that the expression levels of P2X7R were significantly upregulated in mature osteoclasts compared with those in the control group (Fig. 1A and B). In addition, Ca^{2+} concentration was significantly increased in mature osteoclasts compared with that in the control group (Fig. 1D).

Knockdown of P2X7R inhibits osteoclast bone resorption, Ca^{2+} signaling and autophagy during osteoclast differentiation. Based on the aforementioned results, the present study knocked down P2X7R expression using adenovirus-mediated transduction of BMMs with P2X7-shRNA, and induced overexpression of P2X7R in BMMs using HBLV-m-P2Rx7-3xfag-2s Green-Puro. Knockdown and overexpression efficiencies were confirmed by western blot analysis (Fig. 2A and B). As expected, P2X7R knockdown significantly reduced the number of TRAP-positive osteoclasts (Fig. 2C) and inhibited bone resorption in the presence of RANKL and M-CSF (Fig. 2D. In addition, P2X7R knockdown in the presence of M-CSF and RANKL significantly downregulated the protein expression levels of osteoclast key genes, including TRAP, CK, CAII, MMP-9, NFATc1 and c-fos compared with in the NC group (Fig. 2A). By contrast, P2X7 overexpression exerted the opposite effects (Fig. 2B-D). These results indicated that the absence of P2X7R may inhibit osteoclast differentiation.

Ca^{2+} signaling is an essential axis of osteoclast differentiation. Expanding on the aforementioned observations of the role of Ca^{2+} signaling during osteoclastogenesis, the present study revealed that knockdown of P2X7R significantly inhibited the expression levels of calcineurin, whereas P2X7R overexpression upregulated the expression of calcineurin (Fig. 2A and B). Moreover, knockdown of P2X7R suppressed Ca^{2+} concentration in M-CSF and RANKL-stimulated BMMs (Fig. 2G). By contrast, P2X7R overexpression exerted the opposite effects (Fig. 2G). These results suggested that the absence of P2X7R suppressed Ca^{2+} signaling during osteoclast differentiation.

The present study also investigated the effects of P2X7R on autophagy. Immunofluorescence analysis revealed that

