Heavily Induced Root Resorption Stimulates Wnt5a Expression in Cementoblasts

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Abstract
Wnt signaling plays a vital role in bone metabolism. However, currently, its role in bone formation and resorption during orthodontic tooth movement is still unclear. This study aimed to investigate in vivo Wnt5a expression in the resorbed root in rats during experimental tooth movement. Eighteen 6-week-old, male Wistar rats were subjected to an orthodontic force of 10 or 50 g for 7 days to induce the mesial movement of the upper first molars. Immunohistochemical analysis was performed to assess tartrate-resistant acid phosphatase, receptor activator of nuclear factor-κB ligand (RANKL), and Wnt5a protein expressions in the alveolar bone. Additionally, the effects of different compression forces (CFs) on Wnt5a production were investigated using human periodontal ligament (hPDL) cells and human cementoblast-like cells in vitro. Resorption lacunae with multinucleated cells in the CF-10 g group under experimental tooth movement conditions in vivo. Moreover, RANKL and Wnt5a immunoreactivity was detected in the alveolar bone-PDL tissues. In the CF-50 g group, resorption lacunae and RANKL and Wnt5a expressions were detected in PDL-cement tissues. In the in vitro study, 1.0- and 4.0-g CF increased Wnt5a production in hPDL cells and HCEMs, respectively, in a time-dependent manner. These results indicate that the Wnt signaling response to heavy orthodontic forces stimulates the root resorption process during orthodontic tooth movement.

Keywords:
Wnt5a, root resorption, compression force, periodontal ligament

Introduction
Root resorption an accident encountered during orthodontic tooth movement (OTM); however, predicting the occurrence of root resorption is challenging, and many researchers have postulated several hypotheses about its cause. Patient-specific factors causing root resorption include allergies (1), chronic asthma (2), abnormal root (3), and the hardness and form of cementitious materials (4).

Conversely, treatment-specific factors include strong loading and/or jiggling force and prolonged treatment period (2, 5). However, the mechanism underlying the occurrence of root resorption is still unclear. Alveolar bone metabolism on the compression side is considered to play a significant role in smooth tooth movement in orthodontic treatment (6, 7). Recently, Wnt signaling has gained attention as a signaling pathway involved in bone metabolism, with more studies stressing on the development of therapeutic drugs relating to abnormal bone metabolism disorders such as osteoporotic rheumatoid arthritis (8). The signaling molecule Wnt is secreted by proteins, for which 19 types of ligands have been identified in humans. Wnt binds to two different receptor complexes: the complex of Frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP 5/6) and that of Frizzled and receptor tyrosine kinase orphan receptors (Rors) (9).

The Wnt receptor and complex binding activate two signaling pathways: β-catenin-dependent canonical (canonical) pathway and β-catenin-independent noncanonical (non-canonical) pathway. Wnt signaling plays several vital roles at the cellular level by regulating cell proliferation, morphology, motility, and fate (10). Furthermore, Wnt signaling plays an essential role that affects the mesenchyme at early developmental stages of the tooth and alveolar bone (11); additionally, it maintains homeostasis in human periodontal ligament (hPDL) cells (12). The canonical
pathway activates osteogenesis by binding Wnt3a to LRP 5/6, and the noncanonical pathway promotes receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis by binding Wnt5a to Ror2(9). Recently, understanding the control mechanism by Wnt signaling-induced bone formation and resorption is rapidly pathway. As mentioned earlier, binding Ror2 to Wnt5a, a member of the noncanonical pathway, activates the noncanonical pathway that is involved in bone remodeling(13). Wnt5a has been reported to promote osteoclast precursor cell differentiation and induce RANKL expression(14).

Previously, we demonstrated the heavy force that was induced by orthodontic root resorption using an animal model according to the procedure reported by Asano et al (15). However, the role of Wnt5a in the past occurrence of root resorption is still unclear. Hence, in this study, we aimed to examine Wnt5a expression in PDL tissues in the rat tooth movement model in vivo and that in hPDL cells and human cementoblast-like cells (HCEMs) subjected to compression force (CF) 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, Matsudo (approval No. AP16 MD013-1). In total, 6-week-old, male Wistar rats (n=6; body weight, 115.4 ± 10.5 g; Sankyo Labo Service, Tokyo, Japan) were used in the study.

  The rats were randomly categorized into three groups (six each): control group, where they received no CF; CF-10 g group, where rats were subjected to 10 g of CF; and CF-50 g group, where rats were subjected to 50 g of CF.

  **Application of orthodontic devices**

  In each group, animals were anesthetized prior to the application of orthodontic devices by intraperitoneally injecting a combination of three anesthetic agents: hydrochloric acid medetomidine, midazolam, butorphanol tartrate (0.15 mg/kg of body weight). Experimental tooth movement was induced using the method reported by Asano et al (15). Briefly, a closed-coiled spring (length: 0.005 inch, diameter: 1/12 inch; Accurate, Inc., Tokyo, Japan) was ligated to the first maxillary molar using a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The other side of the coil spring was also ligated by laterally drilling holes in the maxillary incisors just above the gingival papilla using a 1/4 round bur using the same ligature wire. The first upper right molar was mesially tipped using the closed-coil spring with a 10 g or 50 g of force. The experiment was performed over a period of 7 days (Fig. 1).

