Effect of continuous compressive force on the expression of RANKL, OPG, and VEGF in osteocytes

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ABSTRACT

The present study aimed to investigate the effect of a compressive force (CF) on the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), and vascular endothelial growth factor (VEGF) in murine osteocytes (MLO-Y4) as well as animal study. After application of a CF for 1, 3, 6, and 12 h, gene and protein expression of RANKL, OPG, and VEGF in MLO-Y4 cells were determined by real-time PCR and enzyme-linked immunosorbent assay (ELISA). Furthermore, the effect of a stretch-activated (S-A) channel was examined by gadolinium (Gd³⁺) administration. In an animal experiment, the expression of these factors in osteocytes of alveolar bone was examined after experimental tooth movement in rats. After CF application, significant increases in RANKL, VEGF and RANKL/OPG ratio were shown. The upregulated gene and protein levels of these factors were reduced by Gd³⁺ administration. After tooth movement, upregulated RANKL and VEGF were immunohistochemically shown in osteocytes of alveolar bone. These findings suggest that CF application on osteocytes elevates expression of osteoclast-inducing factor and angiogenesis factor in vivo and vitro.

Bone tissue shows constant remodeling by osteoblastic bone formation and osteoclastic bone resorption (11, 19). Therefore, bone remodeling has been suggested to be regulated by a crosstalk between osteoblasts, osteoclasts, and osteocytes (18). Tumour necrosis factor (TNF) superfamily member 11 (RANKL) (27) and TNF superfamily member 11b (OPG) (28) are crucial factors for osteoclast differentiation. Osteoblasts have been thought to be the main source of RANKL for osteoclastogenesis (4, 22). Vascular endothelial growth factor (VEGF) plays a major role in angiogenesis (8), and it is thought to be an important factor for skeletal development and bone regeneration (3).

It is well known that mechanical stress can influence bone remodeling. Nettelhoff et al. reported that the highest RANKL/OPG ratio was observed after compressive force (CF) application in osteoblasts (13). Tripuwabhrut et al. also suggested that CFs on osteoblasts enhance osteoclastogenesis by an increase in RANKL expression and decrease in OPG expression (21). It has been reported that cyclic tensile forces increase the mRNA and protein expressions of VEGF in osteoblastic MC3T3-E1 cells (9), and this reaction was inhibited by gadolinium (Gd³⁺), a stretch-activated (S-A) channel blocker (12). Reher et al. showed that the production of VEGF in human osteoblasts can be increased by ultrasounds (16). These results suggest that the application of mechanical stress on osteoblasts can affect RANKL,
Osteocytes are derived from osteoblasts and are embedded in bone matrix and act as mechanosensory cells through the lacuno-canaliculat network (1). Recently, it was shown that osteocytes can be a main source of RANKL during bone remodeling (10, 24). However, the effects of mechanical forces on the expression of factors related to bone remodeling as well as angiogenesis and this mechanism are still unclear. Therefore, in this study, we examined the effects of CF and Gd³⁺ treatment on the expression of RANKL, OPG, and VEGF in murine osteocyte (MLO-Y4) cell line. We also observed the expression of these factors in osteocytes after orthodontic tooth movement in rats.

MATERIALS AND METHODS

**Cell culture.** MLO-Y4 cells were obtained from Keraffast (Boston, MA, USA), and cultured on collagen-coated plates (pig tendon type I collagen; IWAKI, Tokyo, Japan) in minimum essential medium Eagle alpha medication (α-MEM) with 2.5% heat inactivated fetal bovine serum (FBS; Daiichi Chemical, Tokyo, Japan) and 2.5% heat inactivated newborn calf serum (Thermo Fisher Scientific, Waltham, MO, USA). The cells were cultured with 240 ng/mL kanamycin (Meiji Seika, Tokyo, Japan), 1 mg/mL amphotericin-B (ICN Biomedicals Corp, Costa Mesa, CA, USA), 500 ng/mL penicillin (Sigma Aldrich, Saint Louis, MO, USA) at 37°C in 5% CO₂. The cells were subcultured by treatment with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) followed by plating at a density of 5 × 10⁴ cells/well in 6-well plates. For all experiments, cells between the 4th and 6th passages were used.

