Available online at www.sciencedirect.com

Integrative Medicine Research

journal homepage: www.imr-journal.com

Original Article

Water extract of *Uncaria sinensis* suppresses RANKL-induced bone loss by attenuating osteoclast differentiation and bone resorption

Hyunil Ha[a,1], *Ki-Shuk Shim*[b,1], *Jin Yeul Ma*[b,*]

[a] *Clinical Research Division, Korea Institute of Oriental Medicine, Daejeon, Korea*
[b] *Korean Medicine Application Center, Korea Institute of Oriental Medicine, Daegu, Korea*

ARTICLE INFO

Article history:
Received 28 August 2017
Received in revised form
12 September 2017
Accepted 13 September 2017
Available online 20 September 2017

Keywords:
Uncaria sinensis
Osteoclasts
Receptor activator for nuclear factor-κB ligand
Nuclear factor of activated T-cells Cytoplasmic 1

ABSTRACT

Background: The hooks and stems of *Uncaria sinensis* have been used to mitigate cardiovascular and central nervous system disorders in Asia traditional medicine. Regulation of osteoclast differentiation and activity is a major target for preventing and treating pathological bone diseases.

Methods: Tartrate-resistant acid phosphatase (TRAP) activity and the number of TRAP-stained multinucleated cells were used to examine receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation. The activation of RANKL-induced signaling pathways and the expression of transcription factors were investigated by western blot analysis and quantitative real-time polymerase chain reaction. The bone resorption activity of osteoclast was studied using a plate coated with hydroxyl-apatite. Trabecular bone destruction was investigated using a RANKL-induced trabecular bone loss mouse model.

Results: We found that water extract of the hooks and stems of *U. sinensis* (WEUS) inhibits RANKL-induced differentiation of murine bone marrow macrophages and RAW264.7 cells into osteoclasts. WEUS inhibited the activation of NF-κB and the expression of nuclear factor of activated T-cells, cytoplasmic 1. In addition, WEUS suppressed the bone resorbing activity of mature osteoclasts without affecting their survival. Furthermore, oral administration of WEUS suppressed RANKL-induced bone loss with a significant amelioration of trabecular bone micro-structures. WEUS also reduced RANKL-induced increase in serum TRAP5b activity and C-terminal cross-linked telopeptide of type I collagen levels.

Conclusion: The present study demonstrates that WEUS has a pharmacological activity that inhibits osteoclast-mediated bone destruction by suppressing osteoclast differentiation and function. These results suggest that *U. sinensis* could be a promising herbal candidate for preventing and treating bone diseases such as osteoporosis.

* *Corresponding author*. Korean Medicine Application Center, Korea Institute of Oriental Medicine, 70 Cheomdan-ro, Dong-gu, Daegu 41062, Republic of Korea.
E-mail address: jyma@kiom.re.kr (J.Y. Ma).
[1] Contributed equally.

https://doi.org/10.1016/j.imr.2017.09.004

1. Introduction

In adult bone remodeling, bone resorption by osteoclasts along with bone formation by osteoblasts regulates bone microstructure by replacing an old bone with a new bone. This is necessary to maintain optimal bone density and integrity.[1,2] Excessive osteoclastic resorption is primarily involved in bone destruction under pathological conditions such as postmenopausal osteoporosis, rheumatoid arthritis, and tumor-induced osteolysis.[3,4] Osteoclasts, multinucleated bone-resorbing cells, are derived from monocyte/macrophage lineage precursor cells. Receptor activator for nuclear factor-κB ligand (RANKL) is a critical cytokine for the differentiation and activation of these cells.[5,6] To develop therapeutic reagents for the treatment of bone diseases caused by deregulated osteoclastogenesis, signaling molecules and transcription factors under RANKL signaling axis for osteoclastogenesis have been extensively investigated.[3,4] Binding of RANKL to its receptor RANK induces the recruitment of adaptor molecules such as TNF receptor-associated factor 6 to the intracellular domain of RANK, which activates various intracellular signaling cascades to induce and activate osteoclastogenic transcription factors including c-Fos and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1).[7–13]

The hooks and stems of *Uncaria sinensis* (US) have been widely used in traditional medicine in East Asian countries to treat cardiovascular and central nervous system ailments such as dizziness, numbness, and hypertension.[14] A number of pharmacological investigations have demonstrated the beneficial neuroprotective effects of US. It has been reported that water extract of US and its phenolic and alkaloid compounds protect glutamate-induced neuronal death by inhibiting Ca^{2+} influx.[15] *In vivo* studies further provided evidences that water extract of US exhibits a protective effect against transient, ischemia-induced, delayed, neuronal death by reducing oxidative damage to neurons in gerbils.[10,16] US hexane extract also exhibits anti-apoptotic properties against glutamate-induced cytotoxicity in cortical neurons[17] and prevents cerebral ischemic damage through an endothelial nitric oxide synthase-dependent mechanism in mice.[18] Although the pharmacological effects of US relevant to its ethnopharmacological uses have been extensively studied, it is less characterized for bone-related pharmacological properties. In this study, we investigated the effect of water extract of the hooks and stems of US (WEUS) on osteoclast differentiation and function *in vitro*. The bone protective effect of WEUS was also assessed in a mouse model of bone loss.

2. Methods

2.1. Reagents

Air-dried US was purchased from Yeongcheon herb (Yeongcheon, Korea). Minimum Essential Medium Alpha modification (α-MEM), Dulbecco Modified Eagle's Medium (DMEM), fetal bovine serum, and penicillin/streptomycin were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Macrophage colony-stimulating factor (M-CSF) and RANKL were obtained as described previously.[19] Antibodies against phospho-c-Jun N-terminal kinase (JNK) 1/2 (Thr183/Tyr185, 9251), JNK (9252), phospho-p38 (Thr180/Tyr182, 9211), p38 (9212), phospho-IκBα (Ser32), and IκBα (4814) were from Cell Singling Technology (Danvers, MA, USA). Antibodies against β-actin (sc-47778), c-Fos (sc-7202), and NFATc1 (sc-7294) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of WEUS

US was extracted by boiling in distilled water and lyophilized as described previously.[20] The lyophilized extract (yield, 9.29%) was suspended in distilled water, centrifuged at 10,000 × *g* for 15 minutes, and filtered through a 0.2 μm sterile filter to prepare WEUS.

