*Original Article*

# Resolvin E1 Inhibits Osteoclastogenesis and Bone Resorption by Suppressing IL-17-induced RANKL Expression in Osteoblasts and RANKL-induced Osteoclast Differentiation

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## ABSTRACT

**Background**　Resolvin E1 (RvE1) derived from the ω-3 polyunsaturated fatty acid eicosapentaenoic acid is known to be a potent pro-resolving lipid mediator that prevents chronic inflammation and osteoclastogenesis. We investigated the inhibitory effects of RvE1 on osteoclastogenesis and bone resorption to clarify its therapeutic potential for rheumatoid arthritis (RA).

**Methods**　Receptor activator of nuclear factor-ϰB ligand (RANKL)-induced osteoclast differentiation was assessed with tartrate-resistant acid phosphatase staining. RANKL-induced bone resorption was assessed by the measurement of pit formation using calcium phosphate-labeled fluorescent polyanionic molecules in RAW264.7 cells as osteoclast precursors. The effects of RvE1 on the RANKL-induced mRNA expression of osteoclast-specific genes and transcriptional factors such as c-fos and nuclear factor of activated T cells c1 (NFATc1) in RAW264.7 cells were measured by quantitative real-time PCR. The distribution of NFATc1 induced by RANKL was evaluated by immunofluorescence staining in RAW264.7 cells. To analyze the mechanism of the inhibitory effect of RvE1 on osteoclastogenesis, we measured IL-17-induced RANKL mRNA expression in MC3T3-E1 osteoblast cells treated with RvE1 using quantitative real-time PCR and determined the level of prostaglandin $E_2$ ($PGE_2$) production by enzyme-linked immunosorbent assay.

**Results**　RvE1 significantly suppressed RANKL-induced osteoclast differentiation and bone resorption. RvE1 inhibited the RANKL-induced mRNA expression of osteoclast-specific genes along with the transcription factors NFATc1 and c-fos. Moreover, NFATc1 translocation from the cytoplasm to the nucleus of RAW264.7 cells was suppressed following RvE1 treatment. RvE1 also inhibited IL-17-induced RANKL mRNA expression and $PGE_2$ production in MC3T3-E1 cells.

**Conclusion**　RvE1 inhibited osteoclastogenesis and bone resorption by suppressing RANKL-induced NFATc1 and c-fos expression in osteoclasts and IL-17-induced RANKL expression through the autocrine action of $PGE_2$ in osteoblasts. Our data suggest RvE1 as a new therapeutic target of RA.

The bone undergoes continuous remodeling to achieve a balance between bone formation and resorption mediated by osteoblasts and osteoclasts, respectively. Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by progressive synovial inflammation and destruction of the joint cartilage and bone.[1] In particular, RA involves a breakdown of metabolic balance in the bone, and is characterized by an increase in bone resorption that results in impaired bone formation.[2] This imbalance is caused by the increase of various inflammatory cytokines, including receptor activator of nuclear factor-ϰB ligand (RANKL) and its competitive inhibitor osteoprotegerin (OPG), in the inflammatory tissue.[3, 4] Thus, anti-inflammatory drugs are most commonly prescribed to treat the symptoms of RA. However, recent evidence points to the beneficial effects of natural compounds on reducing inflammatory cascades, providing new options for RA treatment.

For example, a diet enriched in ω-3 polyunsaturated fatty acids (PUFAs) was found to reduce joint stiffness in the morning and the number of tender joints,[5] and also decreased the levels of inflammatory cytokines in the blood, including tumor necrosis factor-alpha (TNF-α),

interleukin (IL)-1β, and IL-6.[6, 7] Indeed, several randomized clinical studies have revealed that dietary supplementation with ω-3 PUFAs is efficacious in reducing joint pain, the duration of morning stiffness, the number of tender or swollen joints and non-steroidal anti-inflammatory drug usage in RA patients.[8, 9] However, the mechanisms of these clinical effects of ω-3 PUFAs have remained unclear. Recent discoveries demonstrate that ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be enzymatically converted in vivo to novel bioactive lipid mediators termed specialized pro-resolving mediators, including resolvins, protectins and maresins, which promote the resolution of inflammation and have more potent effects than their lipid precursors.[10]

One of the specialized pro-resolving mediators derived from EPA, resolvin E1 (5S, 12R, 18R-trihydroxy-6Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid; RvE1), was originally identified in exudates of the murine dorsal air pouch, an acute inflammation model.[11] Recent findings demonstrated that RvE1 had protective effects in periodontal disease,[12] peritonitis,[13] asthmatic airway inflammation,[14] bacterial pneumonia and acute lung injury in vivo.[15] Accordingly, we speculated that the same bone-protective effects noted in periodontal disease might be applicable to RA. There have been only a few reports on the regulation of RvE1 for bone resorption and osteoclast differentiation,[16] and the effects and mechanism of RvE1 on bone remodeling are still not fully understood. Therefore, in this study, we originally investigated the effects of RvE1 on the inflammation and signaling pathways in osteoclasts and osteoblasts to provide a foundation for RvE1 as a new therapeutic approach in RA treatment. Our results are the first to indicate that RvE1 interacts with osteoclasts and osteoblasts via inhibition of RANKL production in osteoblasts.

RANKL is a member of the TNF superfamily of cytokines and is known to induce osteoclastogenesis from monocytes or macrophages. The binding of RANKL to its receptor RANK, expressed on osteoclast precursors, induces the expression and/or activation of transcription factors, including nuclear factor of activated T cells c1 (NFATc1) and c-fos, which have been shown to be essential for osteoclast differentiation.[17, 18]