**Application of CF and cell viability.** A thin glass plate was placed over a confluent cell layer in 6-well plates at a density of 5 × 10⁴ cells according to the previous study (Fig. 1) (20). Serum-free conditioned media was used for CF experiment in order to eliminate the influence of serum for cytokine expression. In this study, 1.0 g/cm² of CF for various loading time (1, 3, 6, and 12 h) were applied because it was reported that 2.0 g/cm² of CF application induces osteoblast apoptosis (2). At 1, 3, 6, and 12 h after CF application, cells were treated with 0.05% trypsin-EDTA and the number of survived cells was counted with 0.5% trypan blue staining (n = 7, respectively).

**Total RNA extraction and cDNA synthesis.** Total RNA was isolated from the cell cultures using a Quickprep Total RNA extraction kit (Amersham Biosciences, Tokyo, Japan). The quantity and purity of the isolated RNA were checked by reading the absorbance at a wavelength of 260 nm by a spectrophotometer (BioSpec-nano; Shimadzu Corporation, Kyoto, Japan). Single-stranded cDNA was synthesized from 1 μg of total RNA using Oligo (dT) 20 primer (Toyobo, Osaka, Japan) and a ReverTra Ace-α first-strand cDNA synthesis kit (Toyobo). Primers were designed using Oligo primer analysis software (https://www.oligo.net/) to amplify adjacent exonic sequence on each side. 

- **Tnfsf11/Rankl** (NM_011613): 5’- CATCGCTCTGTTCCTGTACTTTC -3’ (forward), 5’- AGGAGTCAGGTAGTGTGTCTTCA -3’ (reverse);
- **Tnfrsf11b/Opg** (NM_008764): 5’- ACCCAGAAAATGGTCATCAGC -3’ (forward), 5’- CTGCAATACACACACTCATCACT -3’ (reverse);
- **Vegf** (NM_01025250): 5’- ATGCGGATCAAACCTCA -3’ (forward), 5’- TTCTGGCTTTGTTCTTCA -3’ (reverse);
- **Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) primers** was used as a control primer: 5’- ATGGCCTGGTGACTCAGC -3’ (forward), 5’- TCTTGGCTTTGTTCTTCA -3’ (reverse);
- **Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) primers** was used as a control primer: 5’- ATGGCCTGGTGACTCAGC -3’ (forward), 5’- TCTTGGCTTTGTTCTTCA -3’ (reverse);
- **Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) primers** was used as a control primer: 5’- ATGGCCTGGTGACTCAGC -3’ (forward), 5’- TCTTGGCTTTGTTCTTCA -3’ (reverse);

**Quantitative real-time polymerase chain reaction (PCR) analysis.** Quantitative real-time PCR was carried out using the SYBR Green I assay in conjunction with an ABI Prism 7700 sequence detection system (Biosystems, Foster City, CA, USA). A template cDNA at a volume of 1 μL was used during the PCR under the following parameters: 2 min at 50°C; 10 min at 95°C; and then 40 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C. SYBR Green I dye intercalation into the minor groove of dou-
ble-stranded DNA reached maximum emission at 530 nm. PCR reactions for each sample were repeated three times for both the target gene and the control. Quantitative results of real-time fluorescence PCR were assessed by a cycle threshold (Ct) value, which identifies a cycle when the fluorescence of a given sample becomes significantly different from the baseline signal. Relative quantifications of the Rankl, Opg, and Vegf signals were normalized and expressed relative to the Gapdh signals (n = 7, respectively).

Measurement of protein concentrations. The culture medium was collected and cleared at 3000 rpm for 5 min and the amount of protein concentration of RANKL (Murine sRANK Ligand Mini ABTS ELISA Development kit; PeproTech Inc, Rocky Hill, NJ, USA), OPG (Osteoprotegerin Mouse Immunoassay kit; R&D Systems, Minneapolis, MN, USA), and VEGF (Murine VEGF Mini ABTS ELISA Development kit; PeproTech Inc) were measured using the quantitative sandwich enzyme immunoassay technique. Standard curves were obtained as usual, and the experiment was repeated three times (n = 7, respectively).

