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Vibration enhances osteoclastogenesis by inducing RANKL expression *via* NF-κB signaling in osteocytes

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ABSTRACT

To shorten the duration of orthodontic treatment it is important not only to reduce risks such as dental caries, periodontal disease, and root resorption, but also to decrease pain and discomfort caused by a fixed appliance. Several studies have investigated the effect of vibration applied to fixed appliances to accelerate tooth movement. Although it was reported that vibration accelerates orthodontic tooth movement by enhancing alveolar bone resorption, the underlying cellular and molecular mechanisms remain unclear. In this study, we investigated the effects of vibration on osteoclastogenesis *in vitro* and *in vivo*. Vibration applied to pre-osteoclast cell line RAW264.7 cells enhanced cell proliferation but did not affect their differentiation into osteoclasts. Osteocytes in bone are known to be mechanosensitive and to act as receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). Therefore, in the present study, vibration was applied to cells from the osteocyte-like cell line MLO-Y4. In MLO-Y4 cells, vibration induced phosphorylation of the inhibitor of NF-κB (IκB) and caused nuclear localization of NF-κB p65. Additionally, vibration increased RANKL mRNA expression, but did not affect osteoprotegerin (OPG) mRNA expression in MLO-Y4 cells, thus resulting in an increased RANKL/OPG ratio. Consistent with these findings, vibration applied during experimental tooth movement increased NF-κB activation and RANKL expression in osteocytes on the compression side of alveolar bone *in vivo*, whereas vibration had no such effects on the tension side. Furthermore, in a co-culture of MLO-Y4 cells and RAW264.7 cells, vibration applied to MLO-Y4 cells enhanced osteoclastogenesis. These findings suggest that vibration could accelerate orthodontic tooth movement by enhancing osteoclastogenesis through increasing the number of pre-osteoclasts and up-regulating RANKL expression in osteocytes on the compression side of alveolar bone *via* NF-κB activation.

1. Introduction

Orthodontic tooth movement requires bone remodeling, which is regulated by bone resorption by osteoclasts on the compression side and bone formation by osteoblasts on the tension side. When orthodontic force is applied to a tooth by an orthodontic apparatus, the periodontal tissue senses this force, bone remodeling is induced, and tooth movement occurs [1,2]. Orthodontic treatment often takes several years, which is one of the reasons why patients hesitate to start such treatment. Long-term treatment is also associated with complications including an increased risk of caries, periodontal disease, and root resorption. Therefore, shortening the duration of orthodontic treatment is important.

Low-magnitude (LM; < 1 g, where g = 9.81 m/s^2), high-frequency (HF; 20–90 Hz) mechanical vibration can stimulate an anabolic response in both weight-bearing [3] and non-weight-bearing bone [4] as found from studies using animals. LMHF mechanical vibration has also been clinically applied to humans for medical purposes as a safe and non-invasive approach, for example, as treatment to prevent bone loss in postmenopausal woman [5] and also to increase the bone density in

Abbreviations: RANKL, receptor activator of nuclear factor kappa B ligand; NF-κB, nuclear factor kappa B; IκB, inhibitor of NF-κB; OPG, osteoprotegerin; M-CSF, macrophage colony stimulating factor; TRAP, tartrate-resistant acid phosphatase

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children with cerebral palsy in a disabling condition [6]. In recent years, mechanical vibration has gained interest as one method of accelerating orthodontic tooth movement. Several prospective randomized controlled clinical trials have investigated the effects of vibration applied with fixed appliances on orthodontic tooth movement. Although some of these studies showed that vibration increases the rate of tooth movement during orthodontic treatment with fixed appliances [7], others did not [8,9]. These differences in treatment outcomes may be caused by variations in the characteristics of the vibration, such as force magnitude, frequency, exposure duration, and timing. Recently, optimum vibratory conditions for the acceleration of experimental tooth movement in rats were demonstrated to be 3 gf at 70 Hz for 3 min once a week, and this accelerated tooth movement was achieved by enhancement of bone resorption by osteoclasts not only around the tooth root but also inside the alveolar bone [10]. However, the underlying cellular and molecular mechanisms of this phenomenon are still unknown.

Osteoclasts are derived from hematopoietic progenitor cells of the monocyte/macrophage lineage and differentiate under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B ligand (RANKL) [11,12]. RANKL, an essential factor for osteoclastogenesis [13], is expressed not only on osteoblasts but also on various kinds of cells such as B lymphocytes [14], T lymphocytes [15], osteocytes [16,17], and periodontal ligament cells [18]. Changes in the amount of RANKL expression on these cells regulate physiological or pathologic alveolar bone resorption [19]. In recent years, it has been shown that osteocytes, representing > 90% of all bone cells, express much higher levels of RANKL and have a greater capacity to support osteoclastogenesis *in vitro* than osteoblasts [17]. Furthermore, osteocytes show a characteristic morphology with many cellular dendritic processes, by which they communicate with each other and with osteoblasts, lining cells, and osteoclasts *via* interconnecting canaliculi [20–23]. Therefore, osteocytes are thought to sense mechanical stress within bone tissue by acting as mechanosensors and to play an important role in mechanotransduction in bone [20,21,24]. However, it is unclear whether osteocytes sense vibration and fulfil a function in vibration-induced bone remodeling.

The transcription factor nuclear factor-kappa B (NF-κB) is activated by various forms of stimulation including mechanical stress, and its activation is followed by the induction of gene expression related to biological phenomena such as cell survival, growth, differentiation, and apoptosis in eukaryotic cells [25]. In bone, it is known that NF-κB is one of the essential nuclear transcription factors in RANKL-induced osteoclast differentiation, and it has been reported that activated NF-κB takes part in bone remodeling by suppressing bone formation and enhancing osteoclast formation in mice [26,27]. Although NF-κB is likely to respond to mechanical stress and have a function in mechanotransduction in osteocytes, there are few studies investigating the role of NF-κB in osteocytes.

In this study, to clarify the molecular mechanism of vibration-induced bone resorption, we investigated the effects of vibration on osteoclast formation *in vitro* by using the pre-osteoclast cell line RAW264.7 and the osteocyte-like cell line MLO-Y4. Furthermore, we applied vibration to an experimental tooth movement model in rats and investigated the NF-κB activation and the RANKL expression in osteocytes on both the compression and tension sides of alveolar bone *in vivo*.

2. Materials and methods

2.1. Cell cultures

We used the osteocyte cell line MLO-Y4 and the pre-osteoclast cell line RAW264.7. MLO-Y4 cells were cultured on type I-A collagen (Cellmatrix, Nitta Gelatin, Osaka, Japan) coated plates in α-MEM medium (Wako, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 5% bovine serum (BS) (Invitrogen, Carlsbad, CA, USA) and 20 μg/ml kanamycin. RAW264.7 cells were cultured in α-MEM medium (Wako) supplemented with 10% FBS (Sigma-Aldrich) and 100 unit/ml penicillin and 100 μg/ml streptomycin (1% P/S) (Thermo Fisher Scientific, Waltham, MA, USA). Both cell lines were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.2. Cell proliferation assays

RAW264.7 cells were seeded at 3000 cells/well in 96-well plates without RANKL, and the plates were pre-incubated for 24 h at 37 °C and 5% CO_2. Vibration (0.5 gf at 48.3 Hz) was then applied on the bottom of the plate using a cylindrical vibration motor (5 mm × 15 mm) for 1 min at room temperature. At 12, 24, or 48 h after vibration, 10 μ1 Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) was added to each well. After color reaction for 1 h, 10 μl of 0.1 M HCl was added to each well to stop the reaction, and the absorbance at 450 nm was measured with a microplate reader.