2.3. HPLC analysis of WEUS

For HPLC analysis of WEUS, we used a Hitachi LaChrom Elite HPLC system (Hitachi High Technologies Corp., Tokyo, Japan). The chromatographic separation was performed using a Luna C_8 column (4.6 mm × 250 mm, 5 μm), and column temperature was kept at 40°C. The mobile phase was 0.1% TFA in deionized water (A) and acetonitrile (B) with a gradient elution as follows: 0–5 minutes, 10–10% B; 5–55 minutes, 10–50% B; 55–57 minutes, 50–10% B; 57–67 minutes, 10–10%. The flow rate and injection volume were 1 mL/min and 10 μL, respectively. WEUS (100 mg/mL), and a mixture of marker compounds (catechin, caffeic acid, and epicatechin; each 200 μg/mL) were dissolved in methanol and filtered through a 0.2 μm syringe filter before injection for HPLC analysis.

2.4. Cell culture and cytotoxicity assay

Mouse bone marrow-derived macrophages (BMMs) were cultured in α-MEM complete medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in the presence of M-CSF (60 ng/mL) as described in our previous study.[20] BMMs were induced to differentiate into osteoclasts by treatment with RANKL (100 ng/mL). Cell cytotoxicity was determined using Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Rockville, MD, USA) after WEUS treatment for 2 days. To induce the differentiation of RAW264.7 cells (ATCC, Manassas, VA, USA) into osteoclasts, the cells were cultured in α-MEM complete medium with RANKL (100 ng/mL).

2.5. Tartrate-resistant acid phosphatase (TRAP) assay

BMMs were fixed with 10% neutralized formaldehyde, permeabilized with 0.1% Triton X-100, and incubated TRAP assay buffer (50mM sodium tartrate and 0.12M sodium acetate, pH 5.2) containing 1 mg/mL p-nitrophenyl phosphate for 10 minutes at 37°C. The reaction was stopped with 0.1M NaOH, and measured at 405 nm. TRAP-positive multinucleated cells (TRAP (+) MNCs) were visualized by TRAP assay buffer containing 0.1 mg/mL naphthol AS-MX phosphate and 0.5 mg/mL Fast Red violet.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated using an RNA-spin total RNA Extraction Kit (Bioneer Inc., Daejeon, Korea), and RNA concentration was measured with NanoDrop 2.0 spectrophotometer (ThermoFisher Scientific, Pittsburgh, PA, USA). Two micro grams of total RNA was used for cDNA synthesis using AccuPower RT-PreMix (Bioneer Inc.). QPCR analysis was performed by CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using AccuPower GreenStar qPCR Master mix (Bioneer Inc.) and the following primers: c-Fos, 5′-CGGGTTTCAACGCCGACTAC-3′ (forward) and 5′-AAAGTTGGCACTAGAGACGGACAGA-3′ (reverse); NFATc1, 5′-AAGACAGCACTGGAGCAT-3′ (forward) and 5′-TCGGGTGGGAAGTCAGAA-3′ (reverse); hypoxanthine-guanine phosphoribosyltransferase (HPRT), 5′-CCTAAGAT GAGCGCAAGTTG-3′ (forward) and 5′-CCACAGGACTAGAACACCTTGCTAA-3′ (reverse). The PCR cycles were performed as an initial denaturation step (95°C for 5 min) and 40 amplification cycles (94°C for 20 s and at 60°C for 40 s). HPRT-normalized gene expression data were analyzed by $2^{-\Delta\Delta CT}$ method using CFX manager software (Version 3.1).[21]

2.7. Western blot analysis

The cells were washed with cold PBS and lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Roche Applied Science, Indianapolis, IL, USA). Whole cell lysates were vortexed and centrifuged at 20,800 × *g* for 15 minutes. The protein lysates (30 μg) were separated on 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and incubated with specific primary antibodies (1/1000 dilution) and horseradish peroxidase-conjugated secondary antibodies (1/5000 dilution). Chemiluminescent signals on the antibodies-reacted membrane were detected with incubation of Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Rockford, IL, USA). The intensities of the bands on membranes were analyzed using Image Lab software (version 5.2.1, Bio-Rad Laboratories, CA, USA).

2.8. Retroviral gene transduction

Retrovirus packaging and BMM infection by using Plat-E retroviral packaging cells and retroviral vectors pMX-IRES-green fluorescent protein (GFP) and pMX-constitutively active (CA)-NFATc1-IRES-GFP were performed as described previously.[20] BMMs transduced with the retroviruses were cultured in the presence of M-CSF (60 ng/mL) for 1 day and then induced to differentiate into osteoclasts with RANKL (100 ng/mL).

2.9. Nuclear factor-kappa B (NF-κB) luciferase reporter assay

RAW264.7 cells were seeded in a 24-well plate at a density of 2×10^5 cells/well in DMEM complete medium. The next day, cells were transfected with NF-κB luciferase reporter vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Eighteen hours after transfection, the culture medium was replaced with α-MEM complete medium, and cells were pretreated with or without WEUS (80 μg/mL) for 3 hours and further incubated with or without RANKL (100 ng/mL) for 1 day. Cells were washed twice with phosphate-buffered saline (PBS) and then lysed in reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured with a luciferase assay system (Promega).

