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Original Article

Punicalagin inhibits RANKL-induced osteoclastogenesis by suppressing NF-κB and MAPK signaling pathways

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Abstract: Osteoclasts, bone-specialized multinucleated cells, play pivotal roles in osteopenic diseases such as osteoporosis and rheumatoid arthritis (RA). Natural plant-derived products have been recognized as important sources for medicaments given their potential therapeutic and preventive activities against osteopenic diseases. In this study, we determined the effects of punicalagin (PUN), from Punica granatum L, on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation *in vitro*. We found that PUN inhibited RANKL-induced osteoclast differentiation from RAW264.7 cells without cytotoxicity. Furthermore, expression of osteoclastogenesis-related marker genes such as nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), matrix metallopeptidase 9 (MMP-9), and tartrate-resistant acid phosphatase (TRAP), were strongly inhibited by PUN. At molecular levels, PUN inhibited RANKL-induced nuclear factor-κB alpha (IκBα) phosphorylation blocked nuclear factor-κB subunit p65 (NF-κB p65) phosphorylation and nuclear translocation. Moreover, PUN inhibited RANKL-induced phosphorylation of p38 mitogen-activated protein kinase ERK and Jun N-terminal kinase. Taken together, our results suggest that PUN might be useful in the development of new agents to prevent osteoporosis and other osteopenic diseases.

Keywords: Punicalagin, osteoclasts, RANKL, MAPKs, NF-κB

Introduction

The adult skeleton constantly undergoes bone remodeling which involves osteoblast bone formation and osteoclast resorption. It is necessary to repair damaged bone and to maintain mineral homeostasis [1, 2]. Excessive osteoclast formation and differentiation leads to an imbalance in bone remodeling, resulting in pathologic bone disorders including osteoporosis, RA, as well as bone tumor metastases, Paget's disease, and periodontitis [3, 4]. Therefore, inhibiting osteoclast formation may represent a treatment option for diseases involving excessive bone resorption. Several pharmacologic anti-osteoporosis drugs such as bisphosphonates, teriparatide, and raloxifene have been widely used. These drugs, however, come with side effects [5, 6]. Consequently, it is urgent that we develop novel agents that have fewer undesirable side effects.

Osteoclasts are derived from hematopoietic stem cells, multinucleated cells formed by the fusion of circulating mononuclear precursor cells responsible for bone-resorbing [7]. Extensive research in recent years has indicated that receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) play a major role in imitating differentiation of osteoclast precursors into osteoclasts *in vitro* and *in vivo* [8]. RANKL is a tumor necrosis factor (TNF) superfamily member. RANKL-induced activation of cell surface receptor RANK leads to stimulation of TNF receptor-associated factors (TRAF) 1, 2, 3, 5 and 6. Activation of TRAFs and the sequential recruitment of several downstream signaling molecules leads to NF-κB and MAPKs activation, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 [9, 10]. Therefore, inhibition of RANKL signaling might become an effective method in treatment of osteoclastogenesis-induced bone loss.

There is accumulating evidence that nuclear factor of activated T cells, cytoplasmic 1 (NF-

Table 1. Primers used in the study

Primer	Sequence
GADPH	F-AACTTTGGCATTGTGGAAGGG
	R-GACACATTGGGGGTAGGAACAC
NFATc1	F-CAACGCCCTGACCACCGATAG
	R-GGCTGCCTTCCGTCTCATAGT
TRAP	F-AAATCACTCTTTAAGACCAG
	R-TTATTGAATAGCAGTGACAG
MMP-9	F-CTGTCCAGACCAAGGGTACAGCCT
	R-GTGGTATAGTGGGACACATAGTGG

ATc1), a key transcription factor of terminal osteoclastogenesis, is activated by NFκB and activator protein (AP)-1. Activation of NFATc1 and other abovementioned signaling pathways directly regulates expression of osteoclast-specific genes such as TRAP, MMP-9, cathepsin K (Ctsk), and calcitonin receptor (Ctr), which play crucial roles in osteoclast differentiation [11].

Recently, numerous evidence has suggested that inflammatory cytokines play an important role in osteoclastogenesis, leading to bone resorption that is frequently associated with osteoporosis [12, 13]. Natural product-derived small molecules have been proven to possess inhibitory effects on osteoclastogenesis [14, 15]. Pomegranate products are one of the most important crude drugs in the prescriptions of traditional Chinese medicine that have shown significant efficacy for treatment of inflammatory bowel disease, cancer, diarrhea, ulcers, and diabetes mellitus [16, 17]. Punicalagin (2,3,hexahydroxydiphenoyl-gallagyl-D-glucose and referred to as PUN) belongs to ellagitannin, an extract from pomegranate polyphenols [18]. Previous research has reported that the water-soluble ellagitanin PUN is toxic to cattle but repeated oral administration of high doses of PUN to rats for 37 days was not toxic [19]. More recently, PUN has been found to have antioxidant activity and cartilage-sparing effects *in vitro*. PUN has been reported to prevent proliferation of several human cancer cell lines and inhibit effects of cardiovascular diseases at high concentrations [20, 21]. It has been shown that PUN has immune-suppressive activity including the inhibition of NFAT activation [22]. Since NFATc1 is an essential factor in the differentiation of osteoclasts, we hypothesized that PUN may inhibit RANKL-induced osteoclastogenesis. In this study, we have investigated the effects of PUN, the major bioactive constituents of pomegranate polyphenols, on osteoclast formation in RAW264.7 macrophages. Moreover, possible mechanisms were also explored.

