Jagged1-Notch2 signaling induces root resorption via RANKL and IL-6

(Jagged1-Notch2 シグナルは RANKL と IL-6 を介して歯根吸収を惹起する)

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Abstract: The Notch signaling plays an important role in osteoblast and osteoclast differentiation. However, the role of Notch signaling in root resorption during orthodontic treatment is not yet fully understood. In this study, we first investigated the expressions of Jagged1, Notch2, receptor activator of NF-kappaB ligand (RANKL) and interleukin (IL)-6 in areas of root resorption during experimental tooth movement in rats *in vivo*. We then assessed the effects of compression forces (CF) with/without γ-secretase inhibitor （GSI; inhibitor of Notch signaling） on Jagged1, RANKL and IL-6 production from human periodontal ligament (hPDL) cells. Twelve male 6-week-old Wistar rats were subjected to an orthodontic force of 50 g in order to induce mesially tipping movement of the upper first molars for 7 days. The expression levels of tartrate-resistant acid phosphatase (TRAP), Jagged1, Notch2, RANKL and IL-6 proteins in the dental root were determined using an immunohistochemical analysis. Furthermore, the effects of the CF on Jagged1, RANKL and IL-6 production were investigated using hPDL cells *in vitro*. The effects of the cell-conditioned medium obtained from the hPDL cells subjected to CF and Jagged1 on osteoclastogenesis of human osteoclast precursor cells (hOCPs) also investigated. Under the conditions of

experimental tooth movement *in vivo*, resorption lacunae with multinucleated cells were observed in the 50 g group. In addition, immunoreactivity for Jagged1, Notch2, RANKL and IL-6 was detected on day 7 in the PDL tissue subjected to the orthodontic force. In the *in vitro* study, the compression force increased the production of Jagged1, RANKL and IL-6 from the hPDL cells, while treatment with GSI inhibited the production of RANKL and IL-6 *in vitro*. The osteoclastogenesis increased by the CFM and rhJagged1, and the increase in the osteoclastogenesis was almost inhibited by GSI. These results suggest that the Notch signaling response to excessive orthodontic forces stimulates the process of root resorption via RANKL and IL-6 production from hPDL cells.

KEY WORDS: Notch signal, RANKL, IL-6, orthodontic force, root resorption

Introduction

Orthodontic treatment has many advantages, including improvements in the masticatory ability [1] and the ability to restore aesthetics; however, there are risks associated with these procedures. For example, orthodontically induced inflammatory root resorption (OIIRR) is one of the most difficult procedure-related adverse events to predict in cases of orthodontic tooth movement; this undesirable result can cause permanent loss of the dental structure of the root apex. In an epidemiological study by Kaley et al. [2], all patients who underwent comprehensive orthodontic treatment presented with root shortening, and 3% of all patients with severe root resorption (shortening by more than one-quarter of the root length) presented with root shortening in the maxillary central incisors. There are many factors involved in the process of root resorption. Causes of this phenomenon are reported to include the use of a heavy force [3], the length of treatment [4], the type of root [5] and genetic predispositions [6]. The inflammation-related cytokines are involved in the process of root resorption [7]. We previously demonstrated that the receptor activator of NF-kappaB ligand (RANKL) and interleukin (IL)-6 are detected in resorbed roots and periodontal ligament (PDL) tissues exposed to an excessive orthodontic force [8, 9]. Furthermore, we reported that these inflammatory cytokines are produced from human periodontal ligament (hPDL) cells under a compression force (CF) [10, 11, 12]. Therefore, RANKL and IL-6 are important factors in the process of OIIRR.

The Notch signaling pathway is a highly conserved cell signaling system that plays

an important role in various cell differentiation processes [13]. Four Notch receptors (Notch1-4) and five ligands (Delta1, 3, 4 and Jagged 1, 2) have been identified in mammals [14]. This signaling pathway is induced by the binding of the Notch extracellular domain (NECD) on one cell with a membrane-bound Notch ligand on a neighboring cell. NECD is then cleaved by tumor necrosis factor-α converting enzyme (TACE) one of a disintegrin and metalloproteinase (ADAM), followed by γ-secretase, resulting in the translocation of the Notch intracellular domain (NICD) into the nucleus and the transcriptional activation of downstream target genes, that is, the Hes and Hey family, which play a role in the development of various organs [14-16].

The involvement of the Notch signaling pathway in bone metabolism has attracted increasing attention. Several reports have suggested that the Notch signaling pathway is implicated in osteoblast and osteoclast differentiation [17, 18]. Meanwhile, Fukushima et al. [19] investigated that the role of Notch signaling in the differentiation of osteoclasts using the osteoclast differentiation induction system. In that study, the inhibition of Notch signaling by a selective γ-secretase inhibitor (GSI) suppressed RANKL-induced osteoclastogenesis, whereas the addition of immobilized Jagged1 and the ectopic expression of intracellular Notch2 promoted RANKL-induced osteoclastogenesis. Furthermore, Nakao et al. [20] demonstrated that Parathyroid hormone-related protein (PTHrP) increases the expression of Jagged1 and promotes RANKL-induced osteoclastogenesis in hPDL cells. Therefore, it is possible that Notch signaling plays partly an important role in the onset of OIIRR.

However, the role of Notch signaling in root resorption during orthodontic treatment is not yet fully understood. Therefore, this study focused on the relationship between OIIRR and Notch signaling. In an *in vivo* experiment, we investigated the expressions of Jagged1, Notch2, RANKL and IL-6 in areas of root resorption during experimental tooth movement in rats. Furthermore, in order to reproduce the conditions of pressure at the time of orthodontic tooth movement, the effects of compression forces with/without GSI on Jagged1, RANKL and IL-6 release were investigated using hPDL cells and human osteoclast precursor cells (hOCPs), instead of odontoclasts, *in vitro*.

Materials and Methods

In vivo studies

Animals

The animal experimental protocol used in this study was approved by the Ethics Committee for Animal Experiments at the Nihon University School of Dentistry at Matsudo (approval No. AP12MD020). A total of 12 male 6-week-old Wistar rats (body weight 180 ± 10 g; Sankyo Labo Service, Tokyo, Japan) were used for the experiments. Rats were randomly assigned into two groups; control group, where rats received no appliances, and 50 g group, where rats subjected to the excessive CF (control = 6, CF = 6).

Application of orthodontic devices

The animals were anesthetized with thiamylal sodium (15 mg/kg body weight) for the application of the orthodontic devices. Experimental tooth movement was induced using the method of Asano et al. [21], with a closed-coil spring (wire size: 0.005-inch, diameter: 1 ⁄ 12 inch; Accurate, Inc., Tokyo, Japan) ligated to the maxillary first molar with a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla using a 1 ⁄ 4 round bur, applying the same ligature wire. The upper right first molar was tipping moved mesially by the closed-coil spring with a force of 50 g. The period of the experiment was 7 days (Fig.1).