2.10. Bone resorption assay

Bone resorption assay was performed as previously described.[20] In brief, mature osteoclasts were generated from the co-culture of mouse bone marrow cells and osteoblasts isolated from neonatal mouse calvaria in α-MEM complete medium containing 10nM 1α, 25-dihydroxyvitamin D3 and 100nM prostaglandin E2 for 6 days on collagen gel. Mature osteoclasts were obtained by digesting collagen, replaced on Corning Osteo Assay Surface (Corning Inc., Corning, NY, USA), allowed to settle for 2 hours, and then cultured for 16 hours with vehicle or WEUS (20, 40, and 80 μg/mL) in the presence of RANKL (100 ng/mL). MNCs were stained for TRAP activity, and resorbed pits were photographed after removing cells by bleaching solution. Three randomly selected fields of each well were analyzed by using Image J software.

2.11. RANKL-induced trabecular bone loss model

Animal experiments were carried out in accordance with the KFDA Guide for the Care and Use of Laboratory Animals. Animal experiments (approval number, 12-121 and 14-085) were conducted according to the protocols approved by the Institutional Animal Care and Use of Committee (IACUC) in the Korea Institute of Oriental Medicine. RANKL-induced mouse trabecular bone loss model was studied as reported in previous studies[20,22] with slight modifications. ICR mice (6-week-old male; Samtako Bio Inc., Seoul, Korea) were acclimatized for 1 week and received intraperitoneal injection of RANKL (1 mg/kg of body weight) or PBS (control) on days 0 and 1. WEUS (0.25 g/kg of body weight, $n = 7$) or vehicle (distilled water, $n = 8$) was orally administered twice daily for 5 days beginning on day −2. The mice were euthanized, and the femurs were isolated on day 3. The distal femurs were scanned using a SkyScan 1076 micro-computed tomography (CT) scanner (SkyScan N.V., Kontich, Belgium). Bone parameters including trabecular bone volume per tissue volume (BV/TV), number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp) were analyzed in the distal femoral metaphysis between 0.54 and 1.46 mm distal to the growth plate. Serum TRAP 5b activity was determined using the fluorogenic substrate naphthol AS-BI phosphate (Sigma–Aldrich, St. Louis, MO, USA) as described previously.[20] Serum C-terminal cross-linked telopeptide of type I collagen (CTX) and osteocalcin levels were determined using a RatLaps EIA kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) and a mouse osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA), respectively.

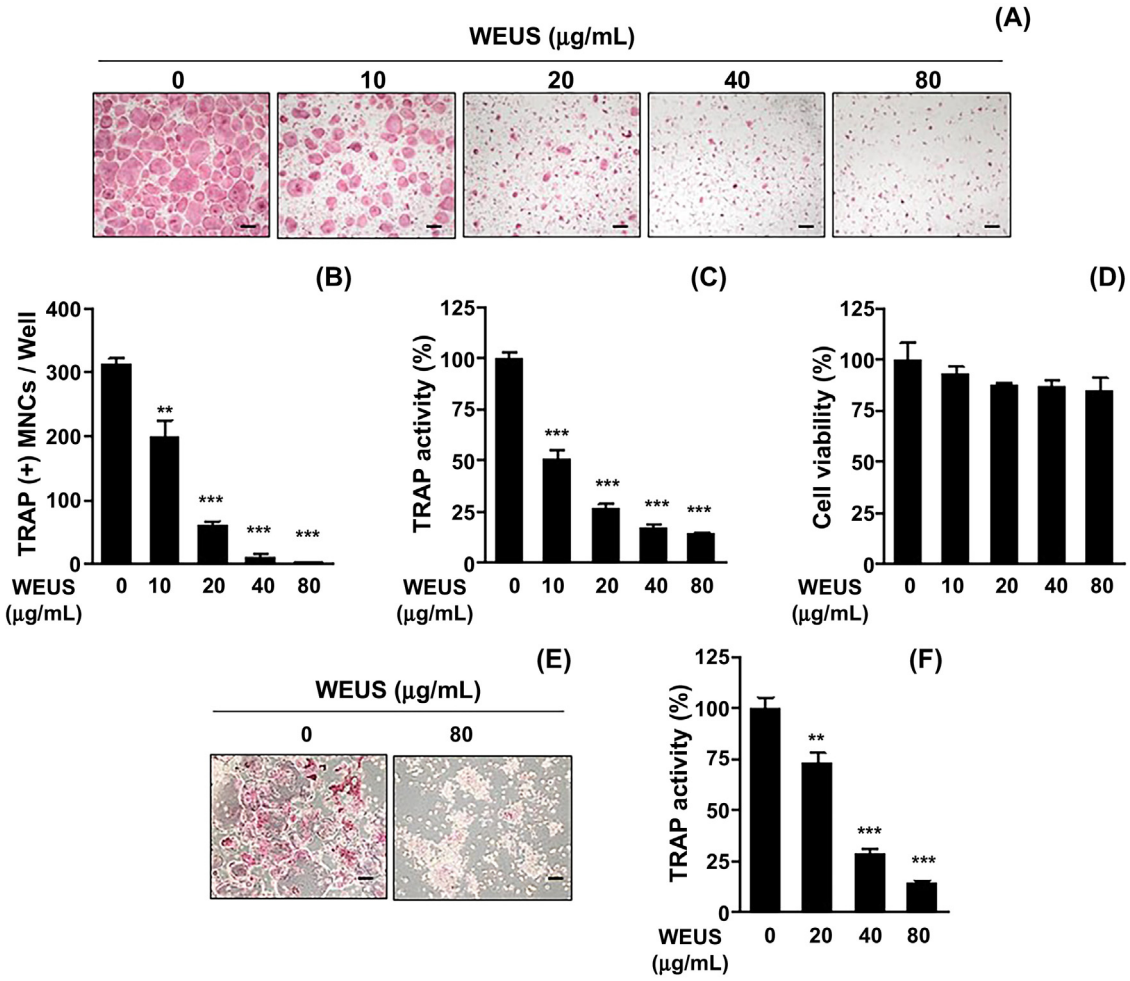

Fig. 1 – WEUS inhibits RANKL-induced osteoclast differentiation. (A–C) BMMs were cultured for 4 days with or without WEUS (10–80 μg/mL) in the presence of M-CSF (60 ng/mL) and RANKL (100 ng/mL). (A) Cells were stained for TRAP activity. Scale bar, 200 μm. (B) The number of TRAP (+) MNCs was counted. $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Total cellular TRAP activity were measured. $^{***}p < 0.001$. (D) Cell viability of BMMs was determined after a 2-day incubation with WEUS in the presence of M-CSF. (E and F) RAW264.7 cells were cultured with or without WEUS (20–80 μg/mL) in the presence of RANKL for 4 days. Cells were subjected to TRAP staining (E) and activity (F). Scale bar, 200 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