Pro-inflammatory molecules play a significant, but primarily indirect, role in osteoclast regulation as they act through modulating RANKL and OPG.[19] TNFα, IL-1, IL-6, IL-11 and IL-17 act on osteoblasts and bone marrow stromal cells to increase RANKL and/or decrease OPG expression in osteoblasts.[20, 21] IL-17 is a pro-inflammatory cytokine induced by a subset of T helper 17 cells.[3, 22] In osteoblasts, RANKL is induced by prostaglandin E2 (PGE$_2$), which is in turn strongly induced by IL-17; however, this PGE$_2$-induced upregulation of RANKL expression could be inhibited by NS398, a selective cyclooxygenase-2 (COX-2) inhibitor.[3, 23] Thus, IL-17 indirectly induces osteoclastogenesis via PGE$_2$-induced RANKL expression in osteoblasts and has been implicated in the promotion of the pathogenesis of RA. Based on this background, in this study, we focused on the potential effects of RvE1 on mediating IL-17-induced osteoclastogenesis to alleviate the inflammation associated with RA. We further examined the potential mechanism underlying the effects of RANKL, IL-17 and/or RvE1 in RAW264.7 and mouse MC3T3-E1 cells as osteoclasts and osteoblasts, respectively. We examined the effect on osteoclastogenesis by determining the expression of matrix metalloproteinase-9 (MMP-9) and cathepsin K, which are highly expressed in osteoclastic cells and considered as markers of mature osteoclasts that play important roles in osteolysis.[24] PGE$_2$ production tends to be induced by endogenous COX-2 and microsomal PGE synthase-1 (mPGES-1). Therefore, the COX-2 and mPGES-1 mRNA expression levels were examined with real-time PCR, and the production level of PGE$_2$ was examined with an enzyme-linked immunosorbent assay (ELISA) in MC3T3-E1 cells to understand the mechanism of the inhibitory effects of RvE1 on osteoclastogenesis. Osteoblasts are involved in osteoclast regulation by expressing RANKL on their membranes or releasing it as a soluble factor.[2] We therefore further verified the mRNA expression of RANKL and its competitive inhibitor OPG in MC3T3-E1 cells by real-time PCR. Together, these in vitro results could provide a foundation for the clinical application of RvE1 in maintaining the balance of bone metabolism and in the treatment or prevention of RA.

## MATERIALS AND METHODS
### Cell culture and reagents
RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA) and were used as osteoclastic cells. Mouse calvarial cells (MC3T3-E1) were purchased from the RIKEN BioResource Center (Tsukuba, Japan) and were used as osteoblastic cells. RAW264.7 cells were cultured in Dulbecco's minimal essential medium (D-MEM; Wako Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum (FBS), 100 µg/mL penicillin and 100 µg/mL streptomycin. MC3T3-E1 cells were cultured in α-MEM (Wako Pure Chemicals) containing 10% FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin. Both cell lines were cultured at 37 °C in a humidified 5% CO$_2$ atmosphere. RAW264.7 cells were cultured with or without

**Table 1. Primers for real-time PCR**

| Genes | Assay ID[a] | RefSeq | Exon Boundary | Product length (bp) |
|---|---|---|---|---|
| NFATc1 | Mm00479445_m1 | NM_00116409.1 | 7-8 | 75 |
| c-fos | Mm00487425_m1 | NM_010234.2 | 1-2 | 59 |
| Cathepsin K | Mm00484039_m1 | NM_007802.4 | 7-8 | 73 |
| MMP-9 | Mm00442991_m1 | NM_013599.3 | 5-6 | 80 |
| RANKL | Mm00441906_m1 | NM_011613.3 | 2-3 | 66 |
| OPG | Mm00435454_m1 | NM_008764.3 | 4-5 | 71 |
| COX-2 | Mm00478374_m1 | NM_011198.3 | 5-6 | 80 |
| mPGES-1 | Mm00452105_m1 | NM_022415.3 | 2-3 | 87 |
| GAPDH | Mm99999915_m1 | NM_001289726.1 | 2-3 | 107 |

COX-2, Cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-9, matrix metalloproteinase-9; mPGES-1, microsomal prostaglandin E synthetase-1; NFATc1, nuclear factor of activated T cells c1; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor ϰB ligand. a: TaqMan Gene Expression Assay (Applied Biosystems)

soluble RANKL (Oriental Yeast, Tokyo, Japan) and RvE1 (Toronto Research Chemicals, Toronto, Canada). MC3T3-E1 cells were cultured with or without IL-17 (Pepro Tech, Rocky Hill, NJ) and RvE1.

### Cell proliferation assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). RAW264.7 and MC3T3-E1 cells were seeded in 96-well plates at $5.0 \times 10^3$ cells/well in D-MEM or MEM-α each containing 10% FBS. Following incubation for 24 h, the cells were treated with 0, 50, 100 or 200 nM RvE1. After 1 h, the cells were treated with 100 ng/mL RANKL (RAW 264.7 cells) or 50 ng/mL IL-17 (MC3T3-E1 cells). After 24 h, 10 µL CCK-8 solution was added to the culture and the cells were further incubated in the dark at 37 °C for 2 h. The plate was then read using a Sunrise microplate analyzer (Tecan, Mannedorf, Switzerland) at 450 nm with a reference at 600 nm. The number of surviving cells was quantified by measuring the absorbance at this wavelength.

### Tartrate-resistant acid phosphatase (TRAP) staining

RAW264.7 cells were plated in 24-well microplates at a density of $1.0 \times 10^4$ cells/mL and left overnight to settle. Conditioned medium containing 50 or 100 ng/mL soluble RANKL was then added to the cells and cultured for 6 days. On day 7 of culture, the cells were fixed and stained using a TRAP staining kit (Wako Pure Chemicals) according to the manufacturer's instructions. The number of osteoclast-like cells per well was then counted (TRAP-positive cells with more than three nuclei). Each experiment was performed in duplicate.

### Quantitative real-time PCR

Total RNA was isolated from cultured cells using the RNeasy Plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The mRNA was reverse-transcribed into cDNA using the Super Script VILO Master Mix (Invitrogen, Carlsbad, CA) and the resultant cDNA was subjected to real-time PCR using the TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA). Specific primers (Table 1) were purchased from Applied Biosystems (Foster City, CA). PCR was performed using the TaKa-Ra PCR Thermal Cycler Dice system (Takara Bio, Kusatsu, Japan) under the following conditions: initial holding at 25 °C for 10 min and then 42.0 °C for 60 min and finally 85.0 °C for 5 min. Real-time PCR was performed on a ViiA7 Real-Time PCR system (Applied Biosystems) for 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The expression levels of NFATc1, c-fos, cathepsin K, MMP-9, RANKL, OPG, COX-2 and mPGES-1 were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All real-time PCR experiments were performed in triplicated and analyzed by the comparative 2-ΔΔCt relative quantification method.