Tissue preparation

The animals were deeply anesthetized using thiamylal sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was immediately dissected and immersed in the same fixative for 18 hours (h) at 4°C. The specimens were decalcified in 10% disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4) solution for four weeks, and the decalcified specimens were subsequently dehydrated using a graded ethanol series and embedded in paraffin. Each sample was sliced continuously into 4-µm sections in the horizontal direction and prepared for hematoxylin and eosin (H.E.) and immunohistochemical staining. The periodontal tissues in the mesial portion of the distal buccal root of the upper right first molar were observed. The animals in which the tooth did not move were assigned to the

control group.

Immunohistochemistry

The tissue sections were deparaffinized, and the endogenous peroxidase activity was quenched via incubation in 3% H_2O_2 in methanol for 30 minutes (min) at room temperature.

After washing in Tris-buffered saline (TBS), the sections were incubated with polyclonal anti-rabbit tartrate-resistant acid phosphatase (TRAP; working dilution, 1:100; Santa Cruz Biotechnology, Inc., CA, USA), polyclonal anti-rabbit Jagged1 (working dilution, 1:100; Santa Cruz Biotechnology) and polyclonal anti-rabbit Notch2 (working dilution, 1:100; Lifespan Biosciences, Inc., WA, USA), polyclonal anti-goat RANKL (working dilution, 1:100; Santa Cruz Biotechnology), polyclonal anti-goat IL-6 (working dilution, 1:100; Santa Cruz Biotechnology) antibodies for 18 h at 4°C. TRAP, Jagged1, Notch2, RANKL and IL-6 were stained using the Histofine Simple Stain MAX-Po kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer's protocol. The sections were rinsed with TBS. The final color reactions were induced using the 3, 3'-diaminobenzidine tetrahydrochloride substrate reagent, and the sections were then counter stained with hematoxylin. As immunohistochemical controls, several sections were incubated with 0.01 M phosphate-buffered saline (PBS) instead of the primary antibody. Negative reactivity was observed in the control samples.

In vitro studies

Human periodontal ligament (hPDL) cell culture

The hPDL cells were prepared according to a modification of the method reported by Somerman et al. [22]. Briefly, hPDL tissues were collected from the roots of premolars extracted from 6 healthy young volunteers (3 males, 3 females; 14-16 years of age) during the course of orthodontic treatment after obtaining informed consent from the donors and used according to a protocol reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019). The hPDL tissue specimens were placed in 35-mm tissue culture dishes and covered with a sterilized glass coverslip. The medium was alpha minimum essential medium (α-MEM) (Wako, Osaka, Japan) supplemented with 100 μg/ml of penicillin-G (Sigma Chemical Co., St. Louis, MO, USA), 50 μg/mL of gentamicin sulfate (Sigma), 0.3 μg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA) and 10% fetal calf serum (FCS) (Cell Culture Laboratories, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medical System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2.

Application of compression force

In order to reproduce the pressure conditions on the periodontal ligament during orthodontic tooth movement, we performed the following *in vitro* experiments, as described by Nakajima et al. [11]. The hPDL cells were continuously compressed using a uniform compression system as a model of the pressure at the site of orthodontic

movement. First, the cells were seeded in 100-mm cell culture dishes and cultured in α-MEM containing 10 % FCS in the presence of 5 % CO_2 at 37°C. Following overnight incubation, the medium for the nearly confluent cells was changed of α-MEM containing 1 % FCS, and the cell layers were positioned in the dishes, with a glass plate placed on top. The cells were subsequently subjected to 1.0 g/cm^2 or 4.0 g/cm^2 of CF for 1, 3, 6, 9, 12, 24 or 48 h.

In some experiments, GSI (R&D Systems Co., MN, USA) was added to the culture medium at a concentration of 5.0 μM in order to inhibit the cleavage of the Notch intracellular domain, in accordance with the method reported by Osathanon et al. [23] and Fukushima et al. [19] before the application of CF. The control cells were subjected to treatment with thin glass plates without lead weight, which produced 0.032 g/cm^2 of CF.

Human osteoclast precursor cells (hOCP) culture

hOCPs were purchased from Lonza (Lonza Walkersville, Inc., MD, USA). We performed the experiments *in vitro*, according to a method reported by Hayashi et al. [9]. The cells were seeded onto 16-well Lab-Tek chamber slides (Nunc, IL, USA) at a density of 1×10^4 cells/100 μl and cultured in either commercial medium (LONZA) including recombinant human Jagged1 (rhJagged1; 1.0 μg/ml) or the hPDL cell culture medium at 37°C for 5 days for TRAP staining. The hPDL cell-conditioned medium

obtained from the hPDL cells was treated with CF and incubated with or without GSI (5.0 μM).

For the pit formation assay, hOCPs were similarly seeded onto chamber slides and cultured in commercial medium for 7 days. After the osteoclasts matured, the commercial medium was changed to either the commercial medium including rhJagged1 (1.0 μg/ml) or the hPDL cell-conditioned medium, and the cells were incubated for another 72 h. The hPDL cell-conditioned medium obtained from the hPDL cells was treated with CF and incubated with or without GSI (5.0 μM).

In each culture group, we added following supernatant and/or reagents as additional treatment groups, (1) Culture supernatant obtained from CF treated hPDL cell culture (CFM), (2) CFM and GSI (5.0 μM) and (3) rhJagged1 (1.0 μg/ml).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the hPDL cells using an RNeasy Mini kit (Qiagen Co., Tokyo, Japan), and aliquots containing equal amounts of mRNA were subjected to real-time PCR. The mRNA was reverse transcribed to cDNA using the Prime Script RT Reagent Kit (Takara Co., Shiga, Japan), according to the manufacturer's protocol. Real-time PCR amplification was performed using SYBR Premix Ex Taq (Takara Co.)

in a thermal cycler (TP-800 Thermal Cycler Dice; Takara). The PCR primers for Jagged, RANKL, IL-6 and GAPDH were purchased from Takara Co. (Takara Co.) and designed with reference to the respective cDNA sequences, as follows:

Jagged1

Fw: 5’-CACCCTGCTGAGTCTGTTCTGG-3’

Rv: 5’-CCAGGTCTTTGAGAACTCCAGATG-3’

RANKL

Fw: 5’-TGGATGCCTTGAATAATAAGCAGGA-3’

Rv: 5’-AATTTGCGGCACTTGTGGAA-3’

IL-6

Fw: 5’-AAGCCAGAGCTGTGCAGATGACTTA-3’

Rv: 5’-TGTCCTGCAGCCACTGGTTC-3’

GAPDH

Fw: 5’-GCACCGTCAAGGCTGAGAAC-3’

Rv: 5’-TGGTGAAGACGCCAGTGGA-3’

Enzyme-linked immunosorbent assay (ELISA)

The culture medium was collected for the ELISA. The levels of Jagged1, soluble RANKL (sRANKL), and IL-6 production into the culture supernatants were measured using a human Jagged1 ELISA kit (Uscn Life Science Inc., TX, USA), human sRANKL ELISA kit (BIOMEDICA Co., Wien, Austria) and human IL-6 ELISA kit (R&D Systems Co., MN, USA), according to the manufacturer’s protocol. In addition, For

ELISA assay of Jagged1, cells were lysed in Mammalian Protein Extraction Buffer (GE Healthcare Co., NJ, USA) before collected.