2.12. *Statistical analysis*

Values are presented as mean ± SD in *in vitro* experiments and mean ± SEM in *in vivo* experiments. Two-group comparisons were performed with Student *t* tests, while multiple-group comparisons were performed with one-way analysis of variance followed by Dunnett test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. *WEUS inhibits osteoclast differentiation*

We first examined the effect of WEUS on osteoclast differentiation. In the presence of M-CSF, RANKL induced differentiation of BMMs into osteoclasts within 4 days, and WEUS markedly inhibited this process in a dose-dependent manner (Fig. 1A). Consistent with these morphological changes, WEUS decreased the number of TRAP (+) MNCs and total TRAP activity (Fig. 1B and C). WEUS exhibited no significant cytotoxic effect on BMMs at concentrations up to 80 μg/mL (Fig. 1D). We next assessed the effect of WEUS on osteoclast differentiation using RAW264.7 cells, which differentiate into osteoclasts in the presence of RANKL through a mechanism independent of M-CSF. WEUS also exhibited an inhibitory effect on osteoclast differentiation and TRAP activity in RAW264.7 cells (Fig. 1E and F). These findings suggest that WEUS could inhibit osteoclast differentiation by targeting RANKL signaling in osteoclast precursor cells.

3.2. *WEUS downregulates NFATc1 expression*

To determine whether inhibition of osteoclast differentiation by WEUS could result from its inhibitory effect on the expression of c-Fos and NFATc1, which are key transcription factors in RANKL signaling axis for osteoclastogenesis,[11,12] we evaluated mRNA and protein levels of c-Fos and NFATc1. Time

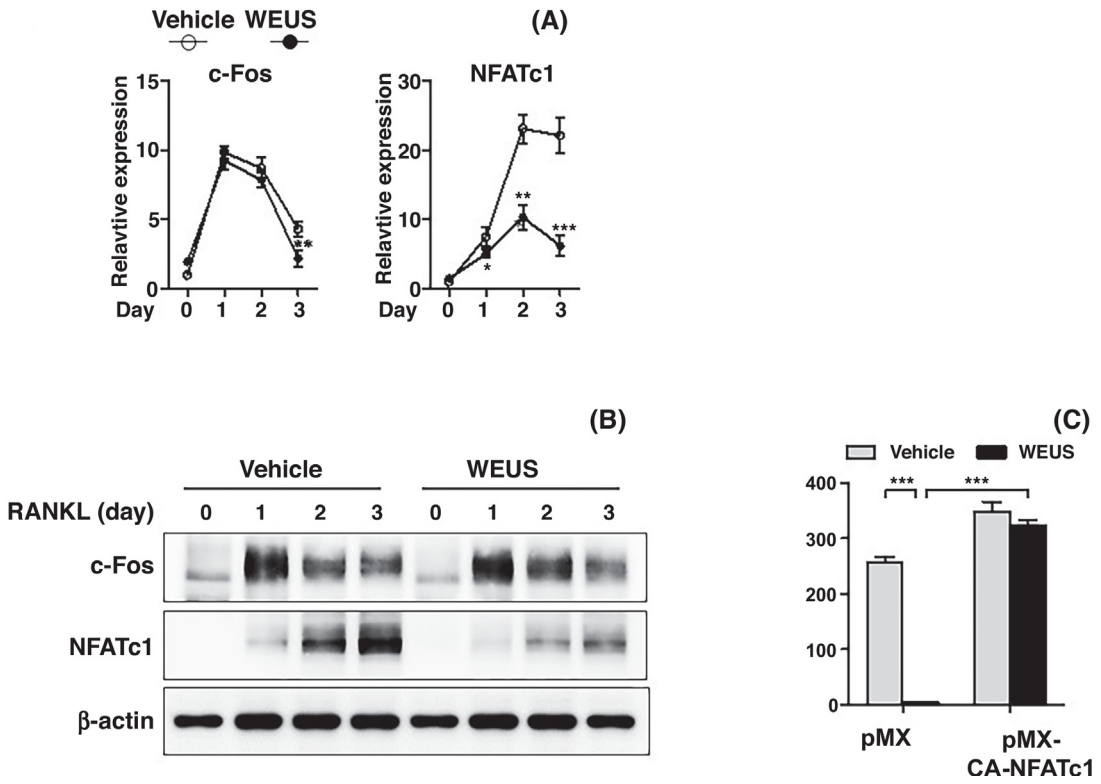

Fig. 2 – WEUS inhibits RANKL-induced NFATc1 expression. (A and B) BMMs were cultured with vehicle (distilled water) or WEUS (80 μg/mL) for the indicated days during RANKL-induced osteoclast differentiation. (A) Relative mRNA expression levels of c-Fos and NFATcl were determined by qPCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) c-Fos and NFATc1 protein levels were determined by Western blot analysis. (C) BMMs transduced with pMX-IRES-GFP (pMX) or pMX-CA-NFATc1-IRES-GFP (pMX-CA-NFATc1) were cultured for 4 days with vehicle or WEUS in the presence of M-CSF and RANKL. The number of TRAP (+) MNCs was counted. ***$p < 0.001$.

course analysis showed that in vehicle-treated cells, c-Fos and NFATc1 levels increased, peaking on days 1 and 2, respectively (Fig. 2A and B). c-Fos mRNA and protein levels were similar in both WEUS- and vehicle-treated cells, whereas NFATc1 levels were markedly diminished by WEUS on days 2 and 3. To examine whether NFATc1 is mainly involved in the inhibitory effect of WEUS on osteoclast differentiation, a constitutively active form of NFATc1 were expressed in BMMs by retroviral gene transduction. Ectopic expression of a constitutively active form of NFATc1 restored the inhibitory effect of WEUS on osteoclast differentiation (Fig. 2C).