2.3. Osteoclast differentiation and TRAP staining

RAW264.7 cells were seeded at 3000 cells/well in 96-well plates with 50 ng/ml RANKL for 24 or 72 h at 37 °C and 5% CO_2. Vibration (0.5 gf at 48.3 Hz) was applied to each plate for 1 min at room temperature. At 24 or 48 h after vibration, cells were fixed in 4% paraformaldehyde for 15 min, and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. Osteoclasts were identified as TRAP-positive cells containing three or more nuclei. To quantify the number of osteoclasts and the number of nuclei in each cell, we used a DP72 microscope (Olympus) with 20× objective.

2.4. Western blot analysis

MLO-Y4 cells were seeded at 1.0×10^4 cells/cm^2 in collagen coated 60 mm dishes, and pre-incubated for 48 h at 37 °C and 5% CO_2. Vibration (0.5 gf at 48.3 Hz) was applied for 1 min at room temperature. The total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Millipore, Burlington, MA, USA) and protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) at 0, 5, 15, 30, 60, or 120 min after vibration. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples (30 μg) were mixed with a 4× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and separated on 4–15% Mini-PROTEAN TGX gels (Bio-Rad) using the PROTEAN Tetra Electrophoresis System (Bio-Rad). The proteins were electrophoretically transferred onto a Trans-Blot Turbo Transfer Pack 0.2 μm PVDF Membrane (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were blocked in 4% (w/v) Block Ace (DS PHAMA BIOMEDICAL, Osaka, Japan) for 1 h, then proved with mouse antibodies to phospho-IκBα (Ser32/36) (Cell Signaling, Danvers, MA, USA), rabbit antibodies to IκB-α (Cell Signaling), and mouse antibodies to β-actin (C4) (Santa Cruz, Dallas, TX, USA) overnight at 4 °C. The membrane was washed in tris buffered saline with Triton X-100 (TBS-T), then incubated with Peroxidase AffiniPure Donkey Anti-Mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA) or Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch) for 1 h. The immunoreactive bands were visualized using the Molecure Imager® VersaDoc™ MP 5000 System (Bio-Rad). The stained bands were quantified with ImageJ (National Institutes of Health, Bethesda, MD, USA). The results were obtained in quadruplicate with the use of 4 separate samples for each test.

2.5. Real-time quantitative polymerase chain reaction (PCR)

MLO-Y4 cells were seeded at 1.0×10^4 cells/cm^2 in collagen-coated

60 mm dishes, and pre-incubated for 48 h at 37 °C and 5% CO_2. Vibration (0.5 gf at 48.3 Hz) was applied for 1 min at room temperature. Total RNA was extracted using the Reliaprep™ RNA Cell Miniprep System (Promega, Madison, WI, USA) at 0, 30, or 60 min after vibration. cDNA was synthesized using the reverse transcription of 2 μg of RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan), and the resultant cDNA mixture was diluted 10-fold in RNase free dH_2O. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Bio) with Thermal Cycle Dice Real Time System TP800 (Takara Bio). Standards and samples were run in triplicate. mRNA levels of each gene of interest were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. The PCR primers were shown as follows:

RANKL (forward, 5′-GAGGGAGCACGAAAAACTGGTC-3′; reverse, 5′-GGAAGGGTTGGACACCTGAATG-3′); OPG (forward, 5′-GAGAGAAG CCACGCAAAAGTG-3′; reverse, 5′-TCTTGGTAGGAACAGCAAAC CTG-3′); and GAPDH (forward, 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse, 5′-TTGCTGTTGAAGTCGCAGGAG-3′).

2.6. Animals

For experiments, 18 25-week-old male Wistar rats were used. The rats were divided randomly into two groups. In one group, the maxillary right first molar was used as a control (C group), and the maxillary left first molar was subjected to tooth movement (TM group). In the other group, the maxillary right first molar was subjected to vibration (V group) and the maxillary left first molar was subjected to tooth movement and vibration (TM + V group). All experimental protocols were approved by the Tohoku University of Science Animal Care and Use Committee.

2.7. Experimental tooth movement and supplementary vibration

Orthodontic force was applied according to the method of Waldo and Rothblatt [28]. Under three types of mixed anesthesia, a piece of an orthodontic elastic module was inserted interproximally between the maxillary first and second molars on the left side (Fig. 1A). Dynamic vibration (3 gf at 70 Hz) was applied to the teeth for 3 min by placing a vibration motor (5 mm × 15 mm) in the palate of the rat and attaching it to the bilateral first molars with a ligature wire.

2.8. Sample preparation and histochemistry

1, 3, or 6 h after the experiments, the anesthetized rats underwent perfusion fixation with 4% paraformaldehyde, and the maxillary bone containing the teeth was dissected out. The samples were decalcified with 20% ethylenediamine tetraacetate (EDTA) solution for 14 days, and paraffin-embedded using the routine method. Serial sections (thickness, 5 μm) were stained with hematoxylin and eosin (H-E). The images were captured with a DP72 microscope. The sections were sliced parallel to the occlusal plane of the maxillary molars, and the level of the sections from the furcation to the apex was calculated by counting the number of serially sliced sections. Three sections per animal were used for quantitative analysis. The first sections were about 125 μm from the furcation of the teeth, the second sections were about 50 μm away, and the third sections were about 100 μm away. The region on the distal side of mesial root of the maxillary first molar was regarded as the tension side with widened periodontal ligament (PDL) width, and the region on the mesial side of mesio-palatal root was regarded as the compression side with narrowed PDL width. The square fields (300 μm in height × 200 μm in width) on both compression and tension sides of alveolar bone adjacent to the PDL, in contact with the line drawn tangentially to the pulp surface of mesial root and mesio-palatal root of the first molar, were set up as the region of interest (ROI) for evaluating NF-κB activation and RANKL expression in osteocytes (Fig. 1B).

2.9. Immunofluorescence

Sections were deparaffinized in xylene, hydrated, washed three times in phosphate buffered saline (PBS). Sections for immunofluorescence of NF-κB p65 were permeabilized in 0.2% Triton X-100 for 15 min at room temperature. Sections for immunofluorescence of RANKL were permeabilized in 0.01 M citrate buffer for 2 h and 0.2% Triton X-100 for 15 min. Then, sections were incubated with anti-NF-κB p65 antibody (c-20; Santa Cruz, CA, USA) at 1:100 dilution or RANKL antibody (FL-317; Santa Cruz) at 1:500 dilution at 4 °C overnight. Negative control was obtained by treating the tissue as described above except that nonimmune rabbit IgG (Sigma-Aldrich) was used instead of NF-κB p65 or RANKL antibody. After being washed with PBS three times, the sections were incubated for 1 h at room temperature with Alexa Fluor 555 anti-rabbit IgG (Invitrogen, CA, USA). Cultured MLO-Y4 cells were fixed at 15, 30, or 60 min after vibration and were incubated with anti-NF-κB p65 antibody (Santa Cruz), followed by Alexa Fluor 555 anti-rabbit IgG (Invitrogen). All samples were sealed using ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) and were examined using confocal laser scanning microscopy (C2si; Nikon, Tokyo, Japan) and NIS-Elements imaging software (version 4.13; Nikon). To quantify the areas of RANKL expression, the fluorescent images obtained from immunostaining were analyzed using ImageJ (NIH) imaging software. Thresholds of 15 and 255 were used to define RANKL expression areas in red channel images.