Materials and methods

Cells and reagents

Raw264.7 cells (ATCC, Manassas, VA) were maintained in α-MEM (Gibco, NY, USA), supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin. Incubations were performed at subconfluence in 5% CO_2 at 37°C and were subcultured every 2-3 days. Punicalagin was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA). Recombinant RANKL (rRANKL) was stored at -80C until use (PeproTech, USA). Antibodies against NF-κB (p-IκBα, p-p65) and MAPKs (p38, p-p38, ERK, p-ERK, JNK and p-JNK) were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Proliferation assays

Cell viability was assessed by cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). RAW264.7 cells were seeded in 96 well plates. Following incubation for 24 hours, cells were treated with various concentrations of PUN, in triplicate, for 1, 3, or 5 days at 37°C. After incubation for the indicated time, CCK-8 solution was added and incubated further for 2 hours. Absorbance values were measured at 450 nm using a microplate reader (Bio-Tek, USA).

Osteoclast differentiation assay

RAW264.7 cells were cultured with RANKL (50 ng/mL) in the presence of different concentrations of PUN (25, 50 or 100 μM) and media were refreshed daily over the course of five days. Cells were then fixed in 4% formaldehyde (Boster Biological Technology, Ltd) for 15 minutes. Fixed cells were subjected to an assay using a TRAP kit, according to manufacturer instructions. We then washed with PBS. The total number of TRAP-positive multinucleated cells (3 or more nuclei) in each well were counted under a light microscope (Olympus IX71, Olympus Corporation, Tokyo, Japan). For each

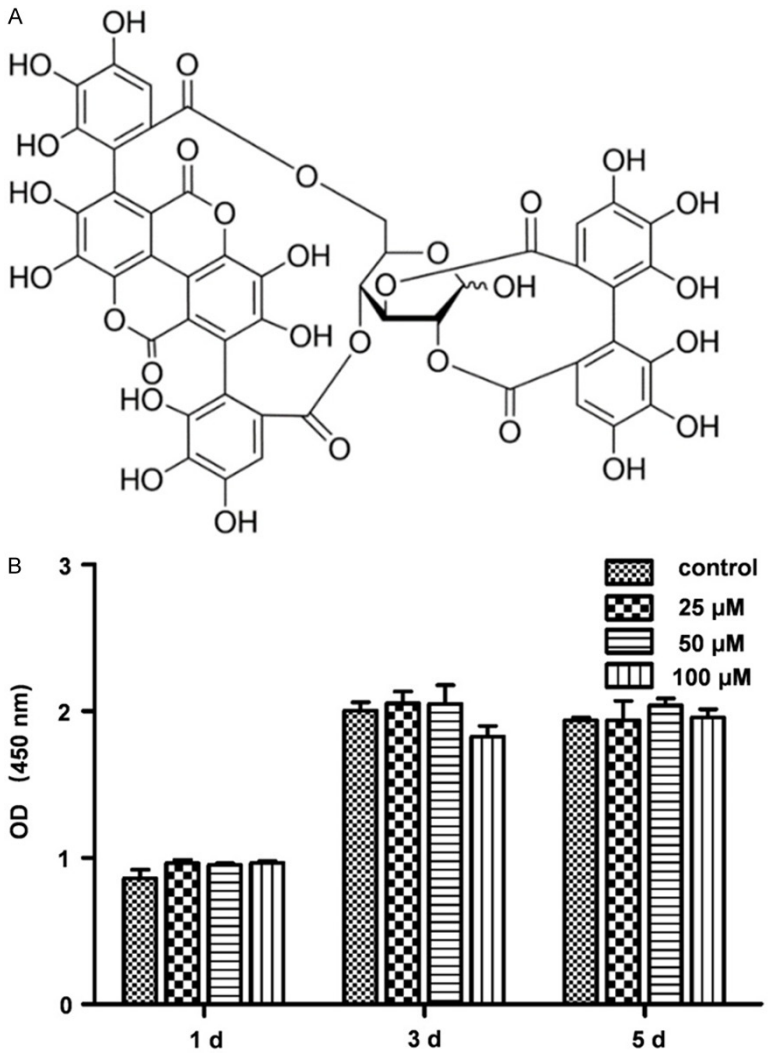

Figure 1. Cytotoxicity of PUN in RAW264.7 cells. A. Chemical structure of PUN. B. RAW264.7 cells were treated with PUN (0, 25, 50 or 100 μM) for 1, 3 or 5 days. Cytotoxicity was determined by a CCK-8 assays. Values are expressed as mean ± SD of triplicate experiments.

sample, three fields of vision were examined. In a separate experiment to evaluate TRAP activity, using a commercially available ELISA kit (Sigma, Mo, USA), the cell culture supernatant was used to measure TRAP activity at 450 nm by spectrophotometer.

Bone resorption assay

Cells were seeded on the fluoresceinamine-labeled chondroitin sulfate-labeled and CaP-coated plates (Cosmo Bio Co., Ltd., Tokyo, Japan). Cells were cultured with 50 ng/ml RANKL with or without PUN (25, 50 or 100 μM) for 3 days. Then, 100 mL of the conditioned medium was harvested and transferred into a 96-well plate. Bone resorption assay buffer was added and fluorescence intensity was detected by fluorescence spectrophotometer (Biotek Instruments, Winooski, VT, USA), with an emission wavelength of 535 nm and excitation wavelength of 485 nm.

Enzyme linked immunosorbent assay (ELISA)

RAW264.7 macrophages were preincubated on 6-well plates at a concentration of 1 × 10^6 cells per well. Then, they were pretreated with PUN (25, 50, or 100 μM) 24 hours prior to RANKL (50 ng/mL) treatment in a humidified incubator for 16 hours. The medium were then collected and immediately analyzed. The concentration of TNF-α was measured by ELISA kit, according to manufacturer instructions.