3.3. *WEUS inhibits RANKL-induced NF-κB activation*

JNK and p38 mitogen-activated kinases and NF-κB signaling pathways have been shown to mediate RANKL-induced osteoclastogenic gene expression and osteoclast differentiation.[9,13,23] To gain more insight into the mechanism of action of WEUS on NFATc1 expression and osteoclast differentiation, we explored the activation of JNK, p38, and NF-κB. WEUS did not affect RANKL-induced activation of JNK and p38, which were determined by their site-specific phosphorylation statues (Fig. 3A). The NF-κB inhibitory protein IκBα phosphorylation and its degradation induced by RANKL were not altered by WEUS. However, we found that WEUS abrogates RANKL-induced NF-κB transcriptional activity (Fig. 3B).

3.4. *WEUS suppresses bone resorbing function of osteoclasts*

Having found that the inhibitory action of WEUS on RANKL-induced osteoclast differentiation, we next investigate whether WEUS affects bone resorbing activity of mature osteoclasts. When mature osteoclasts were cultured on bone-like mineral surface for 16 hours, resorption pits by osteoclasts were formed. Treatment of mature osteoclasts with WEUS decreased the total resorbed area, while it did not affect the number of mature osteoclasts (Fig. 4A–C).

3.5. *WEUS suppresses RANKL-induced trabecular bone loss*

Because WEUS exhibited an inhibitory action on osteoclast differentiation and its bone resorbing function, we next examined the effect of WEUS on bone destruction using a RANKL-induced trabecular bone loss mouse model.[22] Intraperitoneal injections of RANKL markedly induced trabecular bone loss in the distal femoral metaphysis compared to control, whereas the administration of WEUS obviously suppressed RANKL-induced bone destruction (Fig. 5A). Micro-CT

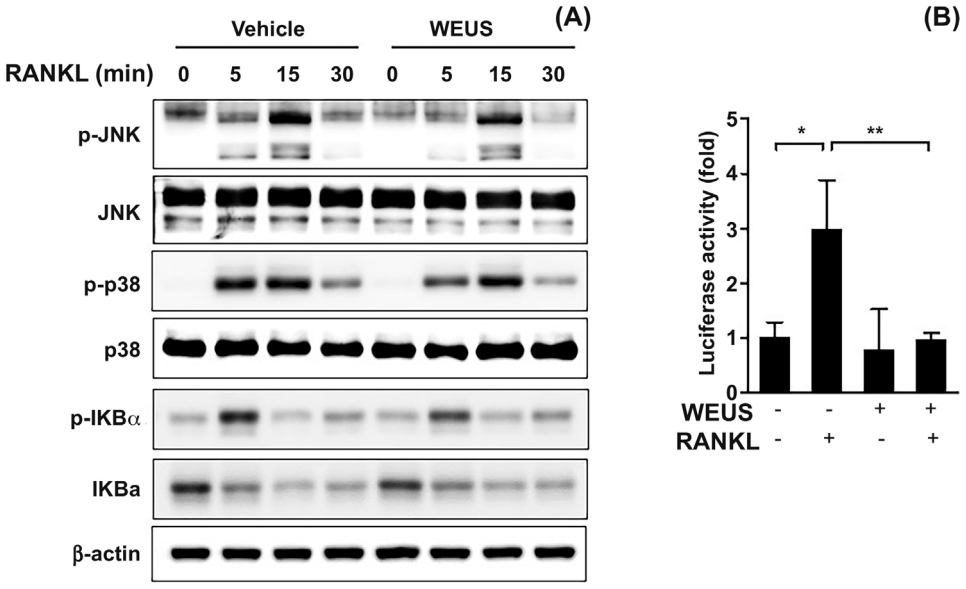

Fig. 3 – WEUS inhibits RANKL-induced NF-κB activation. (A) BMMs pretreated with or without WEUS (80 μg/mL) for 3 h were stimulated with RANKL (100 ng/mL) for 0, 5, 15, and 30 min. Western blotting was carried out with the indicated antibodies. (B) Raw 264.7 cells transfected with NF-κB luciferase reporter vector were pretreated with or without WEUS (80 μg/mL) for 3 h and then stimulated with RANKL (100 ng/mL). Luciferase activity was assessed 24 h later. $^{*}p < 0.05$, $^{**}p < 0.01$.