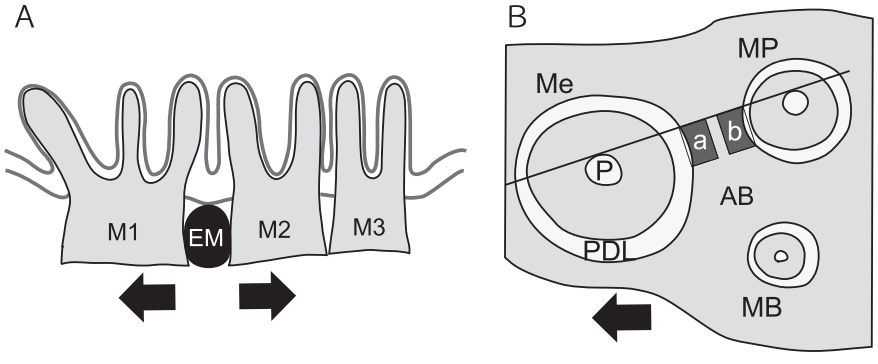

Fig. 1. (A) A schematic drawing of experimental tooth movement using the method of Waldo. An elastic module was inserted interproximally between the maxillary first molar and the second molar. The arrows indicate the direction of the force applied. M1, maxillary first molar; M2, maxillary second molar; M3, maxillary third molar; EM, elastic module. (B) A diagram indicating the area of a horizontal section of the maxillary first molar, periodontal ligament, and alveolar bone. The square fields (300 μm in height × 200 μm in width) on the tension side (a) and the compression side (b) of alveolar bone adjacent to the periodontal ligament, in contact with the line drawn tangentially to the pulp surface of mesial root and mesio-palatal root of the first molar, were set up as the region of interest (ROI) for measurement (dark gray). The arrow indicates the direction of the force applied. AB, alveolar bone; PDL, periodontal ligament; Me, mesial root; MP, mesio-palatal root; MB, mesio-buccal root; p, pulp.

2.10. Co-culture of MLO-Y4 cells and RAW264.7 cells

MLO-Y4 cells were seeded at 1000 cells/cm^2 in collagen-coated 96-well plates (day 0). Vibration (0.5 gf at 48.3 Hz) was applied for 1 min at room temperature on day 2. At 30 min after vibration, RAW 264.7 cells were added at 2500 cells/cm^2 without RANKL and then co-cultured in α-MEM medium supplemented with 10% FBS and 1% P/S for 6 days. The medium was replaced every 2 days. On day 8, the cells were fixed and stained for TRAP. Osteoclasts were identified as TRAP-positive cells containing three or more nuclei.

2.11. Statistical analysis

The data of two-group comparisons were analyzed using Student's *t*-test. Simultaneous comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) followed by *post hoc* analysis using the Tukey-Kramer test. Differences were considered significant at $p < 0.01$ or 0.05. Data was represented as mean ± SD.

3. Results

3.1. Vibration enhances cell proliferation of RAW264.7 cells

We first investigated the effect of vibration on the cell proliferation of pre-osteoclasts. Before and after vibration, no morphological changes were observed (Fig. 2A). To quantitatively evaluate cell proliferation, we used Cell Count Reagent SF. At 12 and 24 h after vibration, the number of RAW264.7 cells had slightly increased in the vibration group compared with that for the control group, but no significant difference was observed (Fig. 2B). However, at 48 h after vibration, the number of cells in the vibration group had increased significantly compared with that found for the control group (Fig. 2B).

3.2. Vibration does not affect osteoclast differentiation of RAW264.7 cells

To examine the effect of vibration on the differentiation of osteoclasts, we applied vibration to RAW264.7 cells and counted the number of TRAP-positive multinucleate cells (TRAP[+] MNCs). In the vibration group in which vibration was applied 24 h after cell seeding, the number of TRAP-positive cells with 3–5 nuclei had increased compared with that for the control group, but no significant difference was observed (Fig. 2C–E). There also was no difference in the number of TRAP-positive cells with 6–9 or ≧ 10 nuclei (Fig. 2C–E). Again, in the case of the vibration group in which vibration was applied 72 h after cell seeding, there was no significant difference in the number of TRAP-positive cells with 3–5, 6–9, or ≧ 10 nuclei at either 24 or 48 h after vibration compared with those numbers for the control group (Fig. 2F–H).

3.3. Vibration activates the NF-κB signaling pathway in MLO-Y4 cells

We next investigated the activation of the NF-κB signaling pathway in vibrated osteocytes *in vitro*. We repeated the same experiment 4 times using 4 separate samples to analyze quantitatively the expression of phospho-IκB (p-IκB) protein. No morphological changes in MLO-Y4 cells were observed before or after vibration (Fig. 3A). IκB phosphorylation was induced immediately after vibration (Fig. 3B), and 15 min after vibration the level of p-IκB in vibrated MLO-Y4 cells was significantly higher than that in the non-vibrated control cells (Fig. 3C, D). By 60 min after vibration, the amount of p-IκB had returned to control levels (Fig. 3C, D). Furthermore, to evaluate NF-κB activation in vibrated osteocytes, we assessed the nuclear localization of NF-κB p65 by using of immunofluorescence. Vibration led to significantly increase in the nuclear localization of NF-κB in MLO-Y4 cells at 15 min after vibration, and further increases were noted at 30 and 60 min (Fig. 3E, F).

3.4. Vibration increases RANKL expression in MLO-Y4 cells

We further examined the change in the amount of RANKL expression in vibrated MLO-Y4 cells. The mRNA level of RANKL in vibrated MLO-Y4 cells increased significantly at 30 min after vibration compared with that for the non-vibrated control cells (Fig. 4A). Although the mRNA level of OPG was not altered (Fig. 4B), the increase in RANKL contributed to a significant increase in the RANKL/OPG ratio at 30 min after vibration (Fig. 4C). By 60 min after vibration, the mRNA level of RANKL and the RANKL/OPG ratio had increased more than those for the non-vibrated control group, but this difference was not significant (Fig. 4A, C).

3.5. Experimental tooth movement and supplementary vibration induces NF-κB activation in osteocytes on the compression side of the alveolar bone

As vibration activated the NF-κB signaling pathway in MLO-Y4 cells *in vitro*, we next investigated the effect of supplementary vibration on NF-κB activation in osteocytes during experimental tooth movement. Osteocytes expressing NF-κB p65 in their nuclei (Fig. 5C) were recognized as being NF-κB-activated osteocytes, and we counted the number of these cells on both compression and tension sides of alveolar bone (Fig. 5A) 1, 3, and 6 h after experimental tooth movement and supplementary vibration. On the compression side, the largest proportion of NF-κB-activated osteocytes was observed in the TM + V group at all times; and it increased over time (Fig. 5B, H–J). The proportion of NF-κB-activated osteocytes in the TM + V group on the compression side was significantly higher than that for the C group at 1, 3, and 6 h, and was also significantly higher than that for the V or TM group at 3 and 6 h (Fig. 5H–J). The proportion on the compression side was significantly greater in the TM group than in the C group at 3 and 6 h, and also than in the V group at 6 h (Fig. 5I, J). In the V group on the compression side, the proportion of NF-κB-activated osteocytes was higher than that for the C group at all times, but it was not significant (Fig. 5H–J). On the tension side, no significant differences were observed in the proportion of NF-κB-activated osteocytes among all groups at all times (Fig. 5B, E–G).These results suggest that supplementary vibration applied during experimental tooth movement synergistically stimulated the activation of NF-κB in osteocytes on the compression side, not tension side, of the alveolar bone. In sections treated with nonimmune rabbit IgG (Fig. 5D), no positive signals were observed.