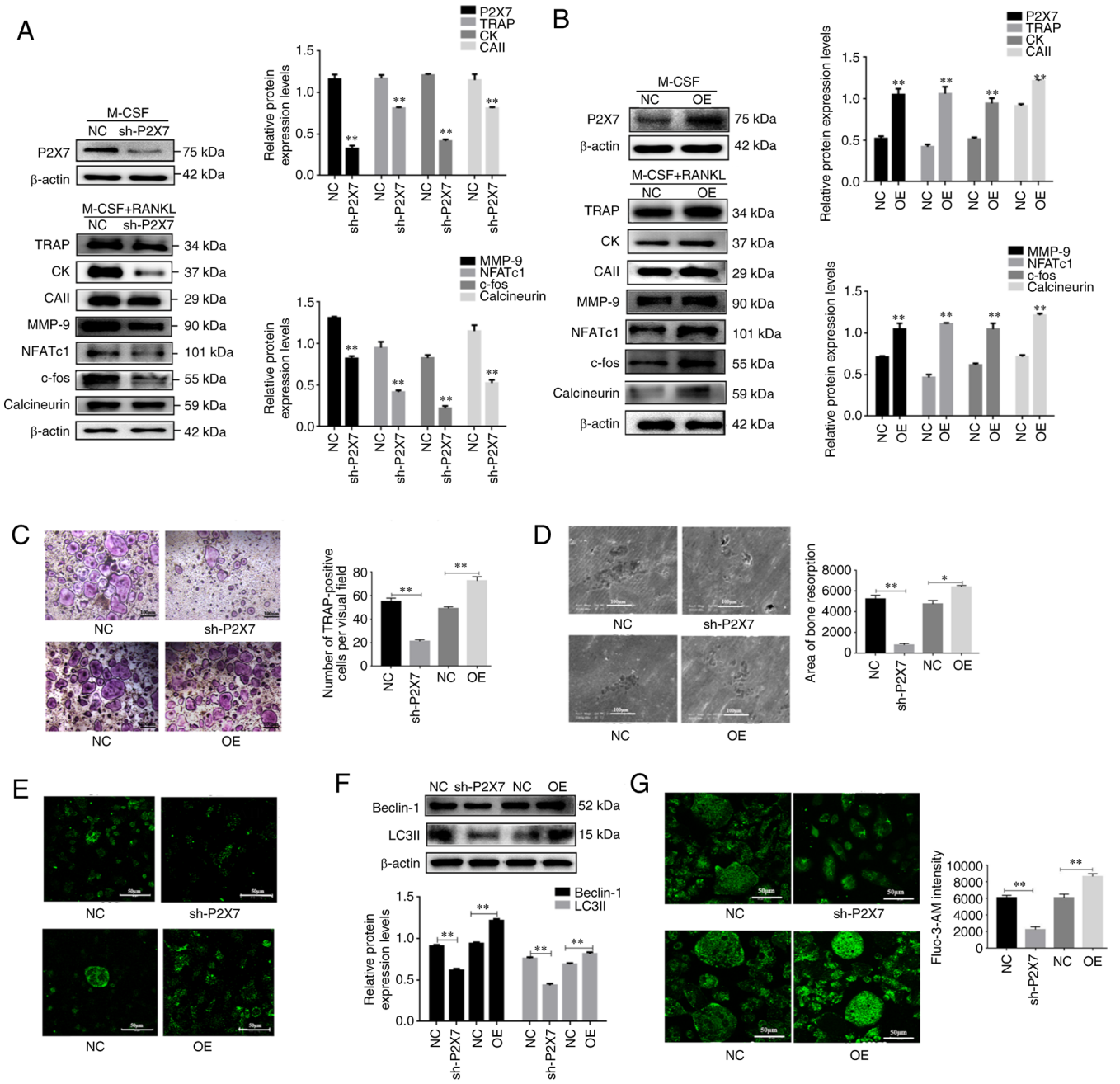

Figure 2. Effects of P2X7 overexpression on osteoclast differentiation, Ca^{2+}/calcineurin/NFATc1 signaling and autophagy. Bone marrow mononuclear macrophages were transduced with sh-P2X7 or transduced with HBLV-m-P2Rx7-3xfag-2s Green-Puro in the presence of M-CSF with or without RANKL. (A and B) Western blot analysis of the protein expression levels of P2X7, TRAP, CK, CAII, MMP-9, NFATc1, c-fos and calcineurin. (C and D) TRAP-positive multinucleated cells were counted as osteoclasts and scanning electron microscopy was used to observe bone resorption lacuna. (E and F) Western blot analysis of the protein expression levels of Beclin-1 and LC3II, and immunofluorescence analysis of LC3 fluorescent puncta. (G) Fluo3-AM staining was performed to detect intracellular Ca^{2+} concentration. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. NC group or as indicated. CAII, carbonic anhydrase II; CK, cathepsin K; M-CSF, macrophage colony-stimulating factor; MMP-9, matrix metalloproteinase-9; NC, negative control; NFATc1, nuclear factor of activated T cells c1; OE, overexpression; RANKL, receptor activator of NF-κB ligand; sh, short hairpin; TRAP, tartrate-resistant acid phosphatase.

knockdown of P2X7R led to a reduction in LC3 puncta in mature osteoclasts, whereas P2X7R overexpression increased LC3 immunofluorescence puncta (Fig. 2E). Furthermore, knockdown of P2X7R significantly reduced the protein expression levels of Beclin-1 and LC3II, as determined by western blotting (Fig. 2F). Taken together, these results suggested that knockdown of P2X7 inhibited autophagy during osteoclast differentiation.