Western blotting

Cells treated at indicated times with various concentrations of PUN were washed twice with cold PBS and then lysed in extraction buffer plus PMSF. Cell extracts were centrifuged at 14,000 × g at 4°C for 10 minutes and supernatants were collected as samples. Equal amounts of protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% skim milk in PBS containing 0.05% Tween 20 at room temperature for 2 hours and probed successively with p-IκB-α, p-p65, p38, p-p38, ERK, p-ERK, JNK and p-JNK at 4°C overnight. Horseradish peroxidase-conjugated goat anti-mice (1:500, LI-COR, Nebraska, USA) or 926-68021 IRDye 680 RD goat anti-rabbit (1:500, LI-COR, Nebraska, USA) were used as secondary antibodies at room temperature for 2 hours. Signals were detected

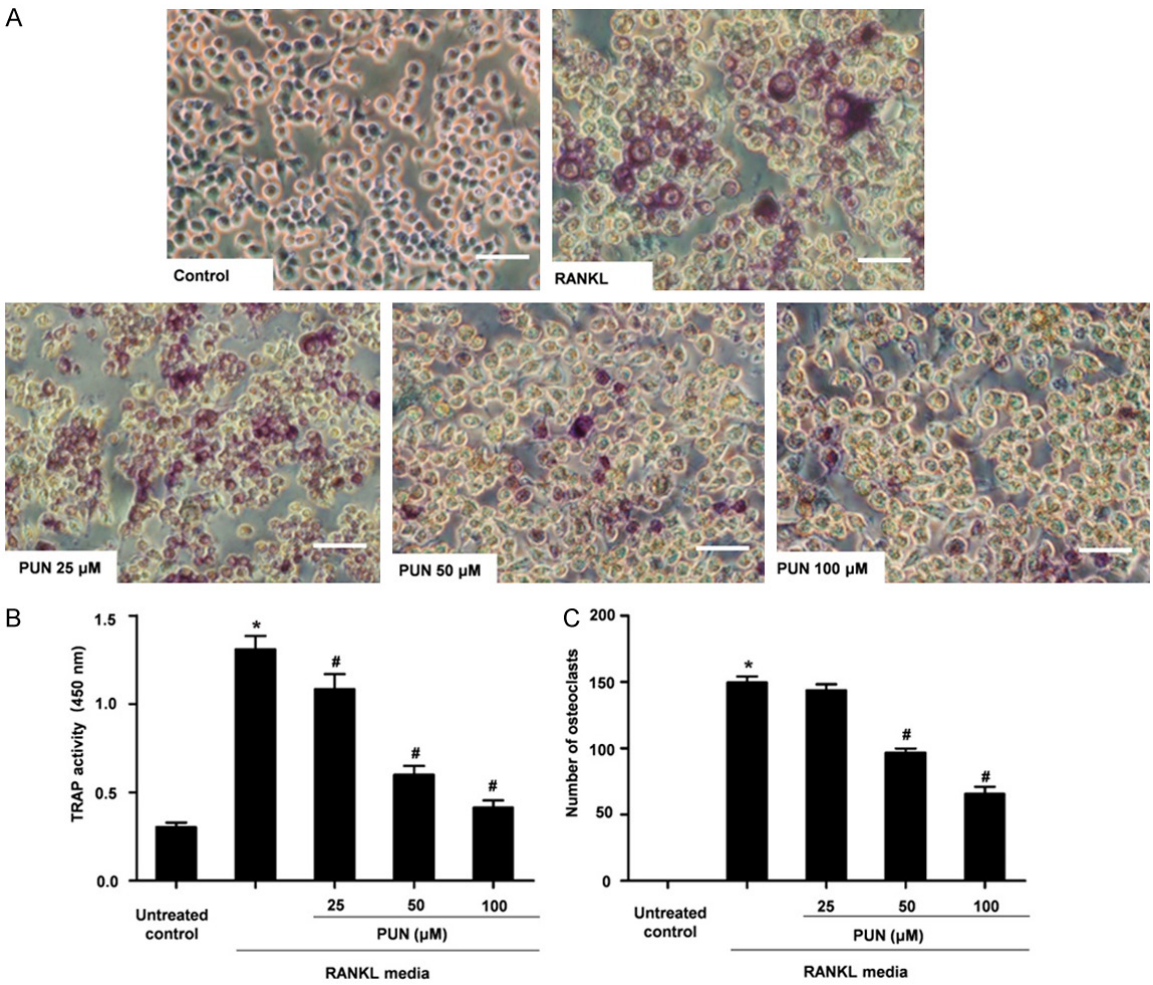

Figure 2. PUN inhibits RANKL-induced osteoclast differentiation in RAW264.7 cells. A. After RAW264.7 cells were cultured for 5 days in the presence of RANKL with different concentrations of PUN (25, 50 and 100 μM), cells were stained using a leukocyte acid phosphatase kit, and TRAP-positive activity was determined. TRAP-positive multinucleated osteoclasts were visualized under light microscopy (magnification × 20). B. Supernatants were collected, and the TRAP concentration in the supernatants was determined in 450 nm by ELISA reader. C. Multinucleated osteoclasts were counted. Values are expressed as mean ± SD of triplicate experiments. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with RANKL group.

by Odyssey infrared laser imaging system (GENE, California, USA).

Immunofluorescence staining

RAW264.7 cells were stimulated with or without RANKL (50 ng/mL) for 30 minutes to induce osteoclast formation in the presence or absence of 100 μM PUN. Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.2% Triton-X 100 for 5 minutes. Cells were then stained by rabbit anti-mouse NF-κB p65 (Cell Signing Technology, USA) for 24 hours at 4°C. Preparations were washed and incubated in donkey anti-rabbit. IgG was added as a secondary antibody. Cells were stained by 4',6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St Louis, MO, USA) solution at room temperature for 10 minutes. Fluorescence staining was viewed under an Axiomager Optional fluorescence microscope (Olympus, Tokyo, Japan).