TRAP staining

After the hOCPs had been cultured for 5 days, the osteoclasts were washed twice with PBS and stained with TRAP using a commercially available staining kit (Hokudo Co., Hokkaido, Japan). TRAP-positive and multinucleated cells that contained three or more nuclei were judged to be osteoclasts.

Pit formation assay

The bone-resorbing activity of mature osteoclasts formed *in vitro* was assessed based on the ability of the cells to form resorption pits on dentin slices (0.15 mm in diameter, 6 mm in thickness). When the incubation schedule was completed, the samples were washed three times with PBS. The slices were placed for 30 min in 1 M of NH_4OH and washed via ultrasonication in order to remove adherent cells and then washed and dried. After drying, the dentin slices were mounted onto stubs and sputter coated with platinum for electron microscopy or placed on glass slides for light microscopy. The entire surface of each dentin slice was examined using a scanning electron microscope (JSM-6340F; JEOL, Tokyo, Japan). The resorption pit area in a single well was determined using an image processing system (Win Roof; Mitani Co., Tokyo, Japan).

Statistical methods

The values in each figure represent the mean ± standard deviation (s.d.) for each group. The Mann-Whitney U-test was used to compare the means of the groups with values of $P<0.05$ and $P<0.01$, which was considered to indicate a significant difference in comparison to the corresponding control.

Results

In vivo studies

Body weights during the experimental period

The body weights of the rats in the experiment group decreased transiently on day 1 after the application of the orthodontic devices and then recovered. No significant differences between the two groups were observed (data not shown).

Histological changes in the periodontal tissues after tooth movement (H.E. staining)

In the control group (0 g), the rat PDL specimens were composed of fibroblasts aligned horizontally from the root cement with relatively dense connective tissue fibers. The root surface was relatively smooth, with a few mononuclear and multinucleated osteoclasts, although no resorption lacunae were observed (Fig. 2A). In the 50 g group, coarse arrangement of fibers with dilated blood capillaries was noted (Fig. 2B). On day 7 after the application of the orthodontic force, many root resorption lacunae with multinucleated odontoclasts were identified on the surface of the root (Fig. 2B).

Immunohistochemical findings of TRAP

In the control group, no resorption lacunae with TRAP-positive multinucleated osteoclasts were observed on the surface of the roots (Fig. 2C). However, in the 50 g group, many resorption lacunae with multinucleated TRAP-positive odontoclasts were identified on the surface of the root on day 7 (Fig. 2D).

Protein expression levels of Jagged1, Notch2, RANKL and IL-6

Immunoreactivity for Jagged1, Notch2, RANKL and IL-6 was examined on day 7 after tooth movement. In the control group, Jagged1-, Notch2-, RANKL- and IL-6-positive cells were rarely observed in the PDL tissues (Figs. 2E, 2G, 2I, 2K). In contrast, in the 50 g group, many Jagged1-, Notch2-, RANKL- and IL-6-positive cells were observed in the PDL tissues (Figs. 2F, 2H, 2J, 2L). Multinucleated cells considered to be odontoclasts were observed in the resorption lacunae (Figs. 2F, 2H, 2J, 2L).

In vitro studies

Effects of different magnitudes of CF on the mRNA and protein expressions of Jagged1 from hPDL cells.

Different CF (1.0 g/cm^2 and 4.0 g/cm^2) was applied to the hPDL cells, and the expression levels of mRNA and proteins for Jagged1 in the hPDL cells were determined using a real-time PCR analysis and ELISA after 1, 3, 6, 9, 12, 24 and 48 h. In the CF (1.0 g/cm^2 and 4.0 g/cm^2) group, the mRNA and protein expression of Jagged1

significantly increased following the application of CF in a time- and force magnitude-dependent manner (Fig. 3A, 3B). In the CF (4.0 g/cm^2) group, the expression of Jagged1 mRNA and protein were significantly increased compared with the CF (1.0 g/cm^2) group .

Effects of CF on the mRNA expressions of RANKL and IL-6 in the hPDL cells, as determined using real-time PCR

CF (4.0 g/cm^2) was applied to the hPDL cells with/without GSI for up to 24 h, and the expression levels of mRNA for RANKL and IL-6 in the hPDL cells were determined using a real-time PCR analysis after 1, 3, 6, 9, 12 and 24 h. The mRNA expressions of both RANKL and IL-6 significantly increased following the application of CF in a time-dependent manner, with peaking at 12 h (Figs.4A, 4C). In contrast, the expressions of RANKL and IL-6 mRNA were significantly decreased in the CF+GSI group compared with that observed in the CF group (Figs.4A, 4C).

Effects of CF on the release of sRANKL and IL-6 by hPDL cells, as determined using ELISA

The release of sRANKL and IL-6 from hPDL cells in response to the application of CF (4.0 g/cm^2) with/without GSI was determined using ELISA. When the hPDL cells were treated with CF for up to 48 h, the release of sRANKL and IL-6 increased in a time-dependent manner compared with that observed in the control group (Figs. 4B, 4D). However, the levels of sRANKL and IL-6 were significantly decreased in the

CF+GSI group compared with that observed in the CF group (Figs. 4B, 4D).

Effects of the hPDL cell-conditioned medium on TRAP staining of hOCPs

The number of TRAP-positive multinucleated cells was significantly increased in the group treated with the cell-conditioned medium obtained from the hPDL cells subjected to CF (CFM group) compared with that observed in the group treated with hPDL cell-conditioned medium without CF (control group) for 5 days. The trend toward an increase was similarly observed in the cells cultured with rhJagged1 (rhJagged1 group). Moreover, the number of TRAP-positive multinucleated cells was decreased in the CFM+GSI group compared with that observed in the CFM group. The number of TRAP-positive multinucleated cells was also counted. In the CFM+GSI group, the increase in the number of TRAP-positive osteoclasts was almost inhibited (Fig. 5A).