3.6. Experimental tooth movement and supplementary vibration increases RANKL expression in osteocytes on the compression side of the alveolar bone

As vibration increased RANKL expression in MLO-Y4 cells *in vitro*, we further investigated the effects of supplementary vibration on RANKL expression in osteocytes during experimental tooth movement on both compression and tension sides of the alveolar bone. RANKL-positive osteocytes in the TM + V group on the compression side were significantly increased in number compared with the number for the C group at 1, 3, and 6 h, and their number was also higher than that for the V or TM group at 3 and 6 h (Fig. 6A, E–G). The V and TM groups on the compression side tended to have more RANKL-positive osteocytes than the C group at all times, but this difference was not significant (Fig. 6E–F). On the tension side, no significant changes were observed in the proportion of RANKL-positive osteocytes among the all groups at all times (Fig. 6A, B–D). These results indicated that supplementary vibration applied during experimental tooth movement synergistically increased the RANKL expression in osteocytes on the compression side, not the tension side, of the alveolar bone.

3.7. Vibration applied to MLO-Y4 cells stimulates osteoclast formation

Given that RANKL expression in osteocytes increased with vibration

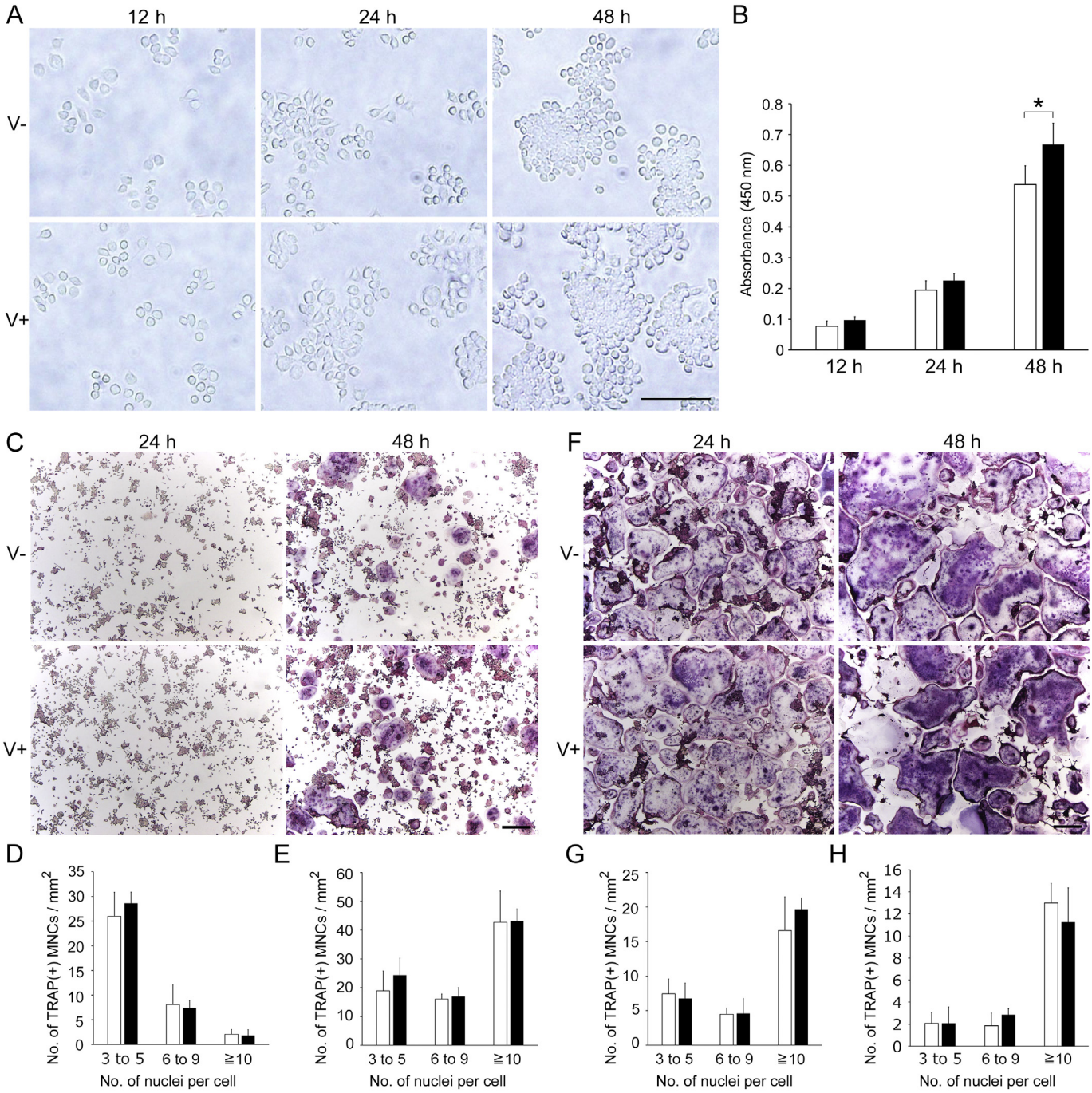

Fig. 2. Vibration enhanced cell proliferation of RAW264.7 cells, but did not affect osteoclast differentiation. (A) Representative phase images of non-vibrated (V−) or vibrated (V+) RAW264.7 cells cultured for the indicated times. Scale bars = 100 μm. (B) Cell proliferation of RAW264.7 cells was evaluated with Cell Count Reagent SF. The data is expressed as the mean ± SD (n = 4). *$p < 0.05$. (C) Representative phase images of non-vibrated (V−) or vibrated (V+) RAW264.7 cells cultured for the indicated times after vibration was applied 24 h after cell seeding. Scale bars = 200 μm. (D, E) The number of TRAP-positive multinucleated cells (TRAP(+) MNCs) counted 24 h (D) or 48 h (E) after vibration was applied 24 h after cell seeding. The data is expressed as the mean ± SD (n = 3). (F) Representative phase images of non-vibrated (V−) or vibrated (V+) RAW264.7 cells cultured for the indicated times after vibration was applied 72 h after cell seeding. Scale bars = 200 μm. (G, H) The number of TRAP(+) MNCs counted 24 h (G) or 48 h (H) after vibration was applied 72 h after cell seeding. The data is expressed as the mean ± SD (n = 3). White bars, non-vibrated group; black bars, vibrated group.

in vivo and in vitro, we investigated whether vibration applied to osteocytes would actually promote osteoclast formation. Vibrated MLO-Y4 cells were co-cultured with RAW 264.7 cells for 6 days. The number of TRAP(+) MNCs containing 3 or more nuclei was significantly higher in the vibration group than in the control group (Fig. 7A, B). This result suggests that vibration to MLO-Y4 cells stimulated osteoclast formation.