RT-PCR analysis

Total RNA was isolated from RAW264.7 cells derived osteoclasts using TRIzol Reagent (Invitrogen, Paisley, UK). The concentration and integrity of extracted RNA was analyzed by measurement of the OD260/280 (Eppendorf 22331, Eppendorf, Hamburg, Germany). Purified RNA was converted into cDNA using a

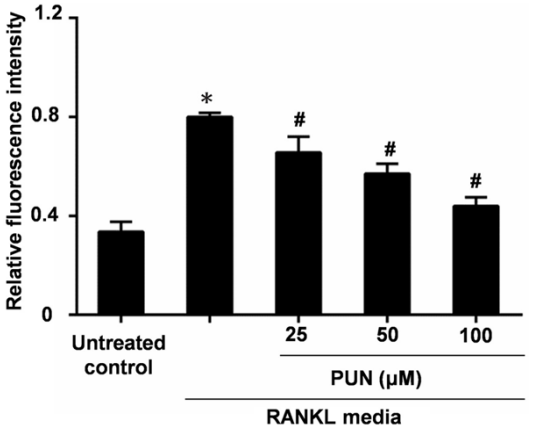

Figure 3. Bone resorption activity was examined by fluorescence intensity. Cells were seeded on the fluoresceinamine-labeled chondroitin sulfate-labeled and CaP-coated plates. After treatments, the conditioned media were harvested fluorescence detection. Multinucleated osteoclasts were counted. Values are expressed as mean ± SD of triplicate experiments. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with RANKL group.

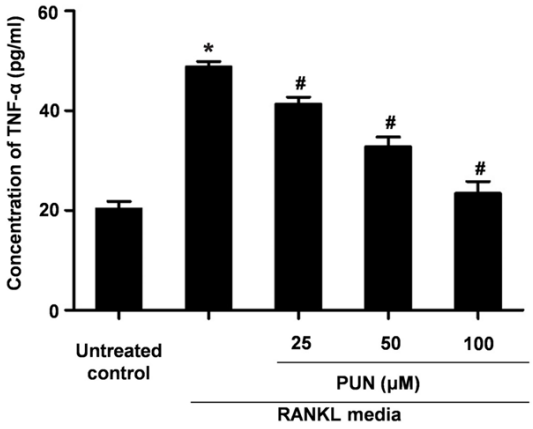

Figure 4. PUN suppresses RANKL-induced TNF-α secretion by RAW264.7 cells. Cells were pretreated with indicated concentrations of PUN (25, 50 and 100 μM) for 2 h, then incubated with RANKL (50 ng/mL) for another 24 h, and supernatants were analyzed for TNF-α by ELISA assay according to the manufacturer's instructions. Data are represented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with RANKL group.

MuLV reverse transcriptase. PCR (Bio-Rad Laboratories, Hercules, CA) was accomplished using mRNA transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NFATc1, TRAP and MMP-9 with an addition of 10 mM dNTP, 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, and 1 unit of Taq DNA polymerase. Each cycle consisted of 30 seconds of denaturation at

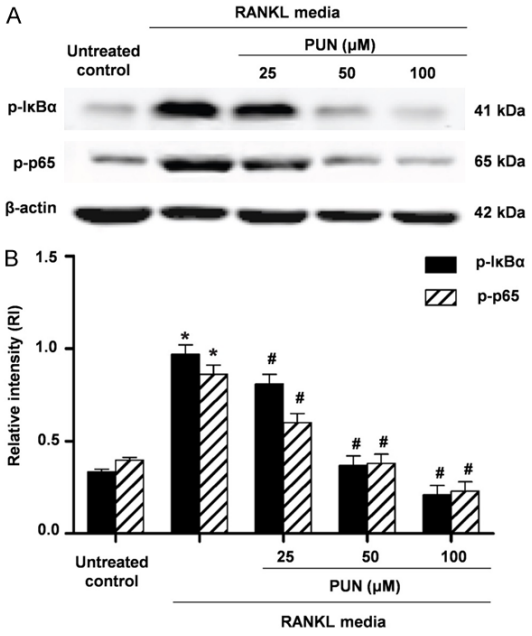

Figure 5. PUN suppresses RANKL-induced NF-κB activation in RAW264.7 cells. A. Confluent RAW264.7 cells were pre-treated with various concentrations of PUN (25, 50 and 100 μM) for 3 hours followed by RANKL (50 ng/mL) for 20 minutes, and IκBα phosphorylation and p65 phosphorylation were determined by Western blot. B. Relative amounts of protein were determined by densitometric analysis. Data are represented as the mean ± SD of three independent experiments. *P < 0.05 compared with control, #P < 0.05 compared with RANKL group.

94°C, 30 seconds at 55°C, and 45 seconds at 72°C. The final extension was 10 minutes at 72°C at the end of 30 cycles. Primers for the selected genes are shown in **Table 1**. An equal volume of each PCR product was electrophoresed on a 2% agarose gel stained with ethidium bromide (0.5 μg/mL).

Statistical analysis

Data are presented as mean ± standard deviation (SD) of values obtained from at least 3 independent experiments, performed in triplicate. All experimental data was determined by Student's t-text and one-way ANOVA. A value of $^{*}P < 0.05$ was considered to be statistically significant.