Pit formation assay

The resorption of mineralized tissue was investigated by measuring the capacity of the osteoclasts formed *in vitro* to resorb dentin. When the osteoclasts were cultured in commercial medium for 7 days and then changed to hPDL cell-conditioned medium on dentin slices, resorption lacunae appeared on the surface of the slices. The area of resorption pits increased significantly in the CFM and rhJagged1 groups compared with that observed in the control group. In contrast, the increase in the resorption area was almost inhibited in the CFM+GSI group (Fig. 5B).

Discussion

In this study, to investigate whether Notch signaling (Jagged1 and Notch2) are involved in root resorption during orthodontic treatment, we induced root resorption by applying excessive orthodontic force in animal models, and examined for cytokine that is expressed in the resorption site.

First, we induced root resorption by applying an excessive orthodontic force in an animal model. On day 7, immunoreactivity for Jagged1 and Notch2 was detected in the rat PDL tissues subjected to the force in the 50 g group (Figs. 2F, 2H). Furthermore, odontoclasts and immunoreactivity for RANKL and IL-6 was detected on the surface of resorbed roots in the 50 g group (Figs. 2J, 2L). Duan et al. [13] suggested that Notch signaling may be involved in pathological bone remodeling (such as that observed in patients with rheumatoid arthritis or periodontitis).

Next, in order to investigate the mechanisms responsible for the alterations in Jagged1, Notch2, RANKL and IL-6 induced by the excessive orthodontic force in the setting of OIIRR during orthodontic tooth movement, the expression levels of Jagged1, RANKL and IL-6 mRNA and protein in hPDL cells were measured. The results indicated that the compression force significantly increased at 6 h the mRNA expression of Jagged1 in a time- and force magnitude-dependent manner (Fig. 3A). Meanwhile, the protein levels of Jagged1 increased at 24 h in a time- and force magnitude-dependent manner (Fig. 3B).

Furthermore, the RANKL and IL-6 expression levels reached a peak at 12 h (Figs. 4A, 4C). Meanwhile, the protein levels of these factors increased at 24 h in a

time-dependent manner (Figs. 4B, 4D). These results suggest that the Notch signaling response to excessive compression forces may be at least partially responsible for the process of root resorption via the induction of RANKL and IL-6 production from hPDL cells. Further studies are necessary to investigate the effect of Notch signaling on the expression of RANKL and IL-6 in orthodontic root resorption by using a Notch knockout mouse.

Finally, TRAP staining and pit formation assays were performed to investigate the effects of CF and Jagged1 on the resorption capacity of osteoclasts. On TRAP staining, the number of TRAP-positive cells increased following treatment with hPDL cell-conditioned medium with CF application (CFM) or rhJagged1. Furthermore, treatment with GSI inhibited the increase of number of TRAP-positive cells in the CFM. In the pit formation assay, the areas of resorption significantly increased in the CFM or rhJagged1 groups. In contrast, in the CFM+GSI group, the increase in the areas of resorption was almost inhibited (Figs. 5A, 5B). However, in the CF group, the depth of resorption lacunae seems to be deep compared with the rhJagged1 group. These results raise the possibility that the other pathway without Notch signaling such as Wnt signaling may involved in osteoclastgenesis. Further consideration will be needed about this phenomenon.

Sethi et al. [24] reported that Jagged1 promotes tumor growth by stimulating IL-6 release from osteoblasts and directly activating osteoclast differentiation. Furthermore, Nakao et al. [20] reported that Jagged1 promotes RANKL-induced osteoclastogenesis, while Fukushima et al. [19] reported that RANKL induces the production of Jagged1

and Notch2 in bone marrow macrophages during osteoclastogenesis. Taken together, these findings and the present results suggest that the increased Jagged1 expression observed in hPDL cells treated with an excessive compression forces activates osteo/odontoclastogenesis.

Duan et al. [13] suggested that Notch signaling may crosslink the immune system with the skeletal system. Our laboratory previously demonstrated that the levels of IL-17 and RANKL are significantly increased in atopic dermatitis (AD) model mice compared with wild-type mice, thus suggesting that Th17 cells are associated with the proceeding of root resorption in AD mice [25]. Therefore, IL-17 may stimulate osteo/odontoclastogenesis via Notch signaling, which may subsequently contribute to the inflammatory response associated with ensuing OIIRR. Further studies are needed to investigate the relationships between IL-17 and Jagged1/Notch2 in the setting of root resorption using *in vitro* and *in vivo* experiments.

In contrast, recent pathological and *in vitro* studies contradict the findings of the present study. For example, Li et al. [26] reported that Notch signaling enhances the osteogenic differentiation of periodontal ligament stem cells in osteoporotic rats, and Ugarte et al. [27] documented that Notch signaling enhances osteogenic differentiation in primary human bone marrow stromal cells. The discrepancies between these previous and the present results may be due to differences in the cell types used and the stimuli applied to the cells. In addition, Yamada et al. [17] reported that Delta1 and Notch1, 2 inhibited the osteoclasts formation. Meanwhile, Fukushima et al. [19] revealed that Jagged1 and Notch2 promoted oasteoclastgenesis. In the osteoblastgenesis, Nobuta et al.

[17] suggested that Delta/Jagged1-activated Notch1 enhances osteoblast differentation. Therefore, Notch signaling may regulate bone homeostasis in a ligand specific manner.

In conclusion, we herein demonstrated that the Notch signaling response to an excessive compression forces may stimulate the process of root resorption via RANKL and IL-6 production from hPDL cells. Our results suggest that Notch signaling plays an important role in the onset of OIIRR, although the involvement of other pathways cannot be ruled out.

Acknowledgments

This article is based on a main reference paper titled, "Notch signaling induces root resorption via RANKL and IL-6 from hPDL cells" in press (2014 Nov5. DOI: 10.1177/0022034514555364) at the *Journal of Dental Research*, and reference paper titled, "Jagged1 stimulates the bone resorption during orthodontic tooth movement" in press at the *International Journal of Oral-Medical Sciences.*

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Figure Legends

Figure 1.

Experimental tooth movement. Diagram of method used for experimental tooth movement. The upper right first molar was moved mesially by a closed coil spring with 50 g (A, B). Schematic illustration showing the area of investigation (shaded box) in the mesial aspect of the distal root of the first molar. The periodontal tissues in the pressure area were quarter of the mesial area facing the distal buccal root (DBR), as determined when linked with the center of the DBR and the mesial root (MR) of the first molar. The large arrow indicates the direction of the force (C). The observation of the positive cells were performed at a 300 μm section (shaded box) of the root from the bifurcation surface on the mesial side, which is the pressure side during tooth movement. The large arrow indicates the direction of the force (D).

Figure 2.