4. Discussion

In the present study, we demonstrated for the first time that vibration enhanced osteoclastogenesis by inducing RANKL expression on osteocytes *in vitro*, possibly as a result of NF-κB activation. We also showed that vibratory stimulation during orthodontic tooth movement significantly increased RANKL expression and nuclear translocation of

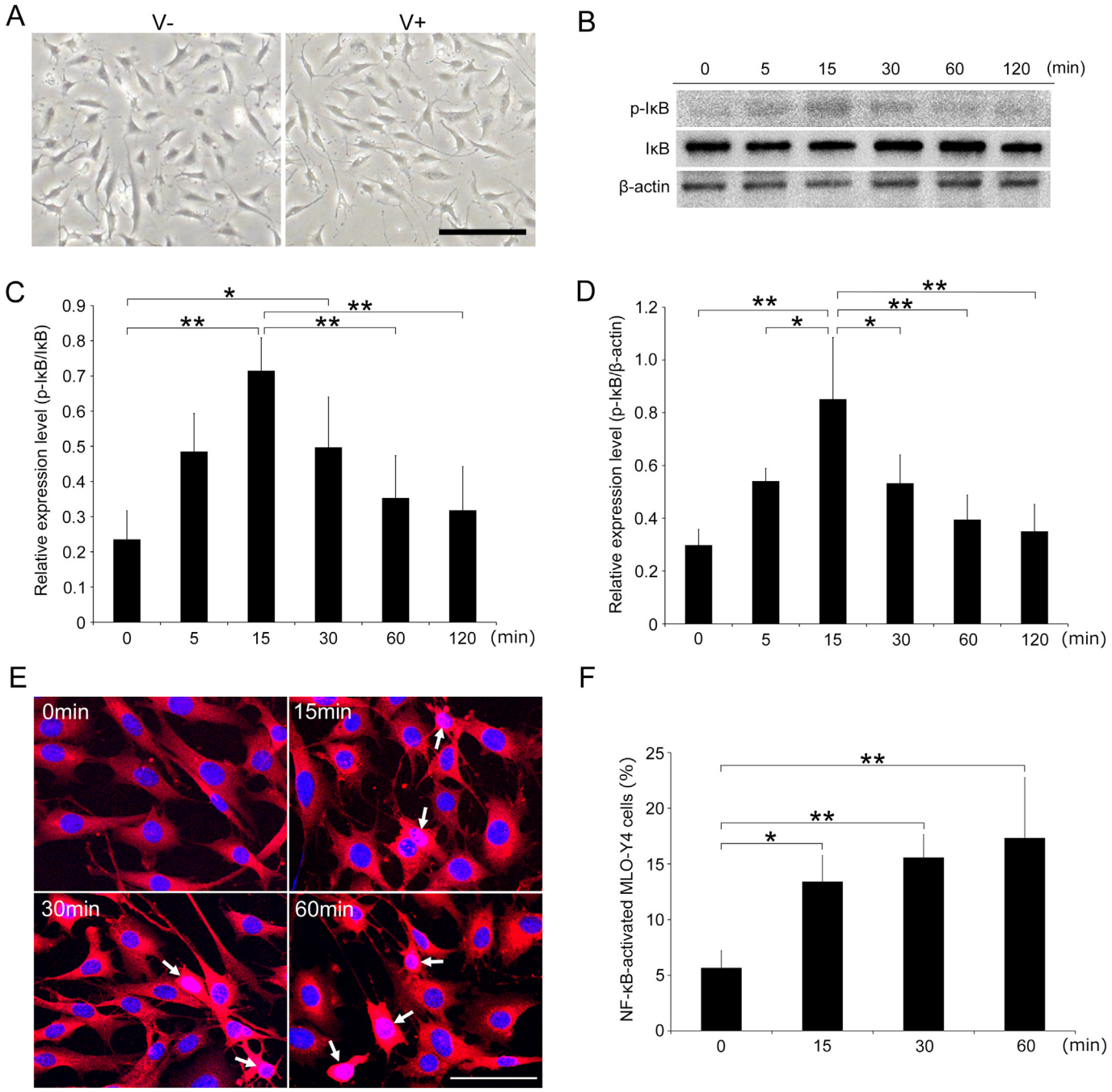

Fig. 3. Vibration activates the NF-κB signaling pathway in MLO-Y4 cells. (A) Representative phase images of MLO-Y4 cells before vibration and at 60 min after vibration. Scale bars = 200 μm. (B) Phosphorylation and degradation of IκB were examined by Western blot analysis. Representative blot data were obtained from 4 independent experiments. β-Actin was used as an internal control. (C, D) Densitometric analysis using ImageJ. The bar graphs represent the ratio of the optical density of the band of p-IκB, normalized to the optical density of IκB or β-actin. The data is expressed as the mean ± SD (n = 4). $*p < 0.05$, $**p < 0.01$. (E) Representative fluorescent images of MLO-Y4 cells at 0, 15, 30, and 60 min after vibration. Arrows indicate NF-κB-activated MLO-Y4 cells. Scale bars = 100 μm. (F) The ratio of NF-κB-activated MLO-Y4 cells to all MLO-Y4 cells. The data is expressed as the mean ± SD (n = 4). $*p < 0.05$, $**p < 0.01$.

NF-κB in osteocytes on the compression side of the alveolar bone in rats. Previously, Takano-Yamamoto et al. reported that vibration (3 gf at 70 Hz) applied to a tooth for 3 min at day 0 and then at day 7 of orthodontic tooth movement resulted in a significant increase in tooth movement on day 9, and a doubling of it on day 21. This increased movement was attributed to enhanced bone resorption due to increasing numbers of osteoclasts not only on the surface of the alveolar bone around the periodontal ligament, but also deep in the alveolar bone [10]. Therefore, vibration could exert a positive effect on osteoclastogenesis by enhancing RANKL expression in osteocytes *via* NF-κB activation on the compression side of the alveolar bone during orthodontic tooth movement *in vivo*, thus leading to acceleration of tooth movement.

Osteocytes are derived from osteoblasts that become buried in the bone matrix. Despite their location in bone, osteocytes sense mechanical stress and transduce mechanical loading through three-dimensional networks that send biochemical signals not only to other osteocytes, but also to osteoclasts and osteoblasts present on the bone surface *via* their cellular dendritic processes [20–22,24,29–31]. Additionally, osteocytes isolated from mice express more RANKL than osteoblasts, and mice in

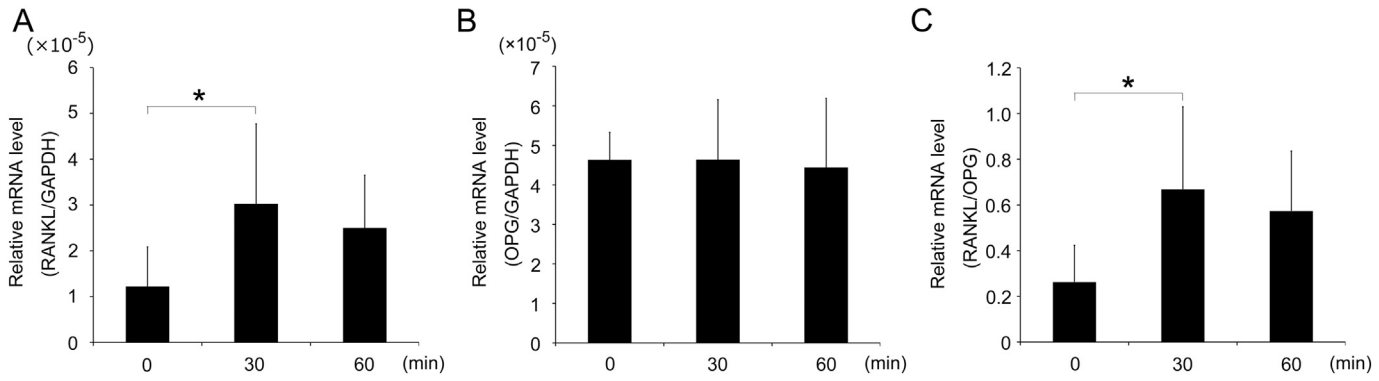

Fig. 4. The effect of vibration on RANKL and OPG gene expression in MLO-Y4 cells. The expression of RANKL, OPG, and RANKL/OPG in vibrated MLO-Y4 cells was determined by real-time quantitative polymerase chain reaction. (A) The expression of RANKL mRNA. (B) The expression of OPG mRNA. (C) The ratio of RANKL/OPG. The data are expressed as the mean ± SD (n = 7) normalized to the expression of GAPDH mRNA. *$p < 0.05$.