Effects of Gadolinium (Gd$^{3+}$). The following 4 groups were examined. 1: CF(−)Gd$^{3+}$ (−) group, 2: CF(−) Gd$^{3+}$ (+) group, 3: CF(+)Gd$^{3+}$ (−) group, 4: CF(+) Gd$^{3+}$ (+) group. Because it has been reported that 1–100 μM gadolinium inhibited S-A channels (26), cells were incubated with 10 μM Gd$^{3+}$ chloride hexahydrate (Wako, Osaka, Japan) for 30 min. Then, CF was applied for 3 h to examine the mRNA expressions of Rankl, Opg, Vegf mRNAs and Rankl/Opg ratio (n = 7, respectively) according to the previous study (9). Furthermore, CF was also applied for 12 h to measure the protein concentration of these factors (n = 7, respectively).

Animal studies. All animal experimental procedures were approved by the Ethics Committee of the Hiroshima University Faculty of Dentistry (A-17-166). Ten 7-week-old Wistar rats were divided into two groups. The first group was without tooth movement (n = 5). The second group underwent tooth movement by insert an elastic band between the upper first and second molars for 5 days (n = 5) (23). The animals were sacrificed under general anesthesia with medetomidine (1.0 mg/mL; Kyoritsu Seiyaku Corporation, Tokyo, Japan), midazolam (5.0 mg/mL; Sandoz, Tokyo, Japan), and butorphanol (5.0 mg/mL; Meiji Seika Pharma, Tokyo, Japan).

Specimens were fixed in 4% paraformaldehyde, decalcified in 14% EDTA (pH 7.4) for 30 days, and embedded in paraffin. The maxillary bones were decalcified and embedded in paraffin. Then, the specimens were sliced into 5-μm thick sections in the frontal direction. For immunohistochemistry, the tissue sections were deparaffinized and incubated with 3% H$_2$O$_2$ in methanol for 30 min. Next, the sections were incubated with primary antibodies against mouse RANKL (ab62516, 1 : 100; Abcam, Cambridge, UK), mouse OPG (ab73400, 1 : 100; Abcam), and mouse VEGF (ab1316, 1 : 200; Abcam) overnight at 4°C. Then, the immunocomplexes were detected using the Histofine Simple Stain MAX-Po rabbit kit (Nichirei, Tokyo, Japan) for RANKL and OPG, and the Histofine Simple Stain MAX-Po mouse kit (Nichirei) for VEGF. The sections were treated with the substrate reagent 3, 3′-diaminobenzidine tetra-hydrochloride. As negative control, isotype control was used instead of primary antibody. All sections were counterstained with hematoxylin, and expressions of RANKL, OPG and VEGF were observed both in the pressure side and tension side of the alveolar bone around the mesial root apex of the first molar.

To investigate the osteoclasts appearance, specimens were subjected to tartrate-resistant acid phosphatase (TRAP) staining. In each specimen, five sections, selected at 35-μm intervals, were examined based on the results of previous study (25).

Statistical treatment. Statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, NC, USA). Statistical significances in mRNA and protein levels after the application of CF were assessed by analysis of variance followed by the
Fisher’s method. A $P < 0.05$ was considered statistically significant.
RESULTS

Time-course effects of CF and cell viability
No significant difference was found in the number of survived cells between control and CF application group, confirming that 1.0 g/cm² of CF was appropriate (Fig. 2). Rankl, Vegf and Rankl/Opg ratio reached maximum levels after 3 h of CF application (Fig. 3). The gene expression level of Opg was significantly lower after 3 h of CF application (Fig. 3). Protein levels of RANKL, VEGF and RANKL/OPG ratio of the CF group were significantly higher than that of the control group at 6 and 12 h; and 1, 3, and 12 h; 3, 6 and 12 h (Fig. 4); respectively. OPG protein was significantly lower in the CF group than that of the control group at 3, 6, and 12 h (Fig. 4). From these results, it is clear that gene and protein expression of RANKL/OPG ratio and VEGF were enhanced significantly by 1.0 g/cm² CF in MLO-Y4 cells.