### Immunofluorescence staining

RAW264.7 cells were cultured in glass chamber slides (Lab-Tek II Chamber Slide w/Cover RS Glass Slide Sterile; Nalge Nunc) for 24 h with or without RANKL plus RvE1. The glass chamber slides were then removed, washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% TritonX-100 for 1 h, incubated with bovine serum albumin for 1 h to block non-specific binding and then incubated with mouse NFATc1 monoclonal antibody (diluted 1:50; Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. The cells were washed again in PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (diluted l:2000; Life Technologies, Carlsbad, CA) for 1 h. The nuclei of cells were stained using the blue fluorescent dye 4',6-diamidino-2-phenylindole in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were obtained with a confocal microscope (TCS-SP2 confocal microscope; Leica, Wetzlar, Germany).
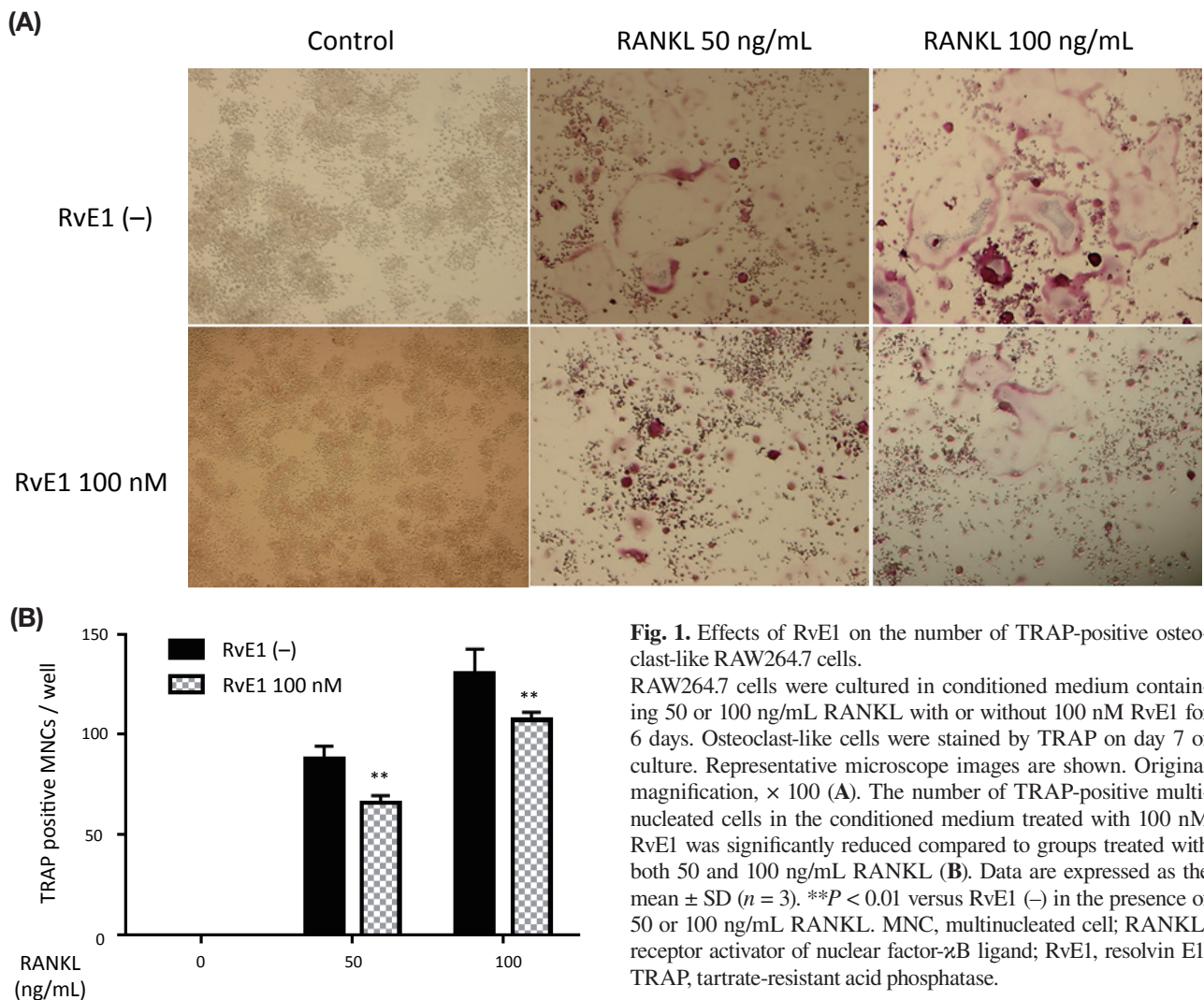
### Bone resorption assay

The bone resorption assay was performed using Bone

Resorption Assay Kit 24 (PG Research, Kodaira, Japan).[25] First, 0.5 mL fluorescein amine-labeled chondroitin polysulfate (Bone Resorption Assay FACS) was added to each well of a calcium phosphate-coated 24-well plate (Bone Resorption Assay 24) and incubated at 37 °C in a humidified 5% $CO_2$ atmosphere for 2 h under a light-shielded condition. After the incubation, each well of the 24-well plate was washed with 1 mL PBS twice and then 1 mL MEM-α without phenol red (Wako Pure Chemicals) containing 10% FBS was added. The RAW264.7 cells were inoculated into each well at a density of $1.0 \times 10^4$ cells/mL and allowed to attach for 4 h before being treated with 50 or 100 nM RvE1 for 1 h, followed by incubation with 10–200 ng/mL RANKL for 6 days without a medium change. RvE1 and RANKL were added at the same dose after 3 days. After 6 days, 100 μL of the culture supernatant from each well was harvested into a 96-well plate and mixed with 50 μL of 0.1 N NaOH (Bone Resorption Assay Buffer). The flu-

orescence intensity of the culture supernatant was measured using a fluorescence plate reader (Infinite F500, Tecan, Mannedorf, Switzerland) with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The remaining plates were washed with PBS and treated with 5% sodium hypochlorite for 5 min. After washing the plates with water and drying them, the pit area was photographed by a fluorescence microscope (BZ-8100, Keyence, Osaka, Japan).