which RANKL in osteocytes is selectively deleted show a marked increase in bone volume and a decrease in the number of osteoclasts with growth [17]. These results indicate that RANKL expressed by osteocytes plays an important role in adult bone remodeling [17]. Therefore, we hypothesized that vibration might regulate RANKL expression in osteocytes during orthodontic tooth movement. It was earlier reported that the expression of RANKL decreases in MLO-Y4 cells with vibratory stimulation (0.3 gf at 30, 60, 90 Hz) for 1 h [32]. In contrast, in another study in which compression force (2.4 g/cm^2) or mechanical scratching using a 21-gauge injection needle was applied to MLO-Y4 cells in 3-dimensional culture, RANKL expression increased [33,34]. Additionally, mice in which RANKL in osteocytes was selectively deleted show decreased osteoclast formation during experimental tooth movement *in vivo*, indicating that RANKL expressed by osteocytes is involved in osteoclast formation induced by tooth movement [29]. In the present study, we applied vibration (0.5 gf at 48.3 Hz) to MLO-Y4 cells for 1 min and found that RANKL expression increased significantly at 30 min after vibration. Consistent with this *in vitro* finding, vibration applied during experimental tooth movement in adult rats increased RANKL expression in osteocytes on the compression side of the alveolar bone by 1 h after the application of vibration compared with the control expression. Importantly, vibration markedly increased RANKL expression in osteocytes in the TM + V group on the compression side compared with that for TM group at 3 and 6 h after the experimental tooth movement and vibration. Our results support the findings of previous reports that mechanical stimulation increases the level of RANKL expression in osteocytes. More importantly, when we co-cultured vibrated MLO-Y4 cells and RAW264.7 cells for 6 days, the number of TRAP(+) MNCs was significantly higher for the vibration group than for the control group. This result corroborated our *in vivo* and *in vitro* findings that vibration increased RANKL expression in osteocytes. Taken together, these findings suggest that vibration may be involved in enhancing osteoclast formation during experimental tooth movement *via* increasing the RANKL expression in osteocytes on the compression side of the alveolar bone.

Because osteoclasts arise from cells of the monocyte/macrophage lineage, we investigated the direct effects of vibration of RAW264.7 pre-osteoclast cells on osteoclast formation. In previous reports, the direct effect on osteoclast formation of mechanical stress, such as periodic vibration [35], periodic tensile force [36], sustained tensile force [37], and microgravity [38,39], applied to osteoclast progenitors was investigated. Vibration (0.3 gf at 45 Hz) for 15 min per day for 4 days or 10% tensile force at 30 cycles per minute (0.5 Hz) for 48 h applied to RAW264.7 cells decreased the number of osteoclasts formed [35,36]. In contrast, a microgravity environment was shown to promote osteoclastogenesis of osteoclast progenitors isolated from mice or RAW 264.7 cells [38,39]; and −100 kPa or lower continuous tensile force on human macrophage cells promoted osteoclastogenesis with induction of osteoclastogenic markers in a dose-dependent manner [37]. Additionally, low-magnitude strain suppressed osteoclast fusion and activation, while high-magnitude strain promoted osteoclast fusion and activation related to a mechanical magnitude-dependent response of receptor activator of nuclear factor kappa B (RANK) expression [40]. Thus, the direct effect of mechanical stress applied to osteoclast progenitors on osteoclastogenesis could vary depending on the type, strength or loading time of the mechanical stress. In the present study, to determine the direct effects of applying vibration to osteoclast progenitors in the early and late stages of osteoclast differentiation, we applied vibration (0.5 gf at 48.3 Hz) to RAW264.7 cells for 1 min at 24 h (early stage of differentiation) or 72 h (late stage of differentiation) after cell seeding. TRAP(+) MNCs were counted at 24 or 48 h after vibration. At the early stage of differentiation, the number of TRAP-positive cells with 3–5 nuclei increased slightly, but not significantly, in the vibration group compared with that for the control group. There was also no significant difference in the number of large TRAP-positive cells with 6–9 nuclei or > 10 nuclei. The application of vibration at the late stage of differentiation did not affect the number of TRAP-positive cells (3–5, 6–9, and ≧ 10) compared with that for the control group. However, when the same vibration was applied to RAW264.7 cells without RANKL, the cell number was significantly higher at 48 h after vibration compared with the control group. Previously, Takano-Yamamoto et al. reported that when vibration (3 gf at 70 Hz) was applied to adult rats, the number of pre-osteoclasts increased significantly compared with that for the control group but that the number of TRAP-positive cells with 3 or more nuclei was unaffected [10]. Taken together, these findings suggest that vibration could enhance the proliferation of osteoclast progenitors, but not affect osteoclast differentiation.

It is known that mechanical stress activates NF-κB by degrading the inhibitor of NF-κB (IκB) to allow nuclear translocation of NF-κB [25,27]. It was reported that the nuclear translocation of NF-κB in osteoblasts is induced by mechanical extension force (1% elongation, 0.05 Hz), fluid shear stress (12 dyn/cm ^{2}) or microgravity [41,42]. However, little is known about the relationship between mechanical stress and activation of NF-κB in osteocytes. Therefore, we applied vibration (0.5 gf at 48.3 Hz) to MLO-Y4 cells for 1 min and investigated the phosphorylation of IκB and nuclear localization of NF-κB. We observed phosphorylation of IκB from 5 min after the vibration stimulus, with the level of p-IκB reaching a peak at 15 min and decreasing with time thereafter. In addition, immunofluorescence for NF-κB confirmed that vibration led to a significant increase in nuclear localization of NF-κB at 15 min after vibration. These results indicate that vibration activated NF-κB signaling in osteocytes. As well as the *in vitro* findings, the application of vibration during experimental tooth movement in rats *in vivo* significantly increased the nuclear translocation of NF-κB in osteocytes in the TM + V group on the compression side of alveolar bone