Effect of Gd³⁺ treatment
In the control group (non-CF group), the mRNA levels of Rankl, Opg and Vegf were not influenced by Gd³⁺ treatment (Fig. 5). Gd³⁺ also did not have any effect on the number of survived cells in the control group (data not shown). Rankl mRNA and the Rankl/Opg ratio in the CF group were significantly reduced by Gd³⁺ (Fig. 5). Opg gene expression in the CF group was significantly increased by Gd³⁺ (Fig. 5). Vegf in the CF group was slightly reduced but not significantly by Gd³⁺ (Fig. 5). Protein levels of RANKL, VEGF and RANKL/OPG ratio were significantly lower in the Gd³⁺ treatment group than in the non-treatment group (Fig. 6). OPG protein was significantly higher in the Gd³⁺ treatment group than in the non-treatment group (Fig. 6). Based on these results, Gd³⁺ treatment reduced the up-regulated gene and protein expression of RANKL/OPG ratio and VEGF by 1.0 g/cm² CF application through S-A channel in MLO-Y4 cells.

Immunohistochemical and TRAP staining
RANKL and VEGF expression was not observed in the non-tooth movement groups (Fig. 7A and 7G). Five days after tooth movement, many RANKL and VEGF positive osteocytes were observed in the pressure side of the alveolar bone (Fig. 7B and 7H). On the other hand, OPG was not expressed in both groups (Fig. 7D and 7E). RANKL, OPG and VEGF positive osteocytes were not observed in the tension side (Fig. 7C, 7F and 7I). As well as osteocytes, many RANKL and VEGF positive osteoblasts were observed in the pressure side of the alveolar bone of tooth movement group (Fig. 7B and 7H).

In the control group, small number of osteoclasts were detected in the alveolar bone (Fig. 7J). However, many osteoclasts were found in the tooth movement group after five days in the alveolar bone on the pressure side (Fig. 7K).

DISCUSSION
Osteocytes are differentiated from osteoblasts, which are embedded in the bone matrix with extended dendritic processes. For crosstalk among the bone component cells, the dendritic processes are thought to be essential for osteocytes to connect each other and adjacent other cell types (1). Although the importance of cellular reaction for bone remodeling is well known, the mechanism that osteocytes promote cytokines for bone remodeling after receiving mechanical stress, especially CF, is still unclear. Nettelhoff et al. reported that the mRNA expression of Rankl as well as the Rankl/Opg ratio peaked in osteoblasts after 5% CF application by using the Flexcell Compression Plus System (13). Sanchez et al. also used the Flexcell Compression Plus System, and showed that Opg gene expression was significantly decreased after 4 h of CF in osteoblasts (17). Tripuwabhrut et al. reported that the application of 4.0 g/cm² CF induced significantly increased expression of Rankl mRNA in osteoblasts. They also showed that 2.0 and 4.0 g/cm² CF significantly reduced the expression of Opg mRNA (21). However, most of these previous study described bone metabolism by osteoblasts. Therefore, we focused on osteocytes to examine the relationship between mechanical stress and bone metabolism. In our study, gene and protein expression levels of RANKL in MLO-Y4 cells reached a maximum 3 h after applying a CF, and OPG gene and protein expressions showed a significant decrease. Also in our animal study, osteocytes exhibited high expression of RANKL and low OPG expression after tooth movement and a number of osteoclasts were observed in the tooth movement group. From these findings, it is confirmed that osteocytes can respond to mechanical stress which leads upregulating RANKL and downregulating OPG both in vitro and in vivo.