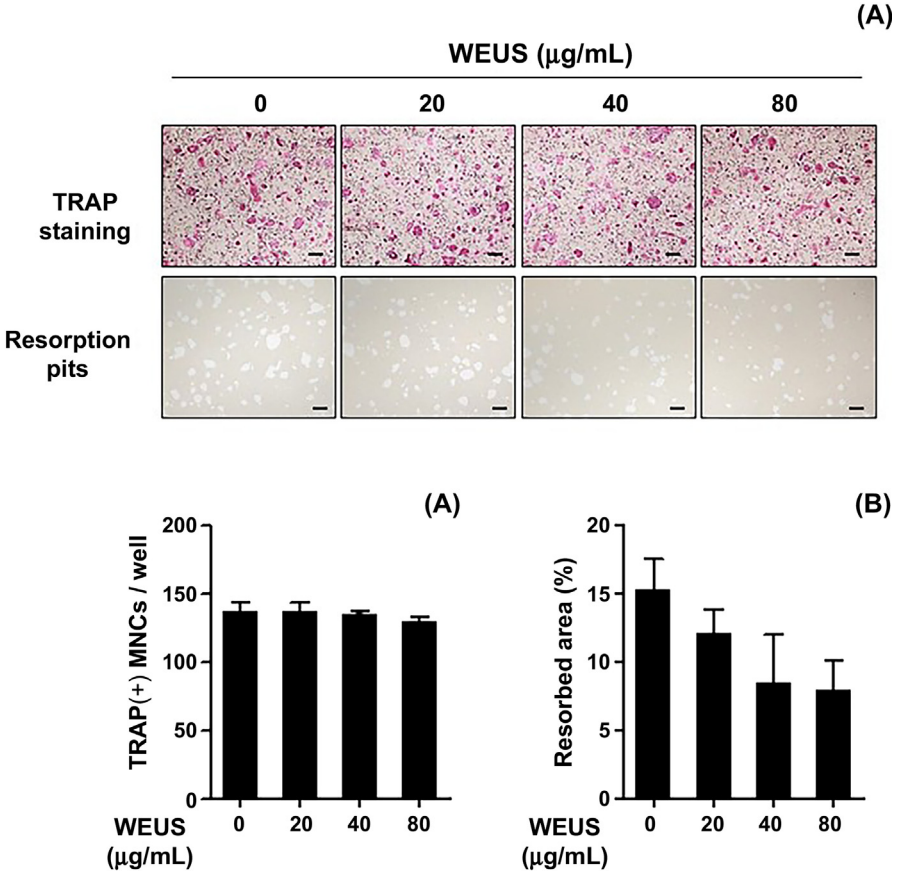

Fig. 4 – WEUS inhibits bone resorbing activity of mature osteoclasts. Mature osteoclasts were seeded on a plate with bone-like mineral surface and cultured for 16 h with or without WEUS (80 μg/mL) in the presence of RANKL (100 ng/mL). (A) Cells were stained for TRAP, and resorption pits were photographed. Scale bar, 200 μm. (B) The number of TRAP (+) MNCs. (C) The percentage of resorbed areas. $^{*}p < 0.05$.

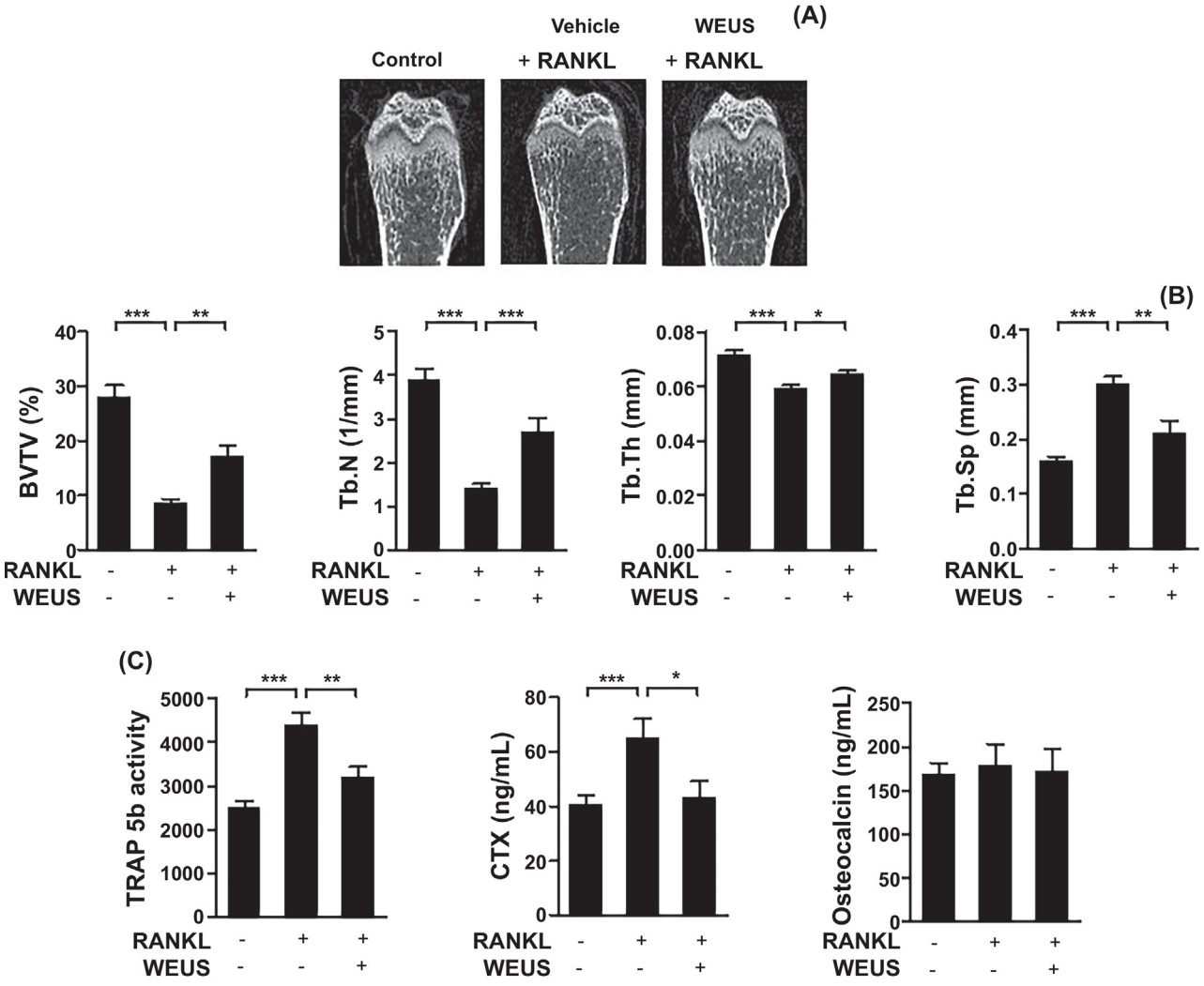

Fig. 5 – WESU suppresses RANKL-induced bone loss in mice. Mice were orally administered vehicle or WEUS (0.25 g/kg) twice daily and received intraperitoneal injections of RANKL (1 mg/kg) as described in Section 2. Sera and femurs were obtained on day 3 after the first RANKL injection. (A) Representative micro-CT images of the distal femoral metaphysis from control, vehicle-treated, and WEUS-treated mice. (B) The bone volume/trabecular volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). (C) Serum TRAP5b activity, CTX levels, and osteocalcin levels. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