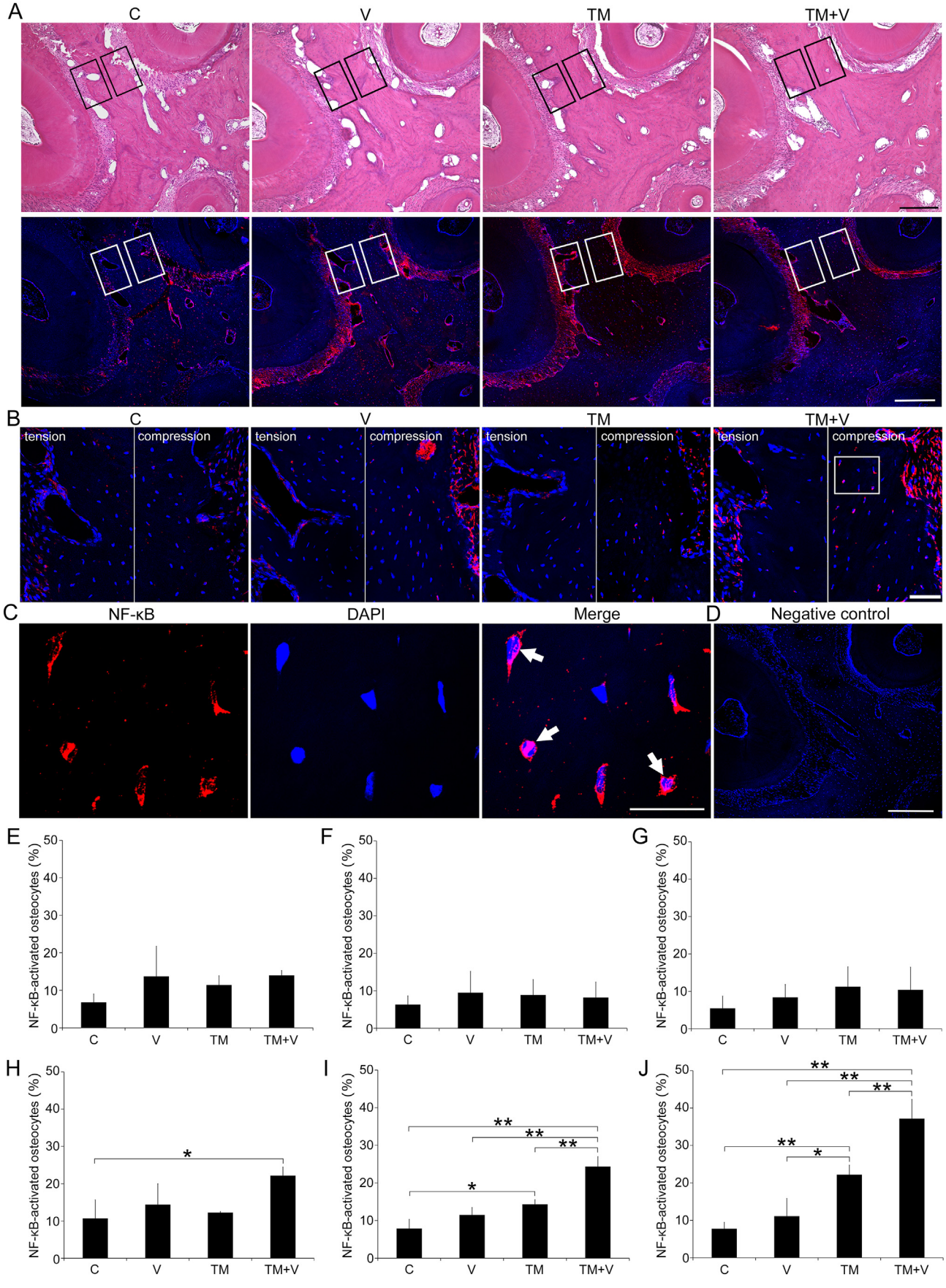

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Fig. 5. Effects of supplementary vibration applied during experimental tooth movement on NF-κB activation in osteocytes. (A) Horizontal sections of the maxillary first molar were obtained from C, V, TM, and TM + V groups at 1, 3, and 6 h after vibration. The 6-hour sections are shown as a representative. Serial sections were stained with hematoxylin and eosin (upper) and treated with anti-NF-κB p65 (lower). The rectangles (300 μm in height × 200 μm in width) indicate the region of interest (ROI) for measurement on both compression and tension sides. Scale bars = 250 μm. (B) High magnification images of ROI on both compression and tension sides shown in panel A. A rectangle indicates the region of magnification shown in panel C. Scale bar = 50 μm. (C) High magnification images of NF-kB-activated osteocytes on the compression side of alveolar bone of the TM + V group. Arrows indicate NF-κB-activated osteocytes. Scale bar = 25 μm. (D) Negative control image of NF-κB p65. Scale bar = 250 μm. (E, F, G) The ratio of NF-κB-activated osteocytes to all osteocytes on the tension side at 1 h (E), 3 h (F), and 6 h (G) after vibration. (H, I, J) The ratio of NF-κB-activated osteocytes to all osteocytes on the compression side at 1 h (H), 3 h (I), and 6 h (J) after vibration. The data are expressed as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$. C, control group; V, vibration group; TM, tooth movement group; TM + V, tooth movement and vibration group.

at 1 h after vibration compared with that for the C group. Then, by 6 h after vibration, the percentage of osteocytes with translocated NF-κB in their nucleus in the TM + V group on the compression side was about 5 times higher than that for the C group. More importantly, the proportion of osteocytes with NF-κB-positive nuclei in the TM + V group was significantly higher than that for the TM group on the compression side at 3 and 6 h after vibration. In contrast, the percentage of osteocytes with translocated NF-κB in their nucleus in the V group on both compression and tension sides was slightly higher than that for the C group at all times after vibration, but the increase was not significant. It is possible that the slight increase in the nuclear translocation of NF-κB induced by vibration *in vivo* was a result of the vibration being transmitted to the alveolar bone *via* the tooth or the periodontal ligament *in vivo*, suggesting that vibratory stimulation was distributed throughout the alveolar bone heterogeneously. In contrast, the percentage of osteocytes with nuclear translocation of NF-κB in the TM group on the compression side increased with time, and showed a significant increase at 3 and 6 h after vibration compared with that for the C group, whereas there was no such effect on the tension side. Interestingly, vibration did not affect the nuclear translocation of NF-κB in osteocytes on the tension side during experimental tooth movement. These results suggest that NF-κB in osteocytes was activated by compression force and that vibration could enhance the compression force-induced activation of NF-κB in osteocytes.

Considering the function of NF-κB in bone remodeling, it has been reported that NF-κB in mature osteoblasts is involved in suppression of bone formation [26]. Recently, Pacios et al. reported that bacteria-induced periodontal bone loss was inhibited, along with a reduction in