**ELISA**
The amount of $PGE_2$ in the culture medium was determined using a commercially available ELISA kit (Enzo Life sciences, Farmingdale, NY) according to the manufacturer's instructions, and the data were converted to pg/mL. Finally, duplicate assays were performed on each sample, and the absorbance at 405 nm was recorded.
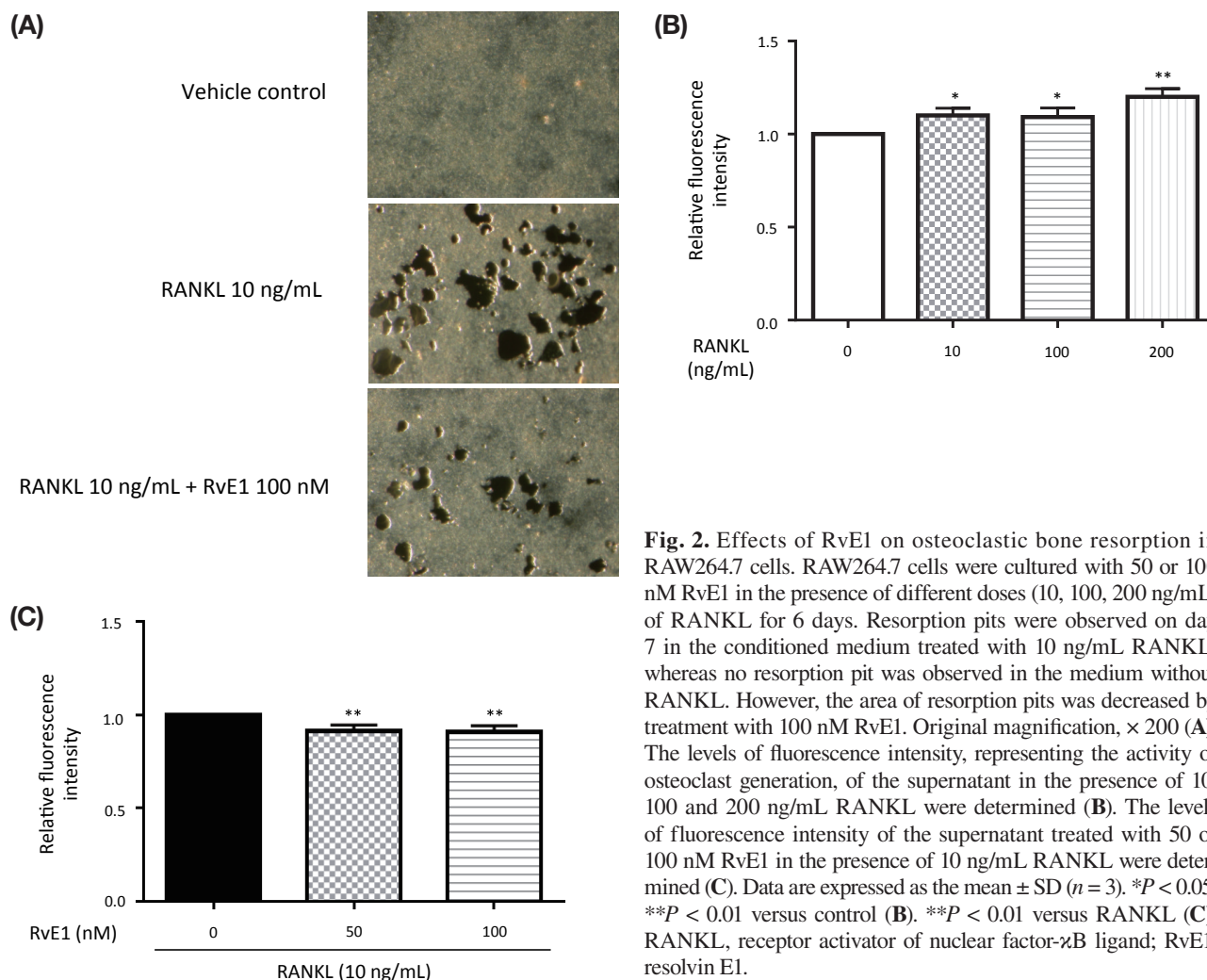


**Fig. 1.** Effects of RvE1 on the number of TRAP-positive osteoclast-like RAW264.7 cells.
RAW264.7 cells were cultured in conditioned medium containing 50 or 100 ng/mL RANKL with or without 100 nM RvE1 for 6 days. Osteoclast-like cells were stained by TRAP on day 7 of culture. Representative microscope images are shown. Original magnification, × 100 (**A**). The number of TRAP-positive multinucleated cells in the conditioned medium treated with 100 nM RvE1 was significantly reduced compared to groups treated with both 50 and 100 ng/mL RANKL (**B**). Data are expressed as the mean ± SD ($n = 3$). **$P < 0.01$ versus RvE1 (–) in the presence of 50 or 100 ng/mL RANKL. MNC, multinucleated cell; RANKL, receptor activator of nuclear factor-κB ligand; RvE1, resolvin E1; TRAP, tartrate-resistant acid phosphatase.

**(A)**

Vehicle control

RANKL 10 ng/mL

RANKL 10 ng/mL + RvE1 100 nM



**(B)**



**(C)**



**Fig. 2.** Effects of RvE1 on osteoclastic bone resorption in RAW264.7 cells. RAW264.7 cells were cultured with 50 or 100 nM RvE1 in the presence of different doses (10, 100, 200 ng/mL) of RANKL for 6 days. Resorption pits were observed on day 7 in the conditioned medium treated with 10 ng/mL RANKL, whereas no resorption pit was observed in the medium without RANKL. However, the area of resorption pits was decreased by treatment with 100 nM RvE1. Original magnification, × 200 (**A**). The levels of fluorescence intensity, representing the activity of osteoclast generation, of the supernatant in the presence of 10, 100 and 200 ng/mL RANKL were determined (**B**). The levels of fluorescence intensity of the supernatant treated with 50 or 100 nM RvE1 in the presence of 10 ng/mL RANKL were determined (**C**). Data are expressed as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ versus control (**B**). **$P < 0.01$ versus RANKL (**C**). RANKL, receptor activator of nuclear factor-ϰB ligand; RvE1, resolvin E1.

## Statistical analysis

The experimental data were analyzed using Graphpad Prism 6 (Graphpad Software, San Diego, CA). All experiments were conducted separately at least three times, and all data are presented as the mean ± SD. Statistically significant differences were assessed by analysis of variance (followed by Bonferroni multiple comparisons test) or Student's $t$-test with P-values < 0.05 considered significant.

## RESULTS
### RvE1 reduced the number of TRAP-positive osteoclast-like RAW264.7 cells

The CCK-8 assay confirmed that any effects of RvE1 on osteoclastogenesis would not be due to the cytotoxicity of this compound, since no change in cell viability was noted in RAW264.7 cells and MC3T3-E1 cells treated with RvE1 (data not shown).

TRAP-positive osteoclast-like cells were observed when RAW264.7 cells were cultured in the presence RANKL, and the number of positive cells increased in a dose-dependent manner. However, the number of TRAP-positive cells decreased in the conditioned medium treated with both 100 nM RvE1 and RANKL (Fig. 1A), representing a statistically significant reduction compared to those detected with 50 and 100 ng/mL RANKL alone (Fig. 1B).