Inhibition of autophagy and calcineurin attenuates P2X7R overexpression-induced osteoclast differentiation. To explore whether Ca^{2+}/calcineurin/NFATc1 signaling was involved in P2X7 overexpression-induced osteoclast differentiation and autophagy activation, P2X7R overexpression-induced BMMs were treated with FK506 (a calcineurin inhibitor) in the presence of M-CSF and RANKL. The results confirmed that FK506 significantly inhibited P2X7R overexpression-induced

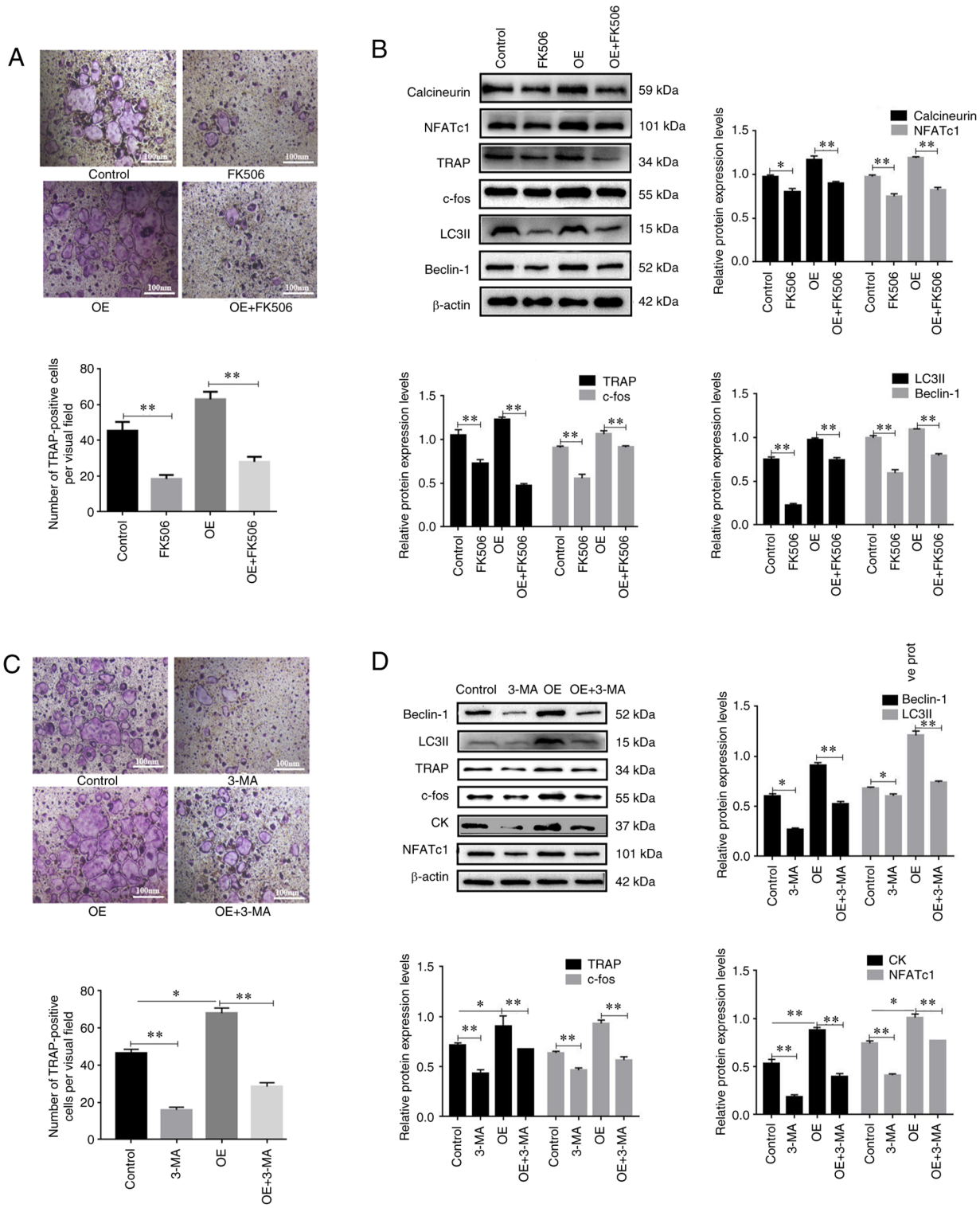

Figure 3. Calcineurin/NFATc1 signaling and autophagy are involved in P2X7R overexpression-induced osteoclast differentiation. Bone marrow mononuclear macrophages were cultured with M-CSF and RANKL. Cells were transduced with HBLV-m-P2Rx7-3xfag-2s Green-Puro in the presence of M-CSF and RANKL, followed by treatment with FK506 (2 μM) for 12 h. (A) TRAP-positive multinucleated cells were counted as osteoclasts. (B) Western blot analysis of the protein expression levels of calcineurin, NFATc1, TRAP, c-fos, Beclin-1 and LC3II. Cells were transduced with HBLV-m-P2Rx7-3xfag-2s Green-Puro in the presence of M-CSF and RANKL, followed by treatment with 3-MA (5 mM) for 12 h. (C) TRAP-positive multinucleated cells were counted as osteoclasts. (D) Western blot analysis of the protein expression levels of NFATc1, c-fos, TRAP, CK, Beclin-1 and LC3II. Data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. NC group or as indicated. CK, cathepsin K; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells c1; OE, overexpression; RANKL, receptor activator of NF-κB ligand; sh, short hairpin; TRAP, tartrate-resistant acid phosphatase.