analysis further revealed osteoporotic bone microarchitecture in RANKL-injected mice, characterized by a 70% decrease in BV/TV, 64% decrease in Tb.N, 19% decrease in Tb.Th, and 88% increase in Tb.Sp (Fig. 5B). Administration of WEUS significantly ameliorated RANKL-induced changes in trabecular bone microarchitecture by increasing BV/TV by 42%, Tb.N by 67%, and Tb.Th by 35%, which consequently decreased Tb.Sp. In accordance with these results, RANKL injections increased serum TRAP5b activity and CTX levels (Fig. 5C), which are markers of osteoclast number and bone resorption, respectively. These increases were greatly suppressed by WEUS. In contrast, neither RANKL nor WEUS affected serum osteocalcin levels, a maker of bone formation.

4. Discussion

Modulation of osteoclast differentiation and/or its bone resorbing function can be a potent therapeutic target for inhibition of pathological bone destruction. Our results demonstrate that WEUS inhibits bone destruction by suppressing both osteoclast differentiation and bone resorbing activity of mature osteoclasts.

Previous phytochemical studies have shown that US contains flavonoids and alkaloids including catechin, caffeic acid, epicatechin, rhynchophylline, and corynoxein.[15,24,25] In line with this, we identified the presence of catechin, caffeic acid, and epicatechin in WEUS, based on their retention times and UV absorption spectra in high performance liquid chromatography analysis (Supplementary Fig. 1). We found that

the phenolics (catechin, caffeic acid, and epicatechin) up to 80 μM does affect RANKL-induced osteoclast differentiation of BMMs (data not shown). Further studies are needed to identify the main active constituents contributing to the anti-osteoclastogenic effect of WEUS.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imr.2017.09.004.

Fig. 5 – Supplementary Fig. 1 HPLC chromatograms of WEUS and a standard mixture of catechin, caffeic acid, and epicatechin.

WEUS inhibited RANKL-induced expression of NFATc1, a master transcription factor for osteoclast differentiation.[11] Ectopic expression of a constitutively active form of NFATc1 in osteoclast precursors restored the inhibitory effect of WEUS on osteoclast differentiation, suggesting that the anti-osteoclastogenic effect of WEUS are attributed to inhibition of NFATc1 expression. RANKL-induced NFATc1 expression requires induction and activation of other transcription factors. The classical NF-κB p65 and p50 subunits are recruited to the NFATc1 promoter in response to RANKL, which induces the initial induction of NFATc1 in cooperation with NFATc1.[23] NFATc1 activated by calcium signals then binds to its own promoter in cooperation with activator protein-1 activated by the induction of c-Fos, leading to induce auto-amplification of NFATc1.[11,23] In the present study, WEUS did not affect RANKL-induced c-Fos expression. We found that WEUS inhibits RANKL-induced NF-κB transcriptional activity without affecting IκBα phosphorylation and degradation. The transcriptional activity of NF-κB can be regulated through multiple post-translational modifications of the components of NF-κB signaling including the NF-κB subunits themselves.[26] In osteoclast precursors, RANKL has been shown to stimulate p65 phosphorylation at Ser536 via a TAK1-MKK6-p38 signaling pathway.[27] However, the role of the phosphorylation of p65at Ser536 in RANKL-induced NF-κB transcriptional activity and NFATc1 induction has not been investigated. Although the molecular mechanism underlying inhibition of RANKL-induced NF-κB transcriptional activity by WEUS remains to be elucidated, our results suggest that inhibition of NF-κB activation by WEUS may contribute to suppression of NFATc1 induction, subsequently inhibiting osteoclast differentiation.

Apart from its role in osteoclast differentiation, the classical NF-κB signaling is also involved in osteoclast activation required for bone resorption. Selective inhibition of NF-κB with by the NEMO-binding domain peptide, which blocks association of NEMO and IKK complex, has been shown to reduce bone resorbing activity of osteoclasts without affecting their survival.[28] It was also reported that the inhibition of NF-κB pathway in osteoclasts by ectopic expression of a dominant-negative form of IKK beta reduces their bone resorbing activity, while overexpression of a constitutively active form of IKK beta increases the activity. Modulation of NF-κB activity by expression of these IKK beta mutants did not affect the survival of osteoclasts.[29] In the present study, we found that WEUS inhibits bone resorbing activity of mature osteoclasts without affecting their survival. Thus, these suggest that the inhibition of NF-κB activation by WEUS might contribute to its inhibitory effect on bone resorbing activity of mature osteoclasts.

Intraperitoneal injections of RANKL into mice induce rapid loss of trabecular bone by stimulating both the differentiation of osteoclast progenitors and the activation of pre-existing osteoclasts.[22] In the present study, two injections of RANKL at a 24-hour interval induced marked trabecular bone destruction and elevated serum TRAP5b activity and CTX levels on day 3 after the first RANKL injection, indicating increased osteoclastic bone resorption. Consistent with the *in vitro* inhibitory effects of WEUS on osteoclast differentiation and activation, the administration of WEUS significantly suppressed RANKL-induced bone destruction and elevation of osteoclast numbers and bone resorption. It has been reported that the elevated bone resorption by RANKL injections at 24-hour intervals for 3 days induces an increase in bone formation, determined by serum alkaline phosphatase levels.[22] However, we did not find a significant change in serum osteocalcin levels by RANKL and WEUS. Therefore, it is likely that the protective effect of WEUS against RANKL-induced bone destruction is mainly due to suppression of bone resorption by inhibition of osteoclast differentiation and activation. Although WEUS did not affect serum osteocalcin levels in the present study, the long-term effects of WEUS on bone formation and remodeling remain to be further studied.