### RvE1 suppressed osteoclastic bone resorption in RAW264.7 cells

To assess the effects of RvE1 on bone resorption, RAW264.7 cells were cultured with or without RvE1 in the presence of different doses (10, 100 and 200 ng/mL) of RANKL. Resorption pits were observed in the conditioned medium of RAW264.7 cells treated with 10 ng/mL RANKL, which were not detected in the medium without RANKL (Fig. 2A). The fluorescence intensity, representing the activity of osteoclast generation, sig-
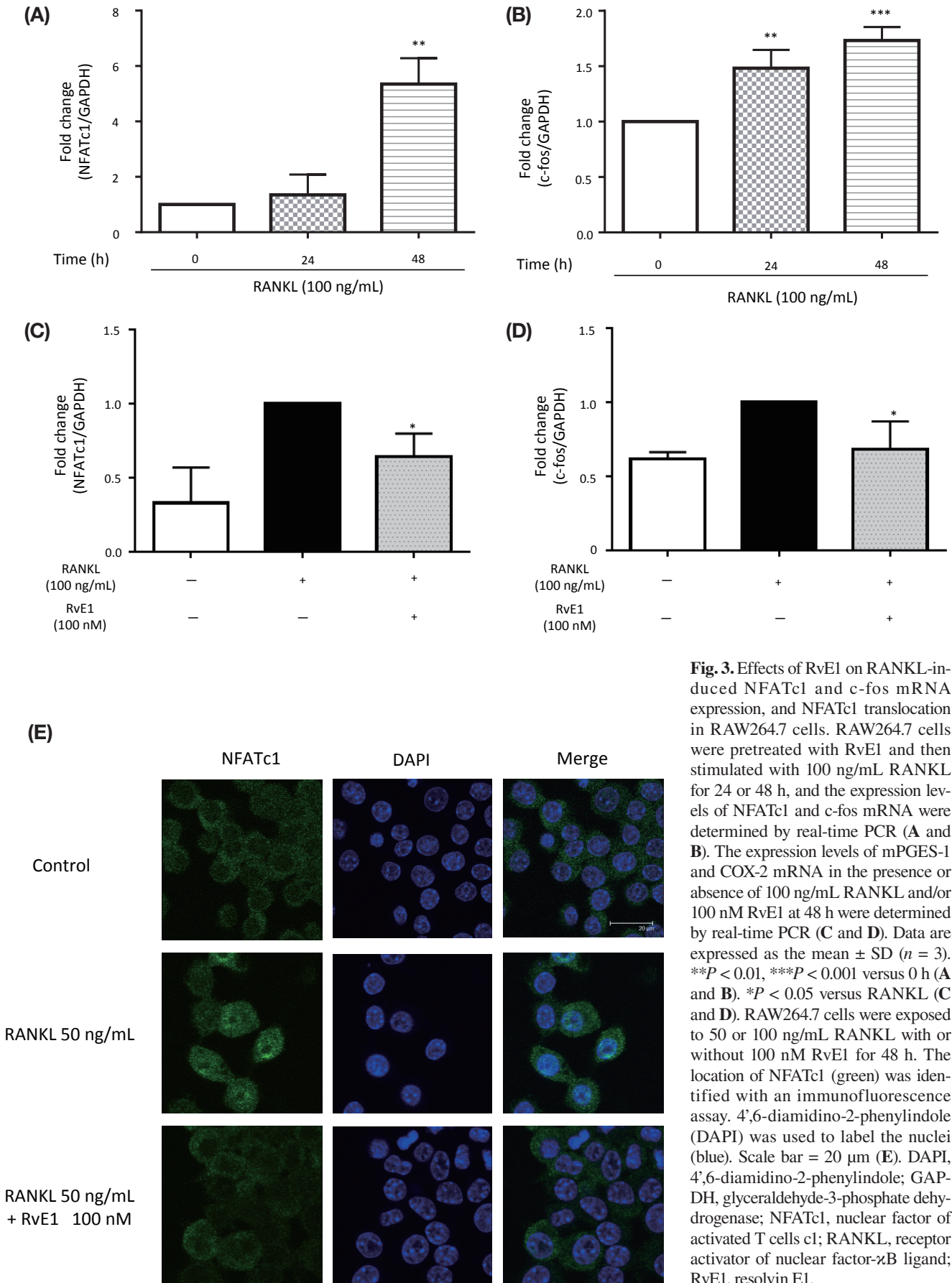
**Fig. 3.** Effects of RvE1 on RANKL-induced NFATc1 and c-fos mRNA expression, and NFATc1 translocation in RAW264.7 cells. RAW264.7 cells were pretreated with RvE1 and then stimulated with 100 ng/mL RANKL for 24 or 48 h, and the expression levels of NFATc1 and c-fos mRNA were determined by real-time PCR (**A** and **B**). The expression levels of mPGES-1 and COX-2 mRNA in the presence or absence of 100 ng/mL RANKL and/or 100 nM RvE1 at 48 h were determined by real-time PCR (**C** and **D**). Data are expressed as the mean ± SD ($n = 3$). **$P < 0.01$, ***$P < 0.001$ versus 0 h (**A** and **B**). *$P < 0.05$ versus RANKL (**C** and **D**). RAW264.7 cells were exposed to 50 or 100 ng/mL RANKL with or without 100 nM RvE1 for 48 h. The location of NFATc1 (green) was identified with an immunofluorescence assay. 4',6-diamidino-2-phenylindole (DAPI) was used to label the nuclei (blue). Scale bar = 20 μm (**E**). DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NFATc1, nuclear factor of activated T cells c1; RANKL, receptor activator of nuclear factor-κB ligand; RvE1, resolvin E1.
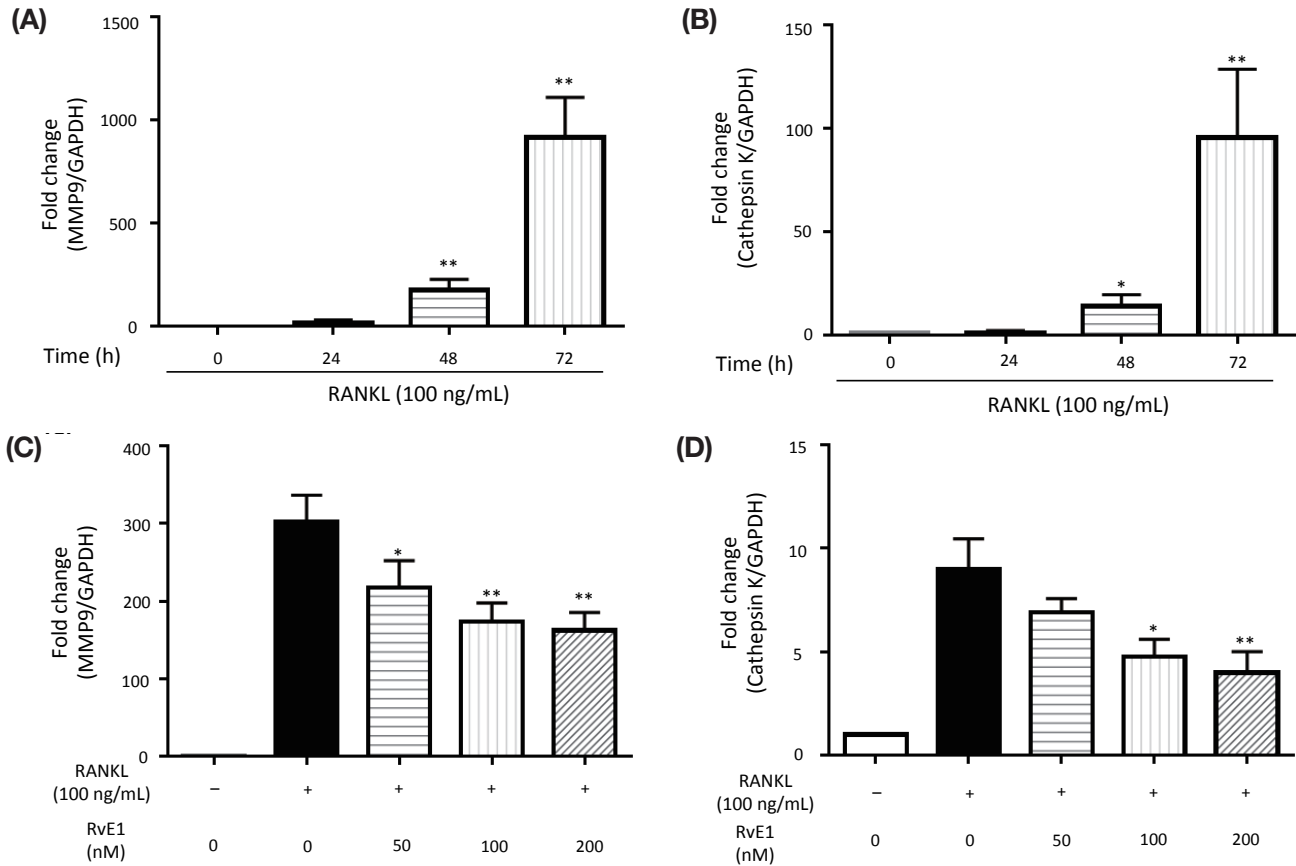
**Fig. 4.** Effects of RvE1 on RANKL-induced MMP-9 and cathepsin K mRNA expression in RAW264.7 cells.RAW264.7 cells were pre-treated with RvE1 and then stimulated with 100 ng/mL RANKL for 24 or 48 h, and the mRNA expression levels of MMP-9 and cathepsin K were determined by real-time PCR (**A** and **B**). The mRNA expression levels of MMP-9 and cathepsin K in the presence or absence of 100 ng/mL RANKL and/or 100 nM RvE1 at 48 h were determined by real-time PCR (**C** and **D**). Data are expressed as the mean ± SD ($n$ = 3), *$P < 0.05$, **$P < 0.01$ versus 0 h (**A** and **B**). *$P < 0.05$, **$P < 0.01$ versus RANKL (**C** and **D**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-9, matrix metalloproteinase-9; RANKL, receptor activator of nuclear factor-ϰB ligand; RvE1, resolvin E1.
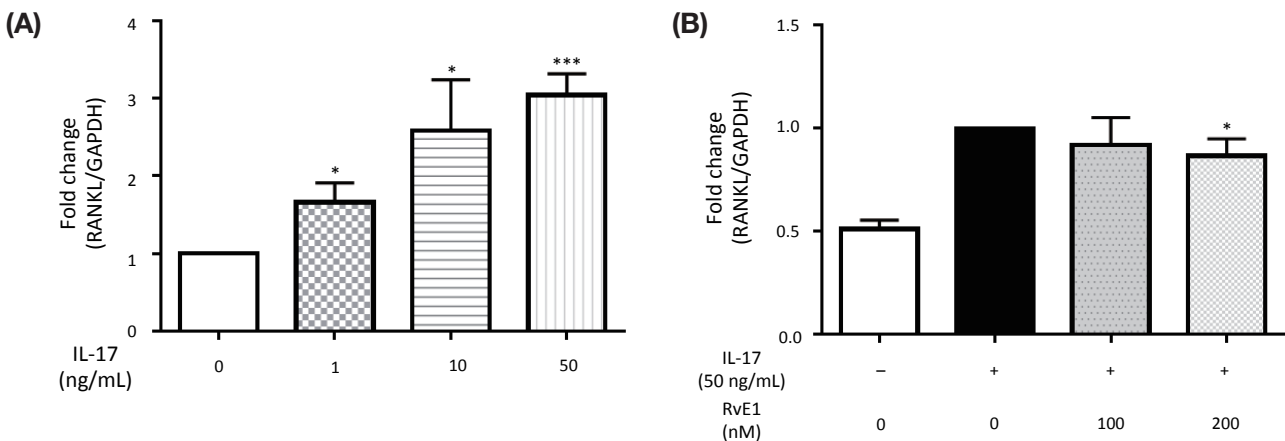