osteoclast differentiation in the presence of M-CSF and RANKL, and decreased the protein expression levels of NFATc1, calcineurin and TRAP (Fig. 3A and B). In addition, FK506 significantly attenuated P2X7R overexpression-induced activation of autophagy, as indicated by a decrease in the protein expression levels of Beclin-1 and LC3II (Fig. 3B). These

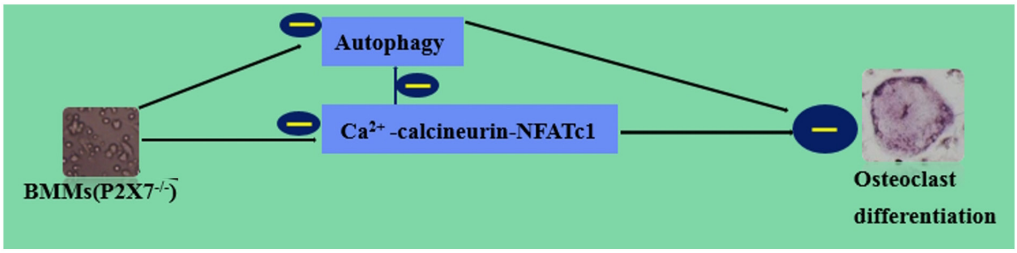

Figure 4. Effect of P2X7 on BMM differentiation. Knockout of P2X7 inhibited BMM differentiation. BMMs, bone marrow mononuclear macrophages; NFATc1, nuclear factor of activated T cells c1.

results indicated that Ca^{2+}/calcineurin/NFATc1 signaling may be involved in P2X7 overexpression-induced osteoclast differentiation and activation of autophagy.

The present study also investigated whether autophagy was involved in P2X7R overexpression-induced osteoclast differentiation. The present results showed that 3-MA (an autophagy inhibitor) significantly inhibited the P2X7R overexpression-induced expression levels of Beclin-1 and LC3II (Fig. 3D). Notably, it was revealed that P2X7R overexpression significantly increased the number of TRAP-positive osteoclasts (Fig. 3C). Furthermore, P2X7R overexpression increased the protein expression levels of osteoclast key genes (Fig. 3D). 3-MA reversed the effect of OE on TRAP cells and protein expression (Fig. 3D). These results indicated that autophagy may be involved in P2X7 overexpression-induced osteoclast differentiation.