Results

Effects of PUN on cell viability

In order to determine whether the effects of PUN on osteoclastogenesis were due to the

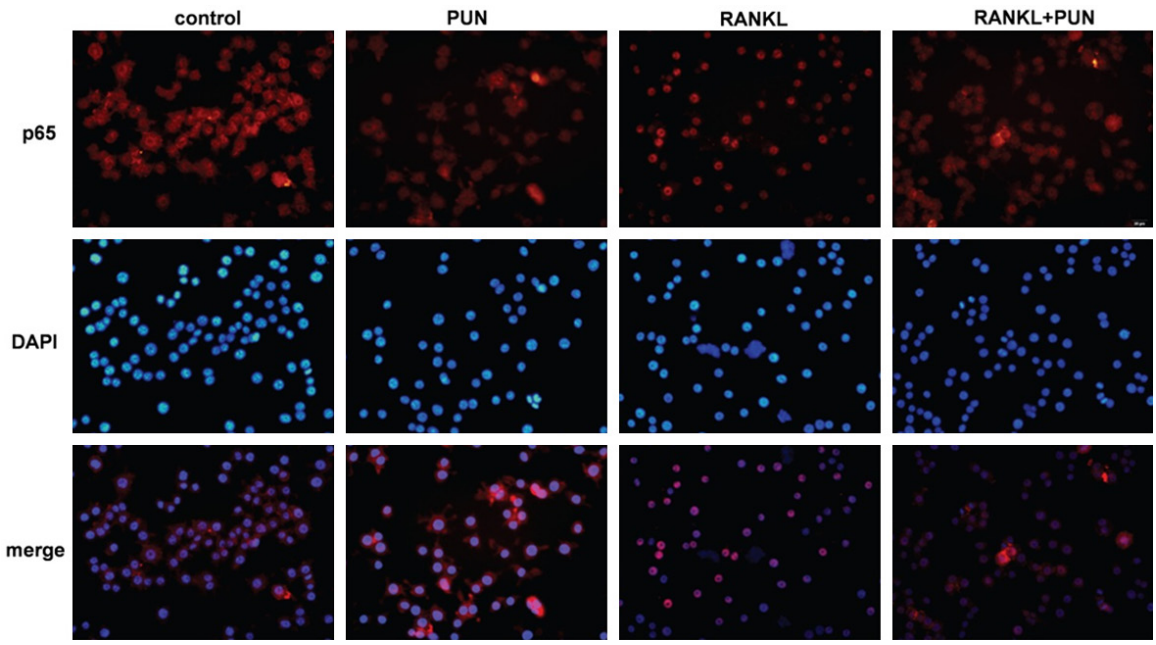

Figure 6. PUN blocks the RANKL-induced NF-κB p65 nuclear translocation in RAW264.7 cells. Cells were pretreated with or without PUN (100 μM) and then stimulated with or without 50 ng/mL of RANKL for 30 min. The localization of p65 was visualized by immunofluorescence staining (magnification × 20). One of three experiments with similar results is shown.

potential toxicity of this drug, the cytotoxicity of PUN was examined using a CCK-8 assay in RAW264.7 cells. Cytotoxicity was very low in cells treated with all concentrations of PUN (25, 50, or 100 μM) for 1, 3, or 5 days (**Figure 1B**), suggesting that the effects of PUN on osteoclast differentiation were not mediated by cytotoxicity of this compound.

Effects of PUN on osteoclast differentiation

In order to examine the effects of PUN on osteoclast differentiation, formation of osteoclast-like cells induced by RANKL (50 ng/mL) stimulation in RAW264.7 cells was examined using TRAP staining. The following doses of PUN were selected for subsequent experiments: 0, 25, 50, and 100 μM. First, we examined the effect of PUN on TRAP activity. TRAP activity of RAW264.7 enhanced by RANKL was dose-dependently diminished in macrophages treated with PUN for 5 days (**Figure 2A**, **2B**). Next, we examined whether PUN has any effect on multinucleated osteoclast-like cell formation. TRAP-positive osteoclasts with multiple nuclei were formed within 5 days in response to RANKL stimulation and this response was inhibited by PUN in a concentration-dependent manner (**Figure 2C**).

Effect of PUN on bone resorption

To further evaluate inhibitory effects of PUN on osteoclast differentiation, we detected the effects of PUN on bone resorption. RAW264.7 cells were cultured on culture plates, coated with calcium phosphate for 5 days, that were treated with PUN (25, 50, or 100 μM) in the presence or absence of RANKL (50 ng/ml). We found that bone resorption was markedly down-regulated by PUN treatments (**Figure 3**).

Effects of PUN on RANKL-stimulated secretion of inflammatory cytokines

Inflammatory cytokines, such as TNF-α, could induce differentiation and activation of osteoclasts [23]. The effects of PUN on production of TNF-α induced by RANKL in RAW264.7 cells were investigated. As shown in **Figure 4**, RANKL promoted production of TNF-α and PUN effectively suppressed TNF-α production, as compared to RANKL treatment.

Effects of PUN on NF-κB activation

Activation of NF-κB is essential in initiating osteoclast differentiation. To determine whether PUN inhibited NF-κB-mediated osteoclast dif-