**Fig. 5.** Effect of RvE1 on IL-17-induced RANKL mRNA expression in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 1, 10 and 50 ng/mL IL-17, and the mRNA expression level of RANKL was determined by real-time PCR (**A**). The cells were also cultured with 50, 100 and 200 nM RvE1 in the presence of 50 ng/mL IL-17. The expression levels of RANKL mRNA in the presence or absence of 50 ng/mL IL-17 and/or 100 nM RvE1 at 24 h were determined by real-time PCR. The value of the fold change in mRNA levels of IL-17 alone was normalized as 1. (**B**). Data are expressed as the mean ± SD ($n$ = 3), *$P < 0.05$, ***$P < 0.01$ versus control (**A**). *$P < 0.05$ versus IL-17 alone (**B**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; RANKL, receptor activator of nuclear factor-ϰB ligand; RvE1, resolvin E1.
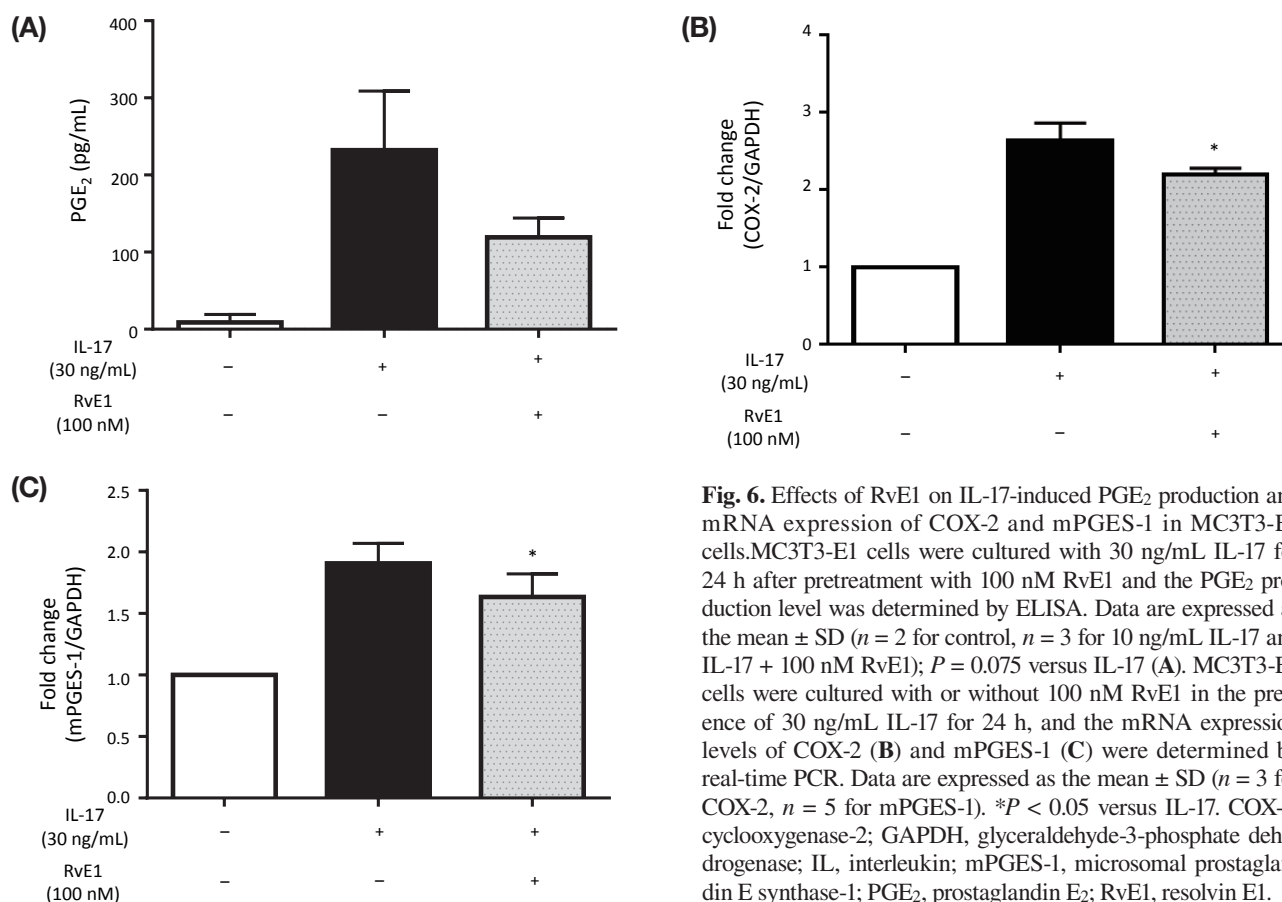
**(A)**