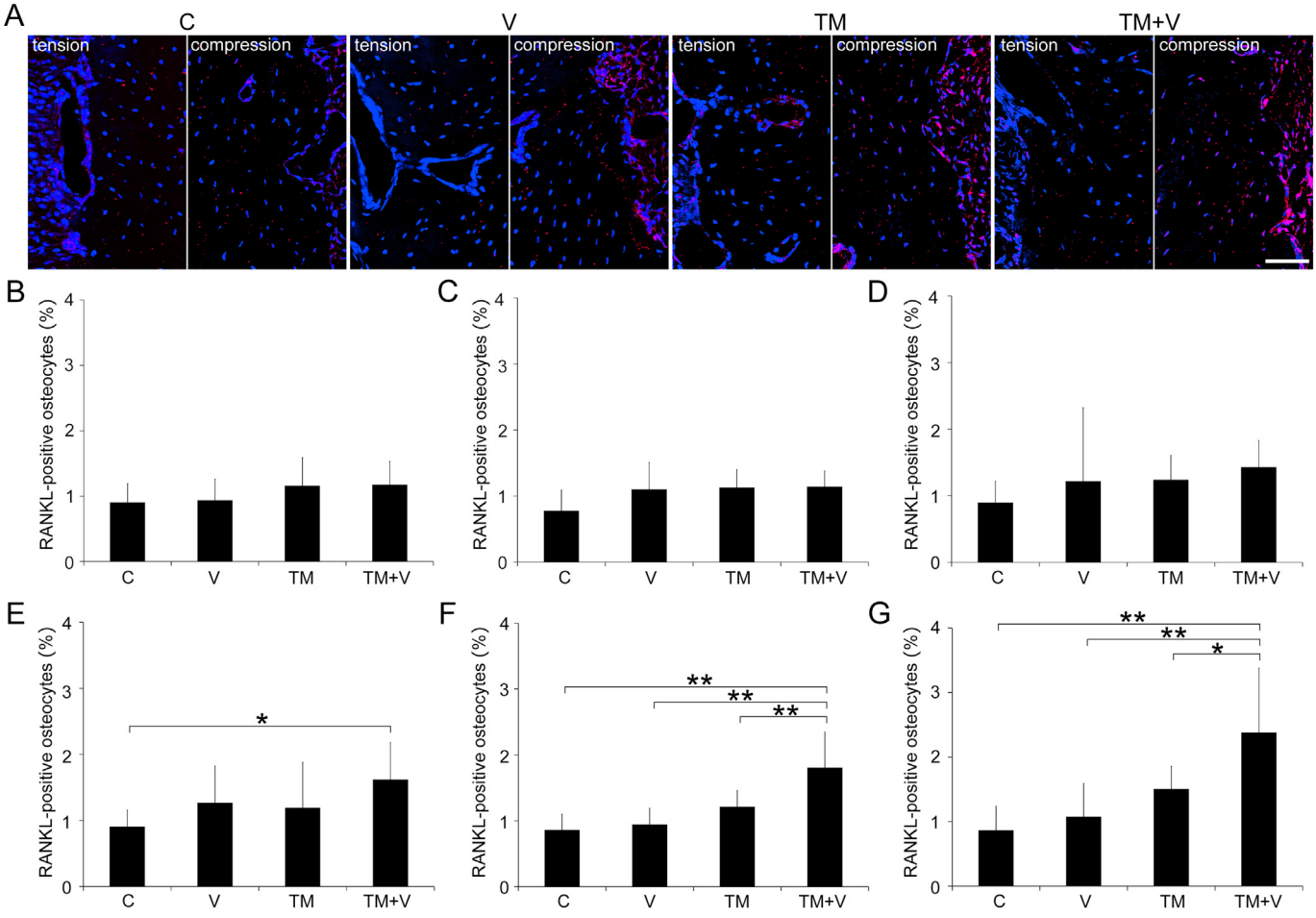

Fig. 6. Experimental tooth movement and supplementary vibration increases RANKL expression in osteocytes on the compression side. (A) Horizontal sections of alveolar bone of the maxillary first molar were obtained from C, V, TM, and TM + V groups at 1, 3, and 6 h after vibration and were treated with anti-RANKL. The 6-hour sections are shown as a representative. Scale bar = 50 μm. (B, C, D) The ratio of RANKL-positive osteocytes to bone volume (BV) on the tension side at 1 h (B), 3 h (C), and 6 h (D) after vibration. (E, F, G) The ratio of RANKL-positive osteocytes to bone volume (BV) on the compression side at 1 h (E), 3 h (F), and 6 h (G) after vibration. The data are expressed as the mean ± SD (n = 9). *$p < 0.05$, **$p < 0.01$. C, control group; V, vibration group; TM, tooth movement group; TM + V, tooth movement and vibration group.

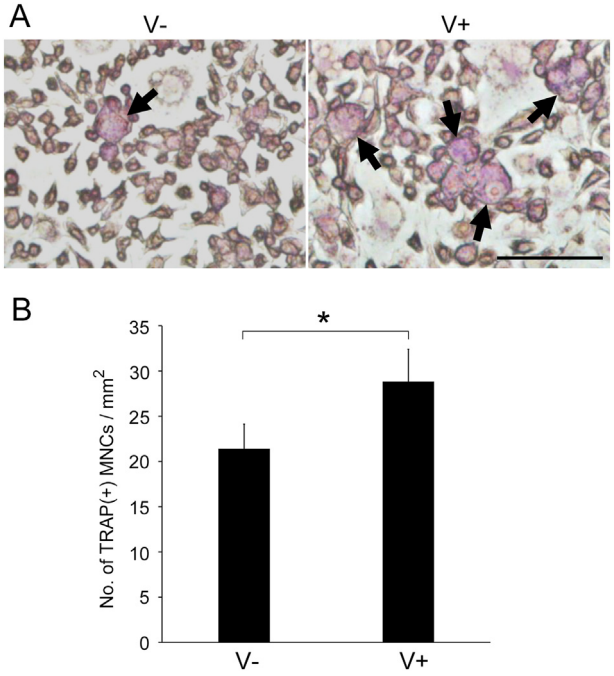

Fig. 7. Vibration to MLO-Y4 cells stimulates osteoclast formation. (A) Non-vibrated (V−) or vibrated (V+) MLO-Y4 cells were co-cultured with RAW264.7 cells for 6 days, then fixed and stained for TRAP. Arrows indicate TRAP-positive multinucleated cells (TRAP[+] MNCs) containing three or more nuclei. Scale bar = 50 μm. (B) The number of TRAP(+) MNCs containing three or more nuclei per unit area at 6 days after co-culture. The data are expressed as the mean ± SD (n = 3). *$p < 0.05$.

the number of RANKL-positive osteoblasts and osteocytes, in transgenic mice that expressed a dominant-negative mutant IκB kinase (IKK) compared with these parameters for the control mice [43]. In addition, TNF-α-stimulated osteoblast cell line MC3T3 cells or MLO-Y4 cells showed the induction of nuclear translocation of NF-κB in either cell type after 1 h of stimulation, which translocation was followed by an increase in the expression of RANKL mRNA [43]. These findings suggest that inflammatory stimulation of alveolar bone could induce nuclear translocation of NF-κB in osteoblasts and osteocytes, leading to RANKL expression, and finally resulting in inflammatory bone resorption. In the present study, we demonstrated that the application of vibration to MLO-Y4 cells activated NF-κB signaling and increased RANKL mRNA expression *in vitro*. Furthermore, we revealed that nuclear localization of NF-κB and RANKL expression in osteocytes were significantly increased in TM + V group compared with TM group on the compression side, not tension side, at 3 and 6 h after experimental tooth movement. Taken together, these findings suggest that vibration may have enhanced osteoclastic bone resorption on the compression side of alveolar bone during orthodontic tooth movement by inducing RANKL expression *via* NF-κB signaling in osteocytes.

It is well known that bone remodeling during orthodontic tooth movement couples bone resorption with bone formation in the alveolar bone around the root of the tooth. In the present study, we showed that vibration did not influence the nuclear translocation of NF-κB or RANKL expression in osteocytes on the tension side of alveolar bone at 1, 3, and 6 h after experimental tooth movement and vibration. In contrast, it is reported that vibration has osteogenic effects in adult sheep [44] and growing mice [45]. In addition, vibration has been reported to induce the osteogenic process in mesenchymal stem cells by increasing the cell proliferation, alkaline phosphatase activity, and mineralization *in vitro* [46,47]. Therefore, vibration would probably have some positive effects on bone formation on the tension side of alveolar bone during orthodontic tooth movement. However, further investigations are needed to clarify the osteogenic effect of vibration during orthodontic tooth movement.

5. Conclusions

Vibration applied to cells of the RAW264.7 pre-osteoclast cell line enhanced cell proliferation, but did not affect osteoclast differentiation. In MLO-Y4 cells, vibration induced phosphorylation of IκB and nuclear localization of NF-κB, as well as increased RANKL mRNA expression. Consistent with the *in vitro* findings, vibration applied during experimental tooth movement increased NF-κB activation and RANKL expression in osteocytes on the compression side of the alveolar bone *in vivo*. More importantly, in a co-culture of MLO-Y4 cells and RAW264.7 cells, vibration applied to MLO-Y4 cells enhanced osteoclastogenesis. These findings suggest that vibration could enhance osteoclastogenesis on the compression side of alveolar bone during orthodontic tooth movement by increasing the number of pre-osteoclasts and up-regulating RANKL expression *via* NF-κB activation in osteocytes.

Declarations of interest

All authors declare that they have no competing interest.

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