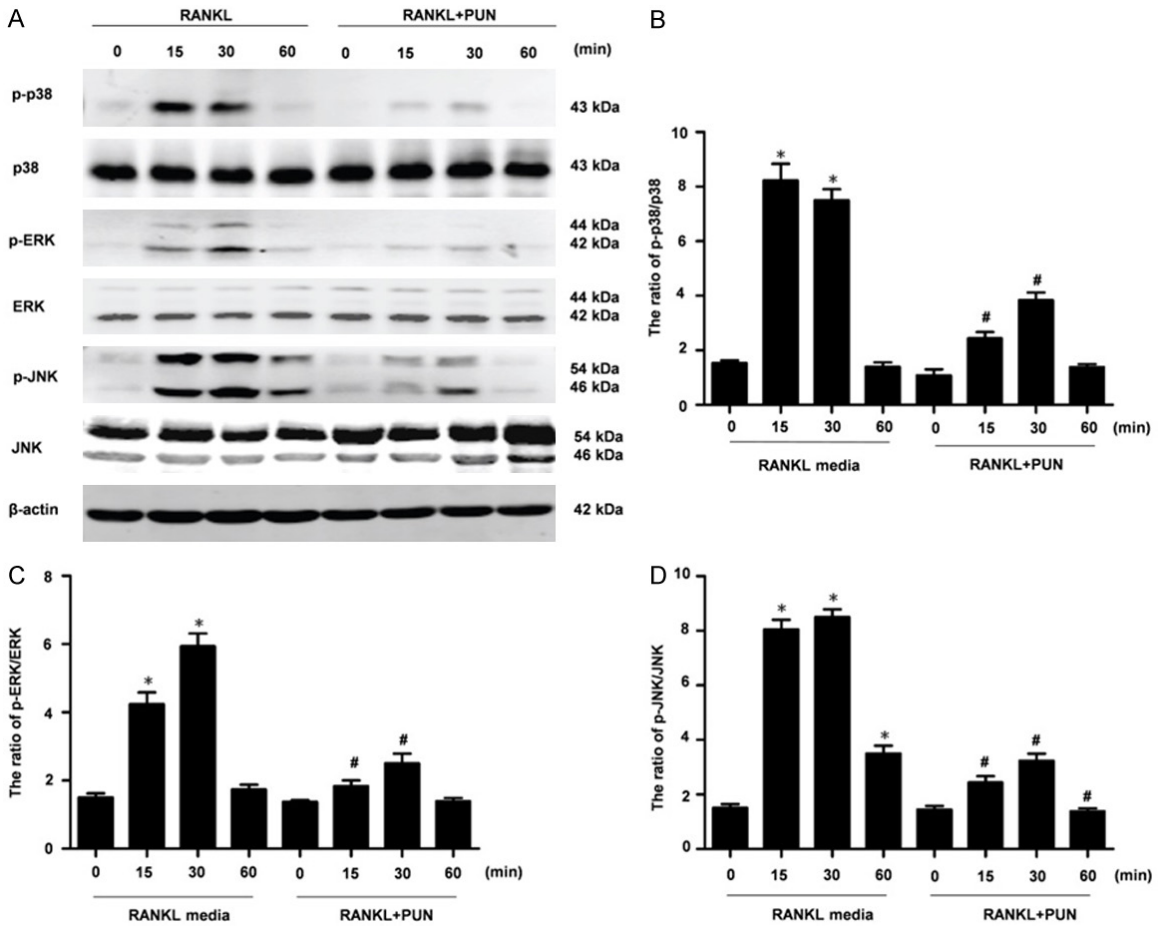

Figure 7. PUN downregulated RANKL induced MAPK phosphorylation in RAW264.7 cells. A. Cells were pretreated with or without PUN (100 μM) for 3 h and then stimulated with RANKL (50 ng/ml) for the indicated times, and the p38, ERK and JNK phosphorylation was determined by Western blot. β-actin was used as the internal control. The relative protein expression level of p-p38, p-ERK, and p-JNK were quantified using Image-Pro Plus 6.0 software and normalized to β-actin. Data are represented as the mean ± SD of three independent experiments (B-D). $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with RANKL group.

ferentiation, we investigated the effects of PUN on activation of NF-κB. Accordingly, RAW264.7 cells were pretreated with vehicle or PUN for 3 hours and phosphorylation of IκBα and NF-κB p65 were determined after 20 minutes of RANKL treatment. PUN was found to significantly suppress RANKL-induced phosphorylation of IκBα and NF-κB p65 (**Figure 5**). Furthermore, the inhibitory effects of PUN on NF-κB activation were further supported by immunofluorescence staining. As expected, upregulated expression of NF-κB p65 protein in the nucleus induced by RANKL was significantly repressed by PUN treatment, whereas expression of NF-κB p65 protein in the cytoplasm was significantly increased by PUN treatment. (**Figures 5**, **6**).

Effects of PUN on activation of MAPKs

Besides NF-κB signaling pathways, activation of MAPK pathways plays important roles in differentiation and formation of osteoclasts. To investigate whether inhibition of NF-κB activation by PUN was mediated through MAPK pathways, we evaluated the effects of PUN on MAPKs following the stimulation of RANKL including p38, ERK and JNK in RAW264.7 cells. We found that these proteins were phosphorylated, following stimulation with RANKL (**Figure 7**). PUN was found to significantly inhibit RANKL-induced phosphorylation of p38, ERK and JNK (**Figure 7**). However, the amount of unphosphorylated ERK, p38 and JNK was unaffected by RANKL and/or PUN treatment. These results