In conclusion, the present study demonstrates for the first time that WEUS has a protective effect against bone destruction by inhibiting osteoclast differentiation and its bone resorbing activity. These findings suggest that WEUS may be useful for the treatment of bone diseases characterized by excessive bone resorption.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by a grant K17281 for the Korea Institute of Oriental Medicine from Ministry of Science, ICT and Future Planning, Korea. We thank to Chung-Jo Lee for technical support.

REFERENCES

1. Eriksen EF. Cellular mechanisms of bone remodeling. *Rev Endocr Metab Disord* 2010;11:219–27.
2. Seeman E, Delmas PD. Bone quality – the material and structural basis of bone strength and fragility. *N Engl J Med* 2006;354:2250–61.
3. Negishi-Koga T, Takayanagi H. Ca^{2+}-NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunol Rev* 2009;231:241–56.
4. Tanaka S, Nakamura K, Takahasi N, Suda T. Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system. *Immunol Rev* 2005;208:30–49.

5. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999;397:315–23.
6. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165–76.
7. Gohda J, Akiyama T, Koga T, Takayanagi H, Tanaka S, Inoue J. RANK-mediated amplification of TRAF6 signaling leads to NFATc1 induction during osteoclastogenesis. *EMBO J* 2005;24:790–9.
8. Walsh MC, Kim GK, Maurizio PL, Molnar EE, Choi Y. TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. *PLoS One* 2008;3:e4064.
9. Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, et al. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J Clin Invest* 2004;114:475–84.
10. Yokoyama K, Shimada Y, Hori E, Nakagawa T, Takagi S, Sekiya N, et al. Effects of Choto-san and hooks and stems of *Uncaria sinensis* on antioxidant enzyme activities in the gerbil brain after transient forebrain ischemia. *J Ethnopharmacol* 2004;95:335–43.
11. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 2002;3:889–901.
12. Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ, et al. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J Biol Chem* 2004;279:26475–80.
13. Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y, Kim HH. Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. *Biochem Biophys Res Commun* 2006;351:99–105.
14. Heitzman ME, Neto CC, Winiarz E, Vaisberg AJ, Hammond GB. Ethnobotany, phytochemistry and pharmacology of Uncaria (Rubiaceae). *Phytochemistry* 2005;66:5–29.
15. Shimada Y, Goto H, Kogure T, Shibahara N, Sakakibara I, Sasaki H, et al. Protective effect of phenolic compounds isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death. *Am J Chin Med* 2001;29:173–80.
16. Yokoyama K, Shimada Y, Hori E, Sekiya N, Goto H, Sakakibara I, et al. Protective effects of Choto-san and hooks and stems of *Uncaria sinensis* against delayed neuronal death after transient forebrain ischemia in gerbil. *Phytomed: Int J Phytother Phytopharmacol* 2004;11:478–89.
17. Jang JY, Kim HN, Kim YR, Hong JW, Choi YW, Choi YH, et al. Hexane extract from *Uncaria sinensis* exhibits anti-apoptotic properties against glutamate-induced neurotoxicity in primary cultured cortical neurons. *Int J Mol Med* 2012;30:1465–72.
18. Park SH, Kim JH, Park SJ, Bae SS, Choi YW, Hong JW, et al. Protective effect of hexane extracts of *Uncaria sinensis* against photothrombotic ischemic injury in mice. *J Ethnopharmacol* 2011;138:774–9.
19. Ha H, An H, Shim KS, Kim T, Lee KJ, Hwang YH, et al. Ethanol extract of *Atractylodes macrocephala* protects bone loss by inhibiting osteoclast differentiation. *Molecules* 2013;18:7376–88.
20. Ha H, Shim KS, Kim T, Lee CJ, Park JH, Kim HS, et al. Water extract of the fruits of *Alpinia oxyphylla* inhibits osteoclast differentiation and bone loss. *BMC Complement Altern Med* 2014;14:352.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* 2001;25:402–8.
22. Tomimori Y, Mori K, Koide M, Nakamichi Y, Ninomiya T, Udagawa N, et al. Evaluation of pharmaceuticals with a novel 50-hour animal model of bone loss. *J Bone Miner Res* 2009;24:1194–205.
23. Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H, et al. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. *J Exp Med* 2005;202:1261–9.
24. Shimada Y, Goto H, Itoh T, Sakakibara I, Kubo M, Sasaki H, et al. Evaluation of the protective effects of alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death in cultured cerebellar granule cells from rats. *J Pharm Pharmacol* 1999;51:715–22.
25. Tan SN, Yong JW, Teo CC, Ge L, Chan YW, Hew CS. Determination of metabolites in *Uncaria sinensis* by HPLC and GC-MS after green solvent microwave-assisted extraction. *Talanta* 2011;83:891–8.
26. Perkins ND. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* 2006;25:6717–30.
27. Huang H, Ryu J, Ha J, Chang EJ, Kim HJ, Kim HM, et al. Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-kappaB transactivation by RANKL. *Cell Death Differ* 2006;13:1879–91.
28. Soysa NS, Alles N, Shimokawa H, Jimi E, Aoki K, Ohya K. Inhibition of the classical NF-kappaB pathway prevents osteoclast bone-resorbing activity. *J Bone Miner Metabol* 2009;27:131–9.
29. Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, et al. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. *J Cell Biol* 2000;148:333–42.