**(B)**



**(C)**



**Fig. 6.** Effects of RvE1 on IL-17-induced $PGE_2$ production and mRNA expression of COX-2 and mPGES-1 in MC3T3-E1 cells.MC3T3-E1 cells were cultured with 30 ng/mL IL-17 for 24 h after pretreatment with 100 nM RvE1 and the $PGE_2$ production level was determined by ELISA. Data are expressed as the mean ± SD ($n = 2$ for control, $n = 3$ for 10 ng/mL IL-17 and IL-17 + 100 nM RvE1); $P = 0.075$ versus IL-17 (**A**). MC3T3-E1 cells were cultured with or without 100 nM RvE1 in the presence of 30 ng/mL IL-17 for 24 h, and the mRNA expression levels of COX-2 (**B**) and mPGES-1 (**C**) were determined by real-time PCR. Data are expressed as the mean ± SD ($n = 3$ for COX-2, $n = 5$ for mPGES-1). *$P < 0.05$ versus IL-17. COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; mPGES-1, microsomal prostaglandin E synthase-1; $PGE_2$, prostaglandin $E_2$; RvE1, resolvin E1.

nificantly increased compared to that of the control in a RANKL concentration-dependent manner (Fig. 2B). However, co-treatment with 100 nM RvE1 decreased the area of the resorption Pits (Fig. 2A) and significantly reduced the fluorescent intensity compared to that of the vehicle control (Fig. 2C).

**RvE1 suppressed RANKL-induced NFATc1 and c-fos mRNA expression and NFATc1 nuclear translocation in RAW264.7 cells**

The NFATc1 and c-fos mRNA levels of RAW264.7 cells treated with 100 ng/mL RANKL increased in a time-dependent manner with a significant increase at 48 h, and at both 24 and 48 h, respectively, compared to 0 h (Figs. 3A and B). However, co-treatment with 100 nM RvE1 significantly reduced the RANKL-induced NFATc1 and c-fos mRNA expression levels compared to those of the vehicle control (Figs. 3C and D). The immunofluorescence assay showed that NFATc1 was mainly localized in the cytoplasm in the control cells, but was distinctly translocated to the nucleus after treatment with 50 ng/mL RANKL for 48 h; however, the nuclear immunostaining intensity was reduced in the presence of 100 nM RvE1 (Fig. 3E).

**RvE1 reduced RANKL-induced MMP-9 and cathepsin K mRNA expression in RAW264.7 cells**

The mRNA expression levels of MMP-9 and cathepsin K significantly increased at 48 and 72 h in RAW264.7 cells treated with 100 ng/mL RANKL compared to 0 h (Figs. 4A and B). RvE1 significantly reduced RANKL-induced MMP-9 and cathepsin K mRNA expression in a dose-dependent manner compared to the vehicle control (Figs. 4C and D).

**RvE1 reduced IL-17-induced RANKL mRNA expression in MC3T3-E1 cells**

After culturing the cells with IL-17 (1, 10 or 50 ng/mL) for 24 h, the RANKL mRNA level was significantly increased in a dose-dependent manner (Fig. 5A). Pretreatment with RvE1 (100 or 200 nM) for 24 h tended to reduce the IL-17-induced RANKL mRNA level in a dose-dependent manner with significant downregulation observed following treatment with 200 nM RvE1 (Fig. 5B). Similarly, the OPG mRNA level was decreased after IL-17 treatment in a dose-dependent manner, and OPG expression was upregulated with RvE1 pretreatment (data not shown). Therefore, RvE1 improved the

imbalance in the RANKL/OPG ratio, which is likely to induce an inhibitory effect on bone resorption.

## RvE1 reduced IL-17-induced PGE2 production and the mRNA expression levels of COX-2 and mPGES-1 in MC3T3-E1 cells

The ELISA results showed that pretreatment of MC3T3-E1 cells with RvE1 inhibited the stimulatory effect of IL-17 on PGE2 production; the PGE2 production levels in the presence of 100 nM RvE1 tended to be reduced compared to those of cells treated with the vehicle control (Fig. 6A). RvE1 treatment also significantly reduced the IL-17-induced expression of COX-2 and mPGES-1 mRNA compared to the vehicle control group (Figs. 6B and C).