Discussion

The present study revealed that P2X7R expression was significantly increased at both the mRNA and protein levels in mature osteoclasts, indicating a potential role for P2X7R during osteoclast differentiation. Accordingly, P2X7R knockdown significantly reduced the number of TRAP-positive osteoclasts and the expression levels of osteoclastogenesis-related genes in the presence of M-CSF and RANKL, whereas P2X7R overexpression exerted the opposite effects. These results suggest that P2X7R may be involved in osteoclast differentiation, and that absence of P2X7R could inhibit osteoclast differentiation.

Given the complexity of cell differentiation, the role of P2X7R during osteoclast differentiation is unclear. However, the present study revealed the effects of P2X7R on osteoclast differentiation, which contrasted with the results of some previous studies. Wang *et al* (17) examined the effect of P2X7R on osteoclast formation and bone loss using a mouse model of osteoporosis. The results of this previous study showed that loss of P2X7R increased estrogen-deficient bone loss, likely due to increased activity of osteoclasts in the absence of estrogen. In this previous study, osteoclast precursors were isolated from $P2RX7^{-/-}$ mice, which generated more mature osteoclasts, but the resorption activity of osteoclasts was significantly reduced. This would suggest that the increased number of osteoclasts observed *in vivo* may well be a compensatory mechanism for their reduced activity. Notably, osteoclasts from $P2RX7^{-/-}$ mice exhibited an inhibition in osteoclast resorption function. By contrast, Agrawal *et al* (18) revealed that a P2X7R-specific antagonist significantly inhibited osteoclast formation and bone resorption. Gartland *et al* (19) also demonstrated that blockade of P2X7R inhibited osteoclast formation *in vitro*. However, in a previous study, P2X7R-deficient mice displayed no overt skeletal problems, and were able to form multinucleated cells (20). It was hypothesized that other regulatory factors may have a compensatory effect, thus short-term loss of P2X7R would not affect osteoclast function. These findings indicated that P2X7R may exert specific effects on osteoclast formation, which require further exploration *in vivo* and *vitro*.

In the present study, the absence of P2X7R inhibited osteoclast formation and bone resorption in BMMs, and how P2X7R affects osteoclastogenesis was examined. NFATc1 has an essential role in osteoclastogenesis. In response to activation of Ca^{2+}/calcineurin signaling, NFATc1 is a key modulator, which activates transcription of a series of key osteoclast genes by translocating to the nucleus (21). NFATc1 is not only required but also sufficient for osteoclastogenesis, as its overexpression in osteoclast precursors has been shown to induce osteoclast differentiation in the absence of RANKL (22). Ca^{2+}/calcineurin/NFATc1 signaling serves an important role in osteoclast differentiation, and can accelerate osteoclast differentiation through inducing an increase in intracellular Ca^{2+} concentration (23). P2X7 directly regulates differentiation and function of osteoclasts by mediating intracellular Ca^{2+} influx. P2X7-mediated Ca^{2+} influx during osteoclast differentiation is critical when ATP or BzATP concentration is relatively high; a previous study revealed that ATP can stimulate the formation and resorption activity of osteoclasts by activating purinergic signaling (24). P2X7-mediated Ca^{2+} influx affects intracellular Ca^{2+} levels, ensuring NFATc1-mediated gene transcription, and thereby regulating osteoclast differentiation; however, it remains to be determined as to whether P2X7 is involved in ATP-induced osteoclast differentiation. In the present study, knockdown of P2X7R was associated with reduced intracellular Ca^{2+} concentration, reduced NFATc1 activity, and reduced osteoclast differentiation and bone resorption. Therefore, based on these observations, the present study explored whether Ca^{2+}/calcineurin/NFATc1 signaling was involved in P2X7-mediated osteoclast differentiation. The results revealed that knockdown of P2X7 significantly inhibited Ca^{2+}/calcineurin/NFATc1 signaling during osteoclast differentiation. By contrast, P2X7R overexpression increased Ca^{2+} concentration and activated calcineurin/NFATc1 signaling. Notably, inhibition of calcineurin abrogated the effect of P2X7R overexpression on osteoclast differentiation.