H.E. and immunohistochemical staining for TRAP, Jagged1, Notch2, RANKL and IL-6. (A, B) An orthodontic force was applied for 7 days, after which root resorption was observed in the 50 g group, but not in the control group. Multinucleated osteoclasts appeared on the dentin surface in the 50 g group according to histomorphometry. (C, D) TRAP immunoreactivity was observed in the multinucleated cells. (E-L) Immunoreactivity for Jagged1, Notch2, IL-6 and RANKL was observed on the root surface and in the PDL tissues (arrows) in the 50 g group. D: dentine, C: cementum,

PDL: periodontal ligament. Original magnification 200×, Bar: 50 μm

Figure 3.

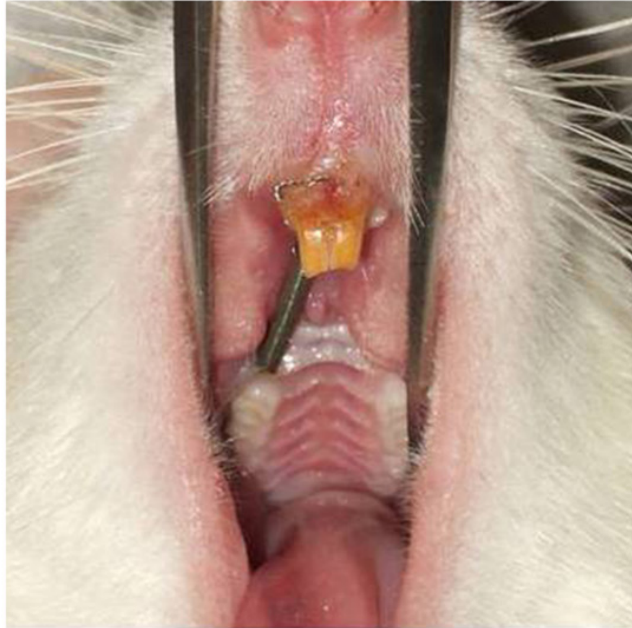
Effects of CF on the mRNA and protein production of Jagged1 by hPDL cells. The mRNA and protein expression levels of Jagged1 was determined using real-time PCR and ELISA after 1, 3, 6, 9, 12, 24 and 48 h. (A) In the CF (4.0 g/cm^2) group, the expression of Jagged1 mRNA was significantly increased compared with that observed in the control and CF (1.0 g/cm^2) group. (B) In the CF (4.0 g/cm^2) group, the release of Jagged1 were significantly increased compared with that observed in the control and CF (1.0 g/cm^2) group (*P < 0.05, **P < 0.01 from the Mann-Whitney U-test, indicating a significant difference from the corresponding control). The data are expressed as the means ± s.d. of six independent experiments.

Figure 4.

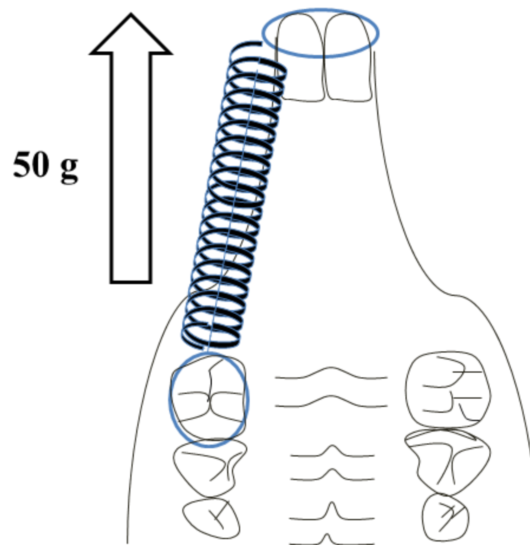
Effects of CF on the mRNA and protein production of RANKL and IL-6 by hPDL cells. The mRNA and protein expression levels of RANKL and IL-6 were determined using real-time PCR and ELISA after 1, 3, 6, 9, 12, 24 and 48 h. The levels of RANKL and IL-6 were significantly increased in the CF (4.0 g/cm^2) group. Moreover, GSI suppressed the CF-induced expression of RANKL and IL-6 (*P < 0.05, **P < 0.01 from the Mann-Whitney U-test, indicating a significant difference from the corresponding control). The data are expressed as the means ± s.d. of six independent experiments.

Figure 5.

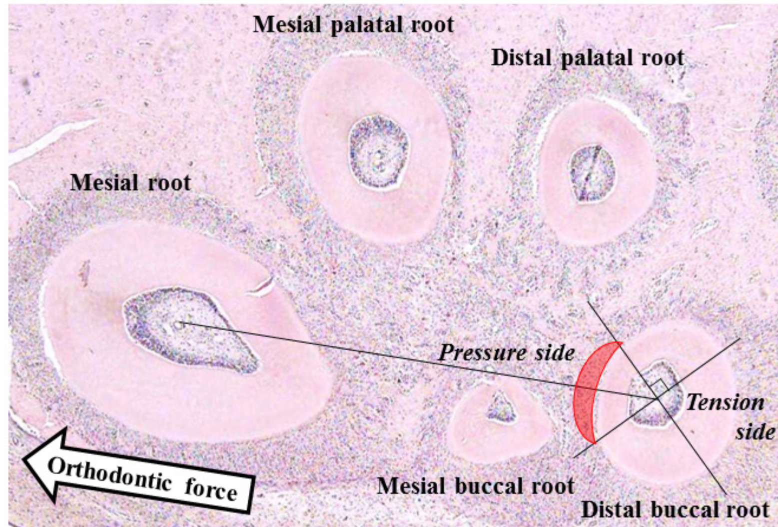
Effects of Notch signaling on osteoclast formation. (A) TRAP staining. hOCPs were cultured in either commercial medium including rhJagged1 (1.0 μg/ml) or hPDL cell-conditioned medium with/without CF (4.0 g/cm^2) and GSI (5.0 μM) for 5 days. The number of TRAP-positive multinucleated cells was significantly increased in the CFM and rhJagged1 groups compared with that observed in the control group. In the CFM+GSI group, the number of TRAP-positive multinucleated cells significantly decreased to the same level as that observed in the control group. (B) Pit formation assay. hOCPs were cultured in commercial medium for 7 days and then changed to hPDL cell-conditioned medium on dentin slices. Resorption pits were then observed using scanning electron microscopy. Significant differences were noted between the group treated with the CFM group and the control group. In the CFM+GSI group, the increase in the areas of resorption was almost inhibited (*P < 0.01 from the Mann-Whitney U-test, indicating a significant difference from the corresponding control). The data are expressed as the means ± s.d. of six independent experiments.