Previous studies have shown that recombinant human VEGF can induce osteoclast generation in osteopetrotic (op/op) mice which were characterized by a deficiency in osteoclasts due to a lack of functional macrophage-colony stimulating factor (M-CSF), and a combination of VEGF and RANKL can sup-
Fig. 5  Mean and standard deviation of effects of Gd$^{3+}$ on the gene expression of Rankl, Opg, Vegf and Rankl/Opg ratio after CF application. Gd$^{3+}$ treatment significantly reduced Rankl mRNA and the Rankl/Opg ratio in the CF group. (•; $P < 0.05$ and **; $P < 0.01$)

Fig. 6  Mean and standard deviation of effects of Gd$^{3+}$ on the protein concentration of RANKL, OPG, VEGF and RANKL/OPG ratio after CF application. Protein expression of RANKL, VEGF and RANKL/OPG ratio were significantly inhibited by Gd$^{3+}$ treatment. (•; $P < 0.05$ and **; $P < 0.01$)
Compressive force on osteocyte

and accelerate the rate of tooth movement (6, 7), showing that VEGF is closely related to bone remodeling by inducing osteoclasts as well as angiogenesis. Therefore, in the present study, we conducted

port the generation of osteoclasts in vitro (5, 14). It was also demonstrated that local administration of recombinant human VEGF during experimental tooth movement can increase the number of osteoclasts

Fig. 7 Immunohistochemical and TRAP staining. (A) non-tooth movement for RANKL, (B) tooth movement in the pressure side for RANKL, (C) tooth movement in the tension side for RANKL, (D) non-tooth movement for OPG, (E) tooth movement in the pressure side for OPG, (F) tooth movement in the tension side for OPG, (G) non-tooth movement for VEGF, (H) tooth movement in the pressure side for VEGF, (I) tooth movement in the tension side for VEGF, (J) non-tooth movement with TRAP staining, (K) tooth movement with TRAP staining. Note that 5 days after tooth movement, many RANKL and VEGF positive osteocytes were observed and many osteoclasts were also detected in the pressure side of the alveolar bone (Fig. 7B, 7F and 7H). (n = 5, Bar denotes 100 μm, Arrows indicate osteoclasts)
to investigate VEGF expression in osteocytes after CF application. As a result, the expression of VEGF in osteocytes was upregulated by CF in vitro, and also in vivo by orthodontic force. So, it was suggested that osteocytes can regulate bone metabolism and angiogenesis due to receiving mechanical stress.

Furthermore, Motokawa et al. reported that cyclic tensile forces enhance the mRNA and protein expressions of VEGF in MC3T3-E1 cells and Gd$^{3+}$ treatment decreased the amount of VEGF expression through the S-A channel (9). Also in our study, 10 μM Gd$^{3+}$ reduced the expression of VEGF as well as RANKL and RANKL/OPG ratio, which was enhanced by CF application in MLO-Y4 cells. Because the mRNA levels of Rankl, Opg and Vegf as well as the number of survived cells were not influenced by Gd$^{3+}$ treatment in the control group, cytotoxicity was not shown due to 10 μM Gd$^{3+}$. Although it is unknown whether the effect of Gd$^{3+}$ is irreversible, it is thought that the blocking effect on the S-A channel can continue for at least 12 h according to the previous study (9). Concerning the blocking S-A channel by Gd$^{3+}$, Naruse et al. explained the mechanism by increased intracellular Ca$^{2+}$ concentration. They showed that the Ca$^{2+}$ response was inhibited by Gd$^{3+}$, and the Ca$^{2+}$ response disappeared when extracellular Ca$^{2+}$ was removed. These results suggest that stretching the membrane primarily induced extracellular Ca$^{2+}$ entry through S-A channel (12). Qin et al. reported that there was a correlation between the degrees of extension of the cell membrane due to the application of CF and the activation of the S-A channel (15), suggesting that osteocytes receive CFs via the S-A channel.

From these findings, it is possible that osteocytes might play an important role in bone metabolism and angiogenesis, as osteocytes regulate the expression of RANKL, OPG, and VEGF via the S-A channel by responding to mechanical stress.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