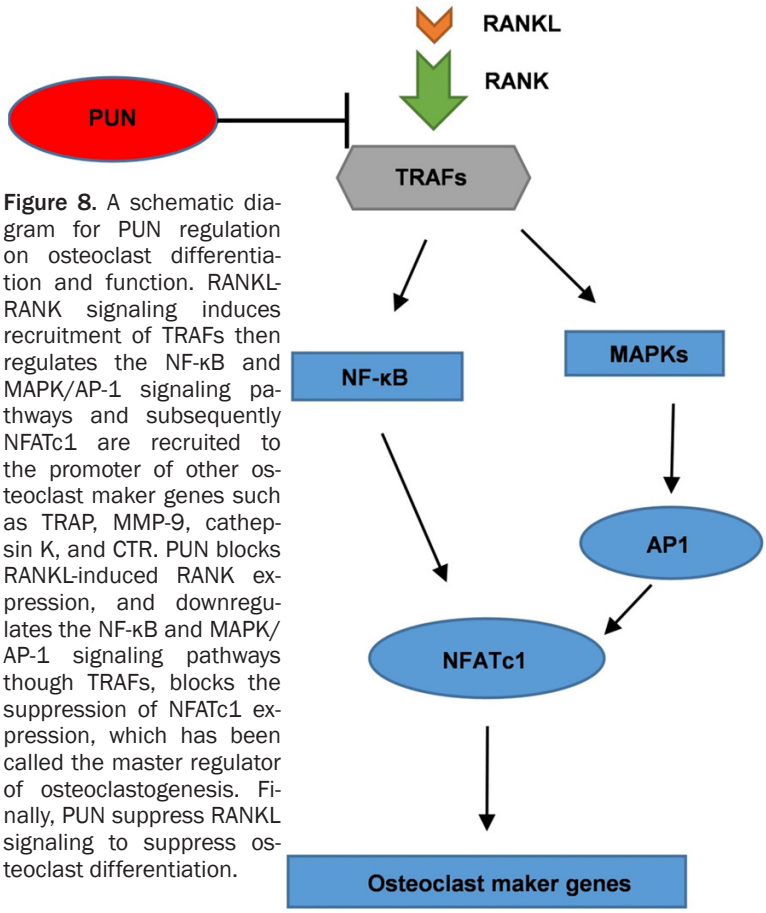

Figure 8. A schematic diagram for PUN regulation on osteoclast differentiation and function. RANKL-RANK signaling induces recruitment of TRAFs then regulates the NF-κB and MAPK/AP-1 signaling pathways and subsequently NFATc1 are recruited to the promoter of other osteoclast maker genes such as TRAP, MMP-9, cathepsin K, and CTR. PUN blocks RANKL-induced RANK expression, and downregulates the NF-κB and MAPK/AP-1 signaling pathways though TRAFs, blocks the suppression of NFATc1 expression, which has been called the master regulator of osteoclastogenesis. Finally, PUN suppress RANKL signaling to suppress osteoclast differentiation.

suggest that PUN can inhibit RANKL-induced activation of MAPKs.

Effects of PUN on expression of osteoclastic marker genes

To further examine its inhibitory effects on osteoclast formation, we examined its effects on expression of osteoclastic marker genes such as NFATc1, TRAP, c-Src, c-fos and cathepsin K, most of which are target genes of NFATc1. In this study, we found that PUN could significantly inhibit NFATc1expression. Expression of NFATc1 is known to be dependent on NF-κB and MAPKs (**Figure 8**). Since PUN can suppress NF-κB and MAPK pathways and NFATc1 expression, we next investigated whether PUN regulates osteoclastogenesis-related marker gene expression as well. Our results indicate that PUN could significantly inhibit RANKL-induced genes levels of TRAP and MMP-9 (**Figure 9**).

Discussion

Polyphenols are a group of plant secondary metabolites. Researchers, in past decades, have paid more attention to the biological activities of polyphenols and have investigated their underlying mechanisms [24]. PUN, one of the most abundant of pomegranate polyphenols, is a kind of ellagitannin possessing anti-inflammatory and antioxidant activities [25, 26]. Recent researches have shown that pomegranate extract and PUN have certain effects on immune-suppressive activity [22]. In this study, the effects of PUN on RANKL-induced osteoclast formation and its function in murine RAW264.7 cells were investigated and signaling mechanisms associated with this process were examined. Our results demonstrated that PUN markedly suppressed RANKL-induced osteoclast differentiation *in vitro*, without detectable cytotoxicity. It was also shown that PUN suppressed expression of NFATc1 and other osteoclast-related genes through inhibition of NF-κB and MAPK signaling pathways. Our study confirms that PUN may serve as a promising drug for the treatment of osteoporosis.

Osteoclasts are formed by monocyte-macrophage lineages [27]. Multinucleated osteoclasts can be induced by RANKL, a key factor that also controls the function and survival of mature osteoclasts. RANKL is considered a convincing inducer of the differentiation and formation of osteoclasts. Thus, agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss. Pro-inflammatory factors and cytokines, such as TNF-α, whose excessive release may induce pre-osteoclast fusion, stimulate differentiation and formation of osteoclasts and support the survival of mature osteoclasts [23]. TNF-α has been regarded as a detrimental master regulator of inflammatory responses and RANK-RANKL pathway has been reported to be involved in TNF-α-induced osteoclast differentiation [28, 29]. PUN has previously been reported to inhibit LPS induced TNF-α, IL-1 and