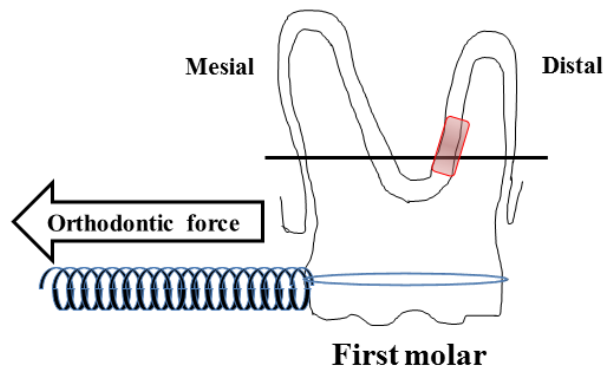
Kikuta *et al*. Fig. 1A



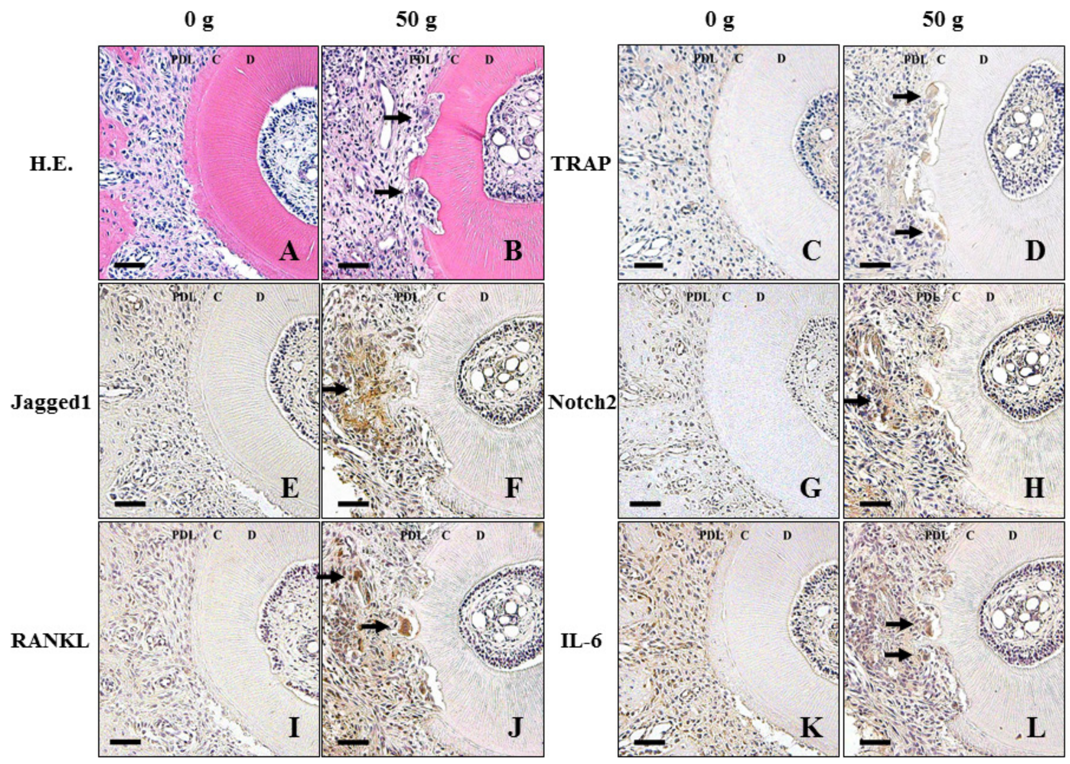
Kikuta *et al*. Fig. 1B



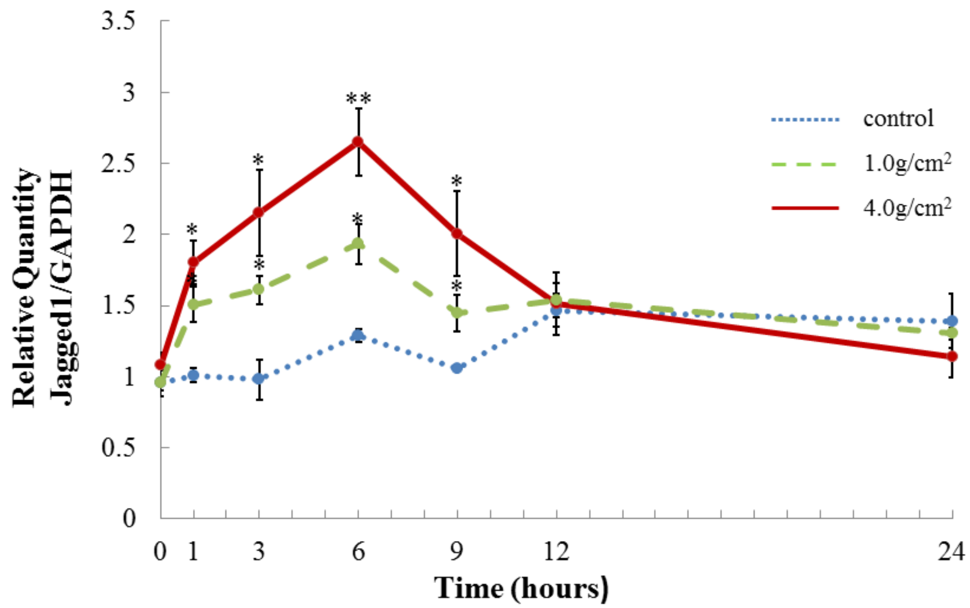
Kikuta *et al*. Fig. 1C



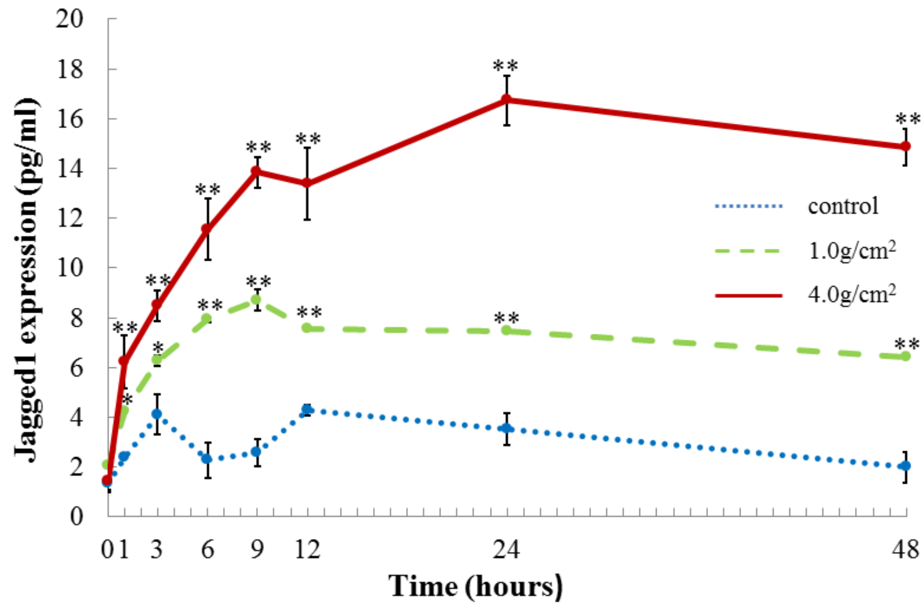