Autophagy is the chief machinery for bulk degradation of superfluous or aberrant cytoplasmic components. Dysregulated

autophagy has been involved in the development of several diseases. Zhang *et al* (25) reported that autophagy was associated with the formation and development of osteoporosis. This previous study indicated that autophagy may have an essential role in modulating bone metabolism and in disease. Although increasing evidence has indicated that OPG may inhibit osteoclast differentiation by promoting autophagy (26,27), other studies have identified a potential mechanism for autophagy in promoting osteoclast differentiation (28,29). Autophagy-related proteins, including Beclin-1and Atg7, have been shown to increase in the osteoclasts of patients with rheumatoid arthritis, leading to bone destruction (30). A previous study suggested that autophagy activation promoted osteoclast formation and differentiation. By contrast, 3-MA (an autophagy inhibitor) interrupted TRPV4 overexpression-induced osteoclastogenesis in a previous study; these results demonstrated that the absence of TRPV4 inhibited osteoclast differentiation (31). The present study revealed that knockdown of P2X7R reduced the expression levels of autophagy-related proteins (LC3II and Beclin-1) during osteoclast differentiation, whereas P2X7R overexpression exerted the opposite effects. Taken together, interrupting autophagy may affect osteoclast formation and differentiation. Notably, a previous study suggested that the overactivation of P2X7R may increase calcium influx, which may activate a series of downstream signaling (32). In general, autophagy may serve a dual role in regulating osteoclast differentiation and cell death. The present study also revealed that inhibition of calcineurin by FK506 abrogated P2X7R overexpression-induced autophagy. Moreover, inhibition of autophagy attenuated P2X7R overexpression-induced osteoclast differentiation. It is well known that bone homeostasis is maintained by the communication between osteoblasts, osteoclasts and osteocytes. Autophagy is a complex process, which exerts dual roles in other bone cells. Another study demonstrated that overactivation of autophagy promoted osteoblast apoptosis and bone loss (33); however, further studies are required to confirm the relationship between autophagy dysfunction, and bone loss and osteoclast differentiation.

In conclusion, the results of the present study indicated that knockdown of P2X7R suppressed osteoclast differentiation by inhibiting autophagy through the Ca^{2+}/calcineurin/NFATc1 signaling pathway (Fig. 4). However, the relationship between the absence of P2X7 receptor and the progression of osteoporosis in mice remains elusive; further study of knockout of P2X7 receptor in a mouse model should be performed to determine the association between osteoporosis and P2X7R. The absence of P2X7R affects osteoclast formation, thereforeP2X7R may be a useful therapeutic target for metabolic bone disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZPL and HYZ conceived and designed the study. YGM and RD performed the cell transduction and culture. YGM and RLS performed the western blotting and immunofluorescence. YGM and HZ performed the TRAP staining and bone resorption. YGM, RD and HYZ analyzed the data. YGM, RD and HZ organized the data. YGM and RD wrote the manuscript. ZPL and HYZ proofread the manuscript and confirm the authenticity of all the raw data. All authors contributed to the article, and read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments and procedures were approved by the Animal Care and Use Committee of Yangzhou University, China [approval number: SYXK (Su) 2016-0020].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Murata K, Fang C, Terao C, Giannopoulou EG, Lee YJ, Lee MJ, Mun SH, Bae S, Qiao Y, Yuan R, *et al*: Hypoxia-sensitive COMMD1 integrates signaling and cellular metabolism in human macrophages and suppresses osteoclastogenesis. Immunity 47: 66-79.e5, 2017.
2. Alves CH, Farrell E, Vis M, Colin EM and Lubberts E: Animal models of bone loss in inflammatory arthritis: From cytokines in the bench to novel treatments for bone loss in the bedside-a comprehensive review. Clin Rev Allergy Immunol 51: 27-47, 2016.
3. Al Faraj A, Shaik AS and Alnafea M: Intrapulmonary administration of bone-marrow derived M1/M2 macrophages to enhance the resolution of LPS-induced lung inflammation: Noninvasive monitoring using free-breathing MR and CT imaging protocols. BMC Med Imaging 15: 16, 2015.
4. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, *et al*: Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17: 1231-1234, 2011.
5. Huang XL, Liu C, Shi XM, Cheng YT, Zhou Q, Li JP and Liao J: Zoledronic acid inhibits osteoclastogenesis and bone resorptive function by suppressing RANKL-mediated NF-κB and JNK and their downstream signaling pathways. Mol Med Rep 25: 59, 2022.
6. Vega D, Maalouf NM and Sakhaee K: Clinical review #: The role of receptor activator of nuclear factor-kappaB (RANK)/RANK ligand/osteoprotegerin: Clinical implications. J Clin Endocrinol Metab 92: 4514-4521, 2007.