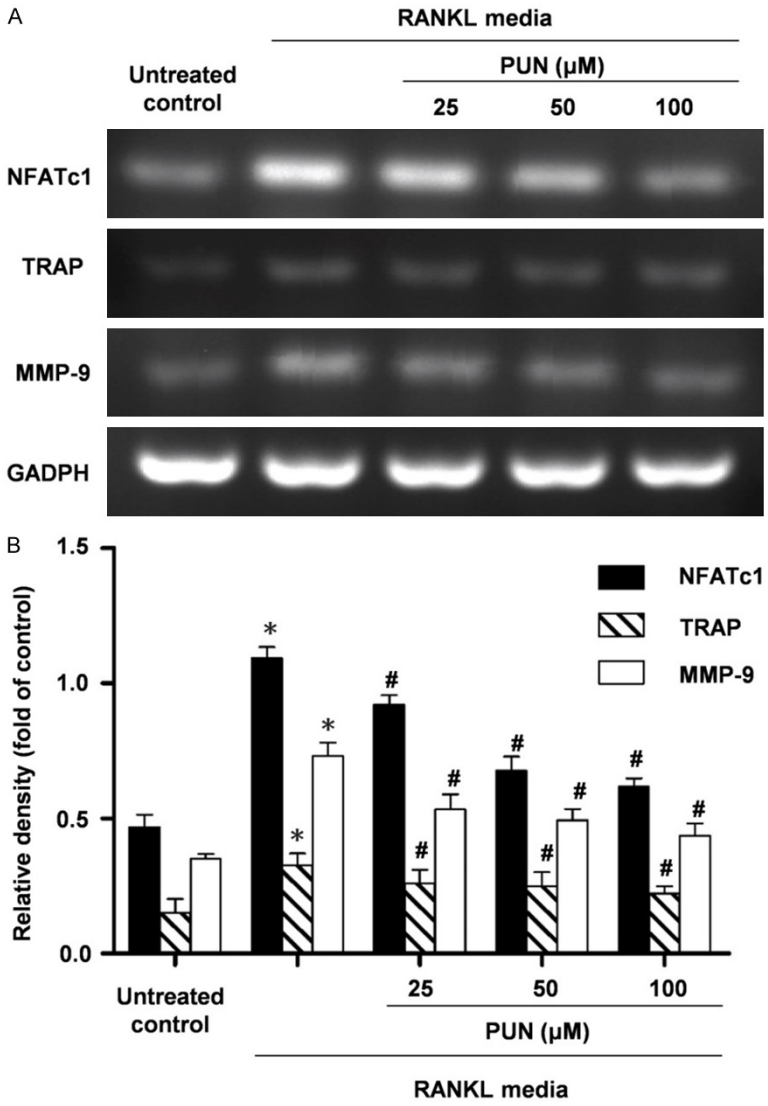

Figure 9. Effects of PUN on mRNA expression of osteoclastic marker genes. A. RAW264.7 cells were cultured in α-MEM and exposed to RANKL (50 ng/mL) in the presence of PUN (25, 50 and 100 μM) for 5 days. B. mRNA expressions of osteoclastogenic marker genes were determined using RT-PCR. Values are expressed as mean ± SD of triplicate experiments. $^{*}P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with RANKL group.

iNOS expression in RAW264.7 cells. Our investigation indicates that PUN has strong inhibitory effects on RANKL-induced TNF-α secretion in RAW264.7 cells, suggesting the possible application of PUN in diseases with inflammatory bone destruction.

An important initial step in osteoclastogenesis is the interaction between RANKL and its receptor RANK, which leads to activation of various downstream signaling pathways including p38, ERK, JNK, Akt and NF-κB, inducing expression of osteoclastogenesis-related genes such as c-Fos, NFATc1, OSCAR and TRAP. In this study, major intracellular signaling pathways NF-κB and MAPK (p38, ERK, JNK) influencing RANKL-mediated osteoclastogenesis were affected by PUN. NF-κB is a major transcription factor for RANKL-activated osteoclastogenesis [30]. NF-κB p50/p52 double knockout mice showed the defects of osteoclast differentiation and severe osteopetrosis [31]. NF-κB is localized in the cytoplasm and is associated with activation of the IκB kinase complex, which phosphorylates IκB and targets it for ubiquitin-dependent degradation [32]. In the alternative IκB-independent pathway, direct phosphorylation of NF-κB subunits p50 and p65 by IKK, meanwhile the dissociative NF-κB subunit p65 translocates from cytoplasm to nucleus and binds to the targeted position of DNA, modulating NF-κB transcription activity [33]. Our results showed that PUN inhibited nuclear translocation of NF-κB p65 by suppressing RANKL-induced phosphorylation of IκBα and NF-κB p65, suggesting that inhibition of NF-κB-dependent pathways is a mechanism underlying the anti-osteoclastogenic effect of PUN.

MAPKs (JNK, ERK and p38) have been identified in mammalian cells and are associated with osteoclastogenesis. Previous studies have demonstrated that MAP kinases regulate NFATc1 during osteoclastogenesis. p38 is important in early stages of osteoclast generation because it regulates NFATc1 expression during osteoclastogenesis [34, 35]. Furthermore, ERK is known to trigger activation of c-Fos for osteoclastogenesis and inhibition of ERK has been shown to suppress osteoclast formation and function. RANKL also stimulates

JNK, which can phosphorylate the transcription of c-Jun. c-Jun forms activator protein-1 (AP-1) complexes with c-Fos, another essential transcription factor for osteoclast formation [36-38]. In the current study, we demonstrated that PUN markedly inhibited phosphorylation of JNK, ERK, and p38 induced by RANKL, suggesting that PUN could suppress MAPK cascades.

Previous studies have demonstrated that NFATc1 is a master regulator in osteoclastogenesis and overexpression of the NFATc1 gene is associated with efficient induction of mature osteoclasts. Expression of this gene is known to be dependent on NF-κB and MAPKs. NFATc1 also regulates expression of a number of osteoclast-specific genes including TRAP, latent transforming growth factor b-binding protein 3 (LTBP3), chloride channel (ClC7), MMP-9, CTR, cathepsin K, and c-Src [39-42]. Our study demonstrated that PUN significantly decreased expression levels of NFATc1, MMP-9, and TRAP in RAW264.7. Our study suggests that PUN affects not only expression of NFATc1 but also its regulated downstream gene expression.

Conclusion

In summary, our results demonstrate that PUN can suppress RANKL-induced osteoclastogenesis and mediate osteoclast activity *in vitro*. Furthermore, we clarified that the inhibitory effects of PUN occur through suppression of NF-κB and MAPK activation, subsequent decreased expression of RANKL-induced osteoclastic maker genes, and suppressed expression of NFATc1, the principle regulator of osteoclastogenesis. Although additional experiments are needed, our results suggest that PUN has a potential indication in treatment of osteoclast-related diseases, especially inflammatory bone destruction.

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Disclosure of conflict of interest

None.

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