1. Bonewald LF (2011) The amazing osteocyte. J Bone Miner Res 26, 229–238.
2. Goga Y, Chiba M, Shimizu Y and Mitani H (2006) Compensative force induces osteoblast apoptosis via caspase-8. J Dent Res 85, 240–244.
3. Hu K and Olsen BR (2016) The roles of vascular endothelial growth factor in bone repair and Regeneration. Bone 91, 30–38.
4. Jimi E, Nakamura I, Amano H, Taguchi Y, Tsurukai T, et al. (1996) Osteoclast function is activated by osteoblastic cells through a mechanism involving cell-to-cell contact. Endocrinology 137, 2187–2190.
5. Kaku M, Niida S, Kawata T, Maeda N and Tanne K (2000) Dose-and time-dependent changes in osteoclast induction after a single injection of vascular endothelial growth factor in osteopetrotic mice. Biomed Res (Tokyo) 21, 67–72.
6. Kaku M, Kohno S, Kawata, T Fujita T, Tokimasa C, et al. (2001) Effects of vascular endothelial growth factor osteoclast induction during tooth movement in mice. J Dent Res 80, 1880–1883.
7. Kohno S, Kaku M, Tsutsui K, Motokawa M, Ohtani J, et al. (2003) Expression of vascular endothelial growth factor and the effects on bone remodeling during experimental tooth movement. J Dent Res 82, 177–182.
8. Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246, 1306–1309.
9. Motokawa M, Kaku M, Tohma Y, Kawata T, Fujita T, et al. (2005) Effects of cyclic tensile forces on the expression of vascular endothelial growth factor (VEGF) and macrophage-colony stimulating factor (M-CSF) in murine osteoblastic MC3T3-E1 cells. J Dent Res 84, 422–427.
10. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-horo M, et al. (2011) Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17, 1231–1234.
11. Nakashima T, Hayashi M and Takayanagi H (2012) New insights into osteoclastogenic signaling mechanisms. Trends Endocrinol Metab 23, 582–590.
12. Naruse K and Sokabe M (1993) Involvement of stretch-activated ion channels in Ca$^{2+}$ mobilization to mechanical stretch in endothelial cells. Am J Physiol 264, 1037–1044.
13. Nettelhoff L, Grimm S, Jacobs C, Walter C, Pabst AM, et al. (2016) Influence of mechanical compression on human periodontal ligament fibroblasts and osteoblasts. Clin Oral Investig 20, 621–629.
14. Niida S, Kaku M, Amano H, Yoshida H, Kataoka H, et al. (1999) Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. J Exp Med 190, 293–298.
15. Qin Y, Zou Y and Hasegawa H (2003) Tensile force and cardiac hypertrophy. Vascular Biology and Medicine 4, 229–235. (in Japanese)
16. Reher P, Doan N, Bradnock B, Meghji S and Harris M (1999) Effect of ultrasound on the production of IL-8, basic FGF and VEGF. Cytokine 11, 416–423.
17. Sanchez C, Gabay O, Salvat C, Henrotin YE and Berenbaum F (2009) Mechanical loading high increases IL-6 production and decreases OPG expression by osteoblasts. Osteoarthritis Cartil 17, 473–481.
18. Seeman E and Delmas PD (2006) Bone quality—the material and structural basis of bone strength and fragility. N Engl J Med 354, 2250–2261.
19. Takayanagi H (2007) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 7, 292–304.
20. Tripuwabhrut P, Mustafa K, Brudvik P and Mustafa M (2012) Initial responses of osteoblasts derived from human alveolar bone to various compressive force. *Eur J Oral Sci* **120**, 311–318.
21. Tripuwabhrut P, Mustafa M, Gjerde CG, Brudvik P and Mustafa K (2013) Effect of compressive force on human osteoblast-like cells and bone remodeling: an in vitro study. *Arch Oral Biol* **58**, 826–836.
22. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, *et al.* (1999) Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor; receptor activator of NF-kappa B ligand. *Bone* **25**, 517–523.
23. Waldo CM and Rothblatt JM (1954) Histologic response to tooth movement in the laboratory rat; procedure and preliminary observations. *J Dent Res* **33**, 481–486.
24. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, *et al.* (2011) Matrix-embedded cells control osteoclast Formation. *Nat Med* **17**, 1235–1241.
25. Yamamoto T, Kaku M, Sumi H, Yashima Y, Izumino J, *et al.* (2018) Effects of loxoprofen on the apical root resorption during orthodontic tooth movement in rats. *PloS One* **13**, e0194453.
26. Yang X and Sachs F (1989) Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. *Science* **243**, 1068–1071.
27. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, *et al.* (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* **95**, 3597–3602.
28. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, *et al.* (1998) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* **139**, 1329–1337.