Kikuta *et al*. Fig. 1D



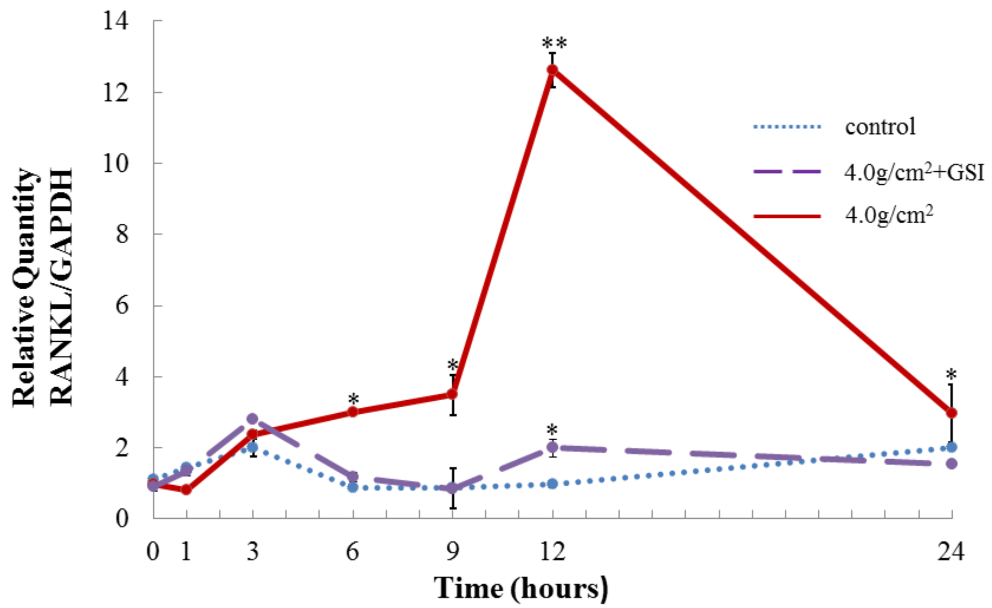
Kikuta *et al*. Fig. 2



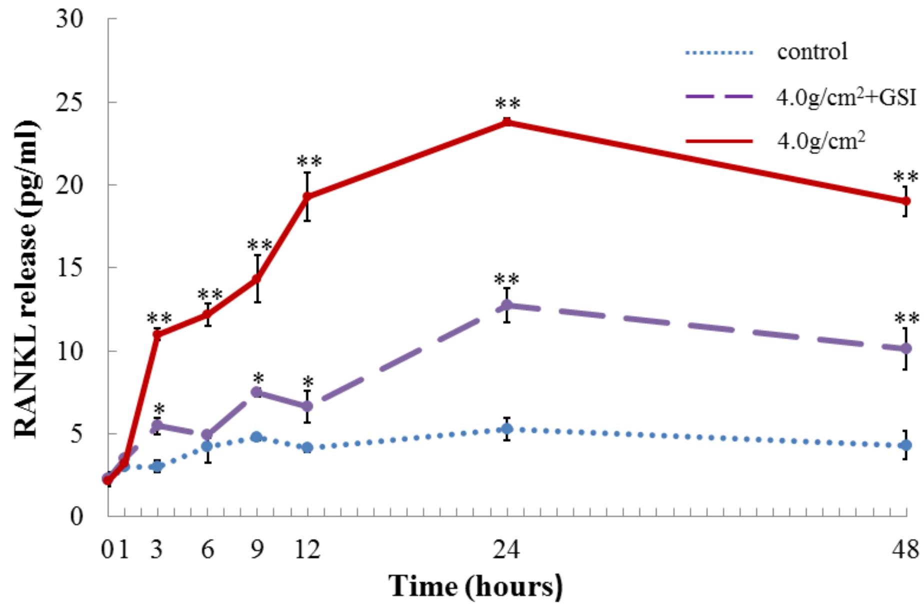
Kikuta *et al*. Fig. 3A



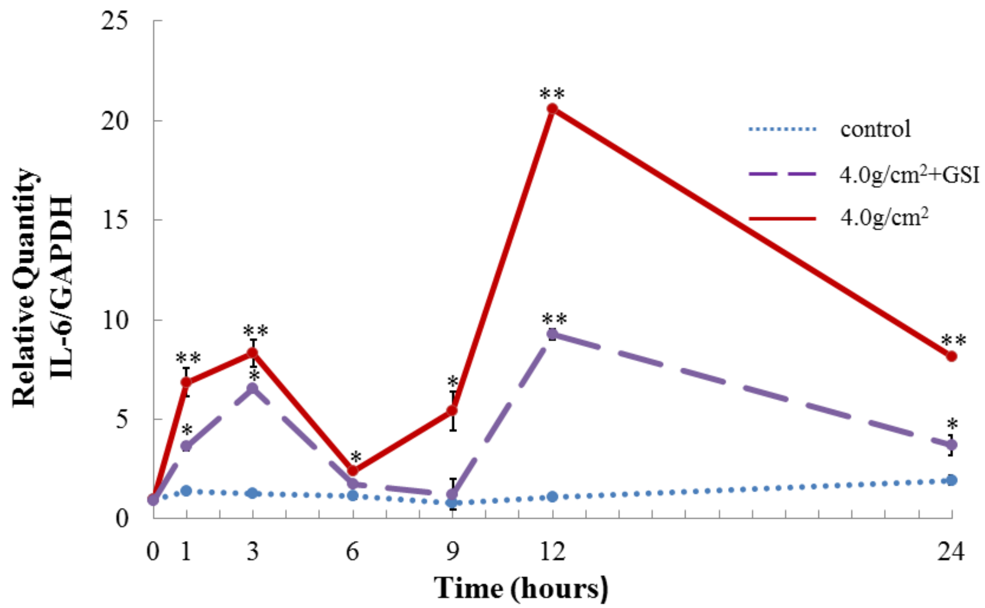
Kikuta *et al*. Fig. 3B



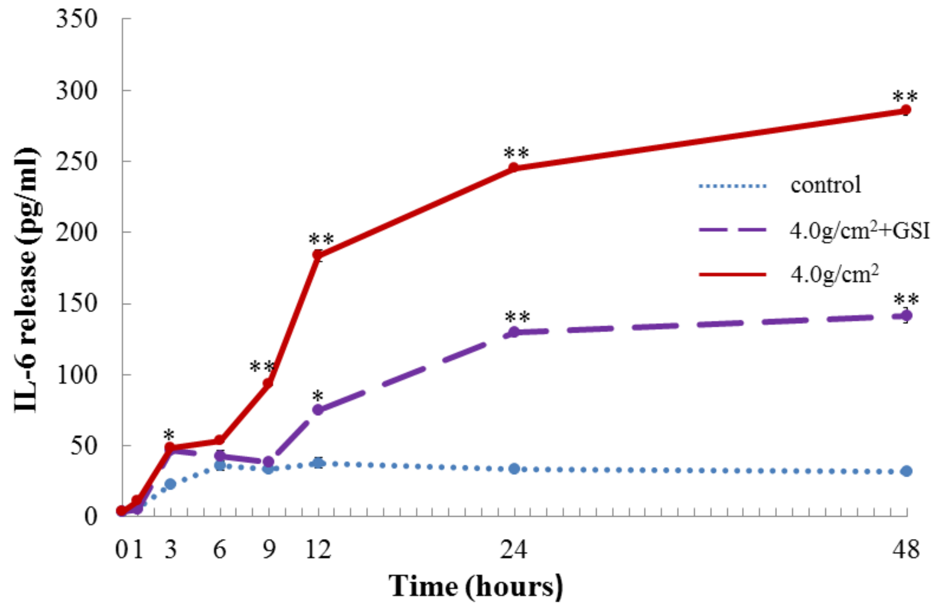