7. Zhou S, Yuan X, Liu Q, Zhang X, Pan X, Zang L and Xu L: BAPTA-AM, an intracellular calcium chelator, inhibits RANKL-induced bone marrow macrophages differentiation through MEK/ERK, p38 MAPK and Akt, but not JNK pathways. Cytokine 52: 210-214, 2010.
8. Jin H, Yao L, Chen K, Liu Y, Wang Q, Wang Z, Liu Q, Cao Z, Kenny J, Tickner J, *et al*: Evodiamine inhibits RANKL-induced osteoclastogenesis and prevents ovariectomy-induced bone loss in mice. J Cell Mol Med 23: 522-534, 2019.
9. Surprenant A, Rassendren F, Kawashima E, North RA and Buell G: The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272: 735-748, 1996.
10. Orioli E, De Marchi E, Giuliani AL and Adinolfi E: P2X7 receptor orchestrates multiple signalling pathways triggering inflammation, autophagy and metabolic/trophic responses. Curr Med Chem 24: 2261-2275, 2017.
11. Jørgensen NR, Syberg S and Ellegaard M: The role of P2X receptors in bone biology. Curr Med Chem 22: 902-914, 2015.
12. Gartland A, Buckley KA, Bowler WB and Gallagher JA: Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts in vitro. Calcif Tissue Int 73: 361-369, 2003.
13. Ke D, Fu X, Xue Y, Wu H, Zhang Y, Chen X and Hou J: IL-17A regulates the autophagic activity of osteoclast precursors through RANKL-JNK1 signaling during osteoclastogenesis in vitro. Biochem Biophys Res Commun 497: 890-896, 2018.
14. DeSelm CJ, Miller BC, Zou W, Beatty WL, van Meel E, Takahata Y, Klumperman J, Tooze SA, Teitelbaum SL and Virgin HW: Autophagy proteins regulate the secretory component of osteoclastic bone resorption. Dev Cell 21: 966-974, 2011.
15. Li Z, Huang Z and Bai L: The P2X7 receptor in osteoarthritis. Front Cell Dev Biol 9: 628330, 2021.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Method 25: 402-408, 2001.
17. Wang N, Agrawal A, Jørgensen NR and Gartland A: P2X7 receptor regulates osteoclast function and bone loss in a mouse model of osteoporosis. Sci Rep 8: 3507, 2018.
18. Agrawal A, Buckley KA, Bowers K, Furber M, Gallagher JA and Gartland A: The effects of P2X7 receptor antagonists on the formation and function of human osteoclasts in vitro. Purinergic Signal 6: 307-315, 2010.
19. Gartland A, Buckley KA, Hipskind RA, Perry MJ, Tobias JH, Buell G, Chessell I, Bowler WB and Gallagher JA: Multinucleated osteoclast formation in vivo and vitro by P2X7 receptor-deficient mice. Crit Rev Eukaryot Gene Expr 23: 243-253, 2003.
20. Ke HZ, Qi H, Weidema AF, Zhang Q, Panupinthu N, Crawford DT, Grasser WA, Paralkar VM, Li M, Audoly LP, *et al*: Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. Mol Endocrinol 17: 1356-1367, 2003.
21. Fujita K, Iwasaki M, Ochi H, Fukuda T, Ma C, Miyamoto T, Takitani K, Negishi-Koga T, Sunamura S, Kodama T, *et al*: Vitamin E decreases bone mass by stimulating osteoclast fusion. Nat Med 18: 589-594, 2012.
22. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, *et al*: Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3: 889-901, 2002.
23. Zhang J, Xu H, Han Z, Chen P, Yu Q, Lei Y, Li Z, Zhao M and Tian J: Pulsed electromagnetic field inhibits RANKL-dependent osteoclastic differentiation in RAW264.7 cells through the Ca^{2+}-calcineurin-NFATc1 signaling pathway. Biochem Biophys Res Commun 482: 289-295, 2017.
24. Morrison MS, Turin L, King BF, Burnstock G and Arnett TR: ATP is a potent stimulator of the activation and formation of rodent osteoclasts. J Physiol 511: 495-500, 1998.
25. Zhang L, Guo YF, Liu YZ, Liu YJ, Xiong DH, Liu XG, Wang L, Yang TL, Lei SF, Guo Y, *et al*: Pathway-based genome-wide association analysis identified the importance of regulation-of-autophagy pathway for ultradistal radius BMD. J Bone Miner Res 25: 1572-1580, 2010.
26. Tong X, Min W, Li S, Chen M, Song R, Bian J, Gu J and Liu Z: Beclin 1 positively regulates osteoprotegerin-induced inhibition of osteoclastogenesis by increasing autophagy in vitro. Differentiation 121: 35-43, 2021.
27. Ji Li, Sun Z, Lin Y, Yan Y, Yan H, Jing B and Han Z: Syndecan 4 contributes to osteoclast differentiation induced by RANKL through enhancing autophagy. Int Immunopharmacol 91: 107275, 2021.
28. Tong X, Gu J, Song R, Wang D, Sun Z, Sui C, Zhang C, Liu X, Bian J and Liu Z: Osteoprotegerin inhibit osteoclast differentiation and bone resorption by enhancing autophagy via AMPK/mTOR/p70S6K signaling pathway in vitro. J Cell Biochem: Sep 6, 2018 (Epub ahead of print). doi: 10.1002/jcb.27468.
29. Chu B, Chen S, Zheng X, Ye J, Cheng X, Zhang L, Guo D, Wang P, Hong D and Hong Z: Nepetin inhibits osteoclastogenesis by inhibiting RANKL-induced activation of NF-κB and MAPK signalling pathway, and autophagy. J Cell Mol Med 24: 14366-14380, 2020.
30. Cejka D, Hayer S, Niederreiter B, Sieghart W, Fuereder T, Zwerina J and Schett G: Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. Arthritis Rheum 62: 2294-2302, 2010.
31. Cao B, Dai X and Wang W: Knockdown of TRPV4 suppresses osteoclast differentiation and osteoporosis by inhibiting autophagy through Ca^{2+} -calcineurin-NFATc1 pathway. J Cell Physiol 234: 6831-6841, 2019.
32. Sekar P, Huang DY, Hsieh SL, Chang SF and Lin WW: AMPK-dependent and independent actions of P2X7 in regulation of mitochondrial and lysosomal functions in microglia. Cell Commun Signal 16: 83, 2018.
33. Ran D, Ma Y, Liu W, Luo T, Zheng J, Wang D, Song R, Zhao H, Zou H, Gu J, *et al*: TGF-β-activated kinase 1 (TAK1) mediates cadmium-induced autophagy in osteoblasts via the AMPK/mTORC1/ULK1 pathway. Toxicology 442: 152538, 2020.