## DISCUSSION

RvE1 is a member of the E series of resolvins that are biosynthesized from EPA.[11] Recent reports identified another member of the E series of resolvins, called resolvin E2 (RvE2) and resolvin E3 (RvE3).[26, 27] Although Barden and colleagues[28] showed that synovial fluid RvE2 was negatively associated with pain score in humans, the effects of RvE2 and RvE3 on osteoclasts and osteoblasts were not fully understood. Thus, we investigated RvE1 as a therapeutic agent for RA. With respect to bone metabolism, RvE1 was shown to inhibit osteoclast growth and bone resorption.[16] Gao and colleagues[29] reported that RvE1 has a direct bone-preserving function via chemokine-like receptor 1, which mediates bone preservation in osteoblasts. Moreover, a recent study indicated that RvE1 influenced pain, a major symptom of RA, showing simultaneous anti-inflammatory and analgesic properties in experimental models closely related to translational sites in humans.[30] These studies have demonstrated the good therapeutic potential of RvE1 in many chronic inflammatory diseases. Indeed, RvE1 (Rx-10001) and its synthetic analog (Rx-10045) are currently under clinical trials for the relief of chronic dry eyes.[31] The aim of our study was to clarify the effect of RvE1 on osteoclastogenesis and determine its feasibility in the development of a new therapeutic approach to RA.

We showed that RvE1 reduced the number of TRAP-positive osteoclast-like cells, which is consistent with a previous report indicating that RvE1 markedly decreased the number of differentiated osteoclasts induced by macrophage colony-stimulating factor and RANKL in primary osteoclast cultures.[16]

Recently, a new assay method of evaluating the bone resorption activity in RAW264.7 cells was reported based on the measurement of pit formation using calcium phosphate labeled with fluorescent polyanionic mole-cules.[25] With this method, we found that RvE1 decreased the bone resorption activity in RAW264.7 cells, in line with a previous report showing that RvE1 diminished dentin resorption in vitro.[16] These results in RAW264.7 cells suggest that RvE1 could inhibit RANKL-induced osteoclastogenesis and bone resorption.

We further found that RvE1 significantly inhibited RANKL-induced NFATc1 and c-fos mRNA expression and the nuclear translocation of NFATc1 from the cytoplasm in RAW264.7 cells, providing a potential mechanism underlying this inhibitory effect of RvE1 on osteoclastogenesis. A previous study showed that RvE1 attenuated the RANKL-induced nuclear translocation of the p50 subunit of NF-κB.[16] Zhu and colleagues[32] reported another mechanism of RvE1 in osteoclasts in bone marrow cells of C57BL/6 mice, in which RvE1 inhibits osteoclast fusion through binding NFATc1 to the pivotal fusion protein DC-STAMP, thereby suppressing RANKL-induced NFATc1 nuclear translocation. Another report showed that Bioaggregate, a laboratory-synthesized and calcium silicate-based nanoparticulate cement, also inhibited the translocation of NFATc1 and c-fos from the cytoplasm to the nucleus in osteoclast precursor cells, which was determined with immunofluorescence analysis.[33]

Our data indicated that RvE1 inhibited RANKL-induced osteoclastogenesis through suppressing the mRNA expression of NFATc1 and c-fos. NFATc1 is known as a master transcription factor that plays a key role in regulating the expression of several osteoclast-specific genes such as TRAP, cathepsin K and MMP-9, which are involved in regulating osteoclast differentiation.[18] Our results suggested that RvE1 significantly inhibited RANKL-induced cathepsin K and MMP-9 mRNA expression by decreasing the translocation of NFATc1 from the cytoplasm to the nucleus.

We further showed that RvE1 might correct the imbalance of RANKL and OPG expression in osteoblasts induced by IL-17 using MC3T3-E1 cells.

PGE2 is well known to be the key mediator of inflammation, pain and joint destruction in RA, and its production is an important target of anti-inflammatory drugs. PGE2 is produced by the conversion of arachidonic acid to prostaglandin H2 by COX-1/COX-2, with the subsequent conversion of prostaglandin H2 to PGE2 by mPGES-1, which is the terminal enzyme in the PGE2 production process at the sites of inflammation, and has an important role in the pathogenesis of RA.[34, 35] PGE2 has several pro-inflammatory effects, including increasing vascular permeability, vasodilation, blood flow and local pyrexia, along with potentiation of pain caused by other agents. Previous studies showed that PGE2 stim-

ulates osteoclast differentiation through the prostanoid receptors EP4 and EP2 by inducing RANKL production and inhibiting OPG expression.[36–38] OPG is a soluble decoy receptor for RANKL that prevents it from binding to RANK. RANKL interacts with its receptor, which is expressed on osteoclast precursors, to induce the differentiation and activation of osteoclasts.[21] A recent study showed that EPA and DHA inhibit $PGE_2$-induced RANKL expression in MC3T3-E1 cells.[39] Similarly, we showed that RvE1 inhibits the IL-17-induced COX-2 and mPGES-1 mRNA expression followed by $PGE_2$ production in MC3T3-E1 cells. These results might indicate that RvE1 indirectly inhibits osteoclastogenesis by reducing IL-17-induced COX-2 and mPGES-1 expression in osteoblasts, suggesting that RvE1, like EPA and DHA, has an anti-inflammatory effect in RA.

We did not examine the effects of RvE1 on the expression of $PGE_2$ receptors in osteoblasts, such as EP4 receptor. However, recent reports showed that $PGE_2$ enhanced osteoclast formation through EP4 receptor activation on osteoblasts.[40, 41] In addition, the effects of RvE1 on the leukotriene B4 receptor subtype 1 (BLT1) expressed on osteoclasts, identified as receptors for RvE1,[42] should be further investigated, as a previous study showed that BLT1 mediates the actions of RvE1 on osteoclasts.[16] Although the major effect of $PGE_2$ on bone resorption is generally considered to occur indirectly via upregulation of RANKL and inhibition of OPG expression in osteoblastic cells,[43] further study is needed to determine whether the EP4 receptor and BLT1 are therapeutic targets of RvE1. The mechanisms of inflammation in RA are complex. In RA, many pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-17 increase RANKL expression, leading to an increased osteoclast differentiation and subsequent bone erosions. Several of these cytokines also act synergistically with RANKL in promoting osteoclast differentiation.[44] Further studies are needed to assess the anti-inflammatory effect of RvE1 in RA.

In conclusion, our results suggest that RvE1 inhibits osteoclastogenesis and bone resorption by suppressing RANKL-induced osteoclast differentiation. The mechanism of action was determined to occur by the downregulation of NFATc1 and c-fos in osteoclasts and suppression of IL-17-induced RANKL expression through the autocrine action of $PGE_2$ in osteoblasts. These findings suggest the potential of RvE1 as a new therapeutic approach to RA, providing the foundation for further preclinical and clinical investigations.

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