Kikuta *et al*. Fig. 4A



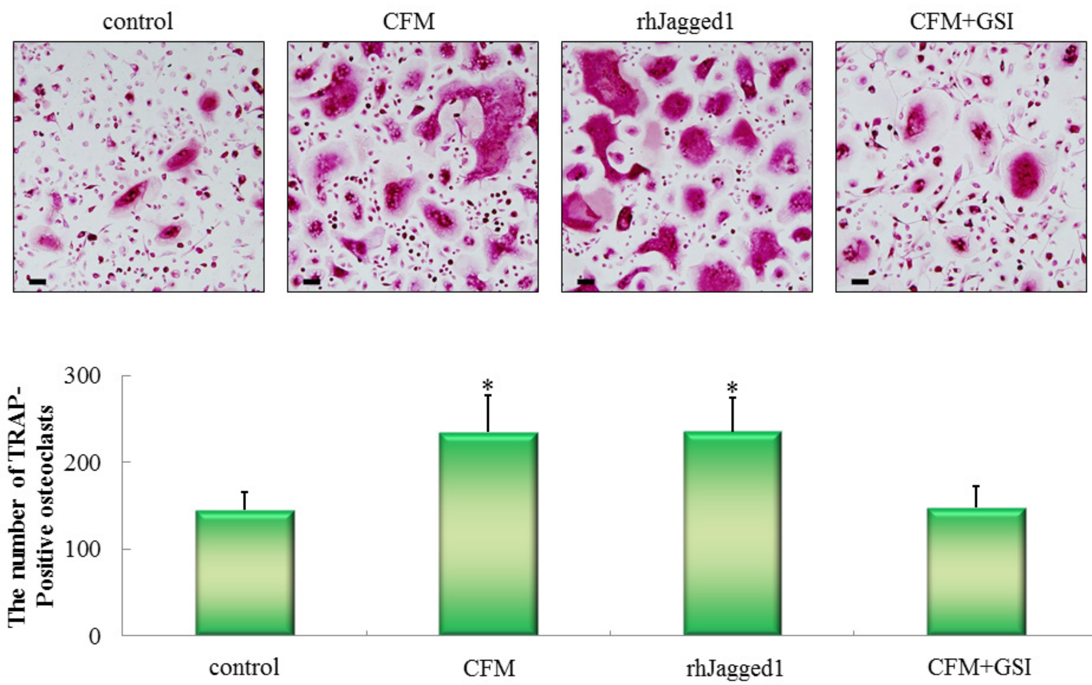
Kikuta *et al*. Fig. 4B



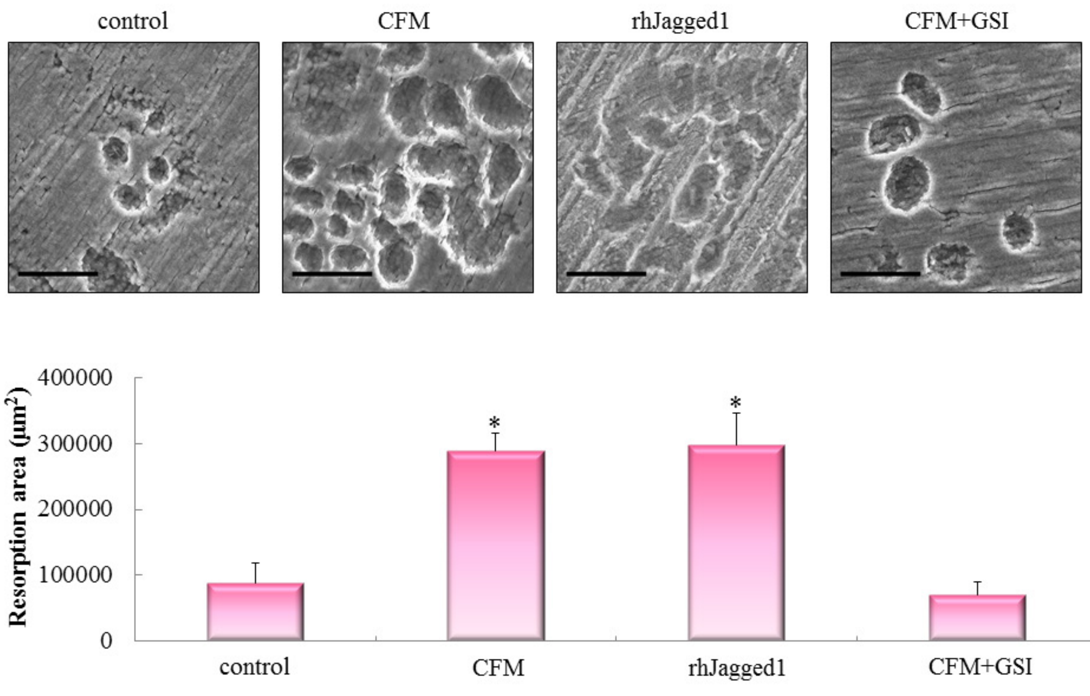
Kikuta *et al*. Fig. 4C



Kikuta *et al*. Fig. 4D



Kikuta *et al*. Fig. 5A



Kikuta *et al*. Fig. 5B