  **Tissue preparation**

  The rats were deeply anesthetized using thiamylal sodium and were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Subsequently, the maxilla was immediately dissected and then immersed in the same fixative for 18 h at 4°C. These specimens were decalcified by immersing in 10% disodium ethylenediaminetetraacetic acid (pH 7.4) solution for 4 weeks, were dehydrated using a graded series of ethanol washes, and were then embedded in paraffin. Each specimen was continuously sliced into 4-μm-thick sections in horizontal direction for hematoxylin and eosin and immunohistochemical staining. Additionally, periodontal tissues in the mesial portion of the distal buccal root of the first upper molar were observed. Rats that exhibited no tooth movement were assigned to the control group.

  **Immunohistochemistry**

  The tissue sections were deparaffinized, activated by heat treatment, and the endogenous peroxidase activity was quenched by incubating in 3% H2O2 and methanol for 30 min at room temperature. The resultant sections were subsequently washed in tris-buffered saline (TBS) and were then incubated with polyclonal anti-rabbit tartrate-resistant acid phosphatase (TRAP), polyclonal anti-goat RANKL, or polyclonal anti-rabbit Wnt5a (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 18 h at 4°C. TRAP and RANKL was determined by staining using the Histofine Simple Stain MAX-Po (G) kit (Nichirei, Co., Tokyo, Japan) and Wnt5a using the Histofine Simple Stain MAX-Po (R) kit (Nichirei, Co., Tokyo, Japan). The sections from control group were observed for negative reactivity.

  **Quantitative evaluations of the number of TRAP-, Wnt5a-, and RANKL-positive cells**

  A binarized image was generated using the positively immunostained region as the region of interest, and a binary image was generated using the cell nucleus as the region of
interest. These two images were superimposed.
Measurement of lightness was performed, and the area
where the two regions were adjacent to each other was
judged to represent positive cells, which were enumerated.
ImageJ (U. S. National Institutes of Health, Bethesda,
Maryland, USA) was used for measurement.

Double staining for Wnt5a and Ror2 or RANK and RANKL
The tissue sections were stained for a combination of
either Wnt5a and Ror2 or RANK and RANKL. Tissue
sections for the aforementioned staining were prepared in
the same manner as that for immunohistochemical staining.
First, the sections were deparaffinized in xylene, then
gradually hydrated through a graded series of alcohol
washes, and finally washed in TBS. The excess liquid was
then aspirated from the slides.

Immunofluorescence staining was performed in accord-
ance with the method proposed by Hirate et al (16). After
washing in TBS, the sections were incubated with polyclonal
anti-rabbit Ror2 antibody (Biorbyt Ltd; working dilution, 1:100) or RANKL polyclonal antibody (Bioworld Technology,
Inc. working dilution, 1:100) for 1 h at room temperature.
Then the sections were incubated with the Alexa Fluor®568 (red) -Fluoro Nanogold™ Fab’ fragment of a
goat anti-rabbit IgG (Invitrogen, Co, CA, USA; working
dilution, 1:100) for 1 h at room temperature. Polyclonal anti-
rabbit Wnt5a antibody or RANK polyclonal antibody these
labeled with Zenon rabbit IgG labeling reagent was used.
These antibody and Alexa Fluor®488 (green) -Fluoro Nanogold™ Fab’ fragment of a goat anti-rabbit IgG
(Invitrogen, Co, CA, USA; working dilution, 1:100) for 1 h at
room temperature. Finally, the sections were counter-
stained using ProLong® Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen, Co.) and were observed using a fluorescence microscope (Olympus, Tokyo, Japan). For immunofluorescent controls, instead of incubating with the primary antibody, several sections were incubated with either non-immune rabbit IgG or 0.01 M PBS.

* In vitro studies

**HPDL cell culture**

HPDL cells were prepared according to the modified method reported by Somerman et al. (17). Briefly, hPDLCs were procured from the roots of premolars extracted from six young, healthy volunteers (three men and three women; age, 18–29 years) during their course of orthodontic treatment were used in the study. Informed consent was obtained from the donors for procuring tissues. The culture protocol was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 18–15–027–1).

**Cementoblast cell culture**

HCEMs

HCEMs (14) were cultured in alpha minimum essential medium (α-MEM; Wako, Osaka, Japan) that was combined with fetal calf serum (FCS) (Cell Culture Laboratories, OH, USA: 10%), penicillin G (Sigma Chemical Co., St. Louis, MO, USA: 100 μg/ml), gentamicin sulfate (Sigma Chemical Co.: 50 μg/mL), and amphotericin B (Flow Laboratories, McLean, VA, USA: 0.3 μg/ml). HCEMs were stored in a humidified incubator (Forma CO₂ incubator MIP-3326; Sanyo Electric Medical System Co., Tokyo, Japan) in 95% air and 5% CO₂ at 37°C.

**CF application**

To reproduce pressure conditions on PDL during orthodontic tooth movement (OTM), we performed the following in vitro experiments based on the method reported by Nakajima et al. (18). hPDLCs were continuously compressed using a uniform compression system as a pressure model at the site of OTM. First, cells were seeded in 100-mm cell culture dishes and cultured in α-MEM containing 10% FCS under the conditions of 5% CO₂ at 37°C. After overnight incubation, the medium for the resultant, almost confluent cells was changed to α-MEM containing 1% FCS. The cell layers were positioned in the dishes, with a glass plate on the top. The cells were subsequently subjected to 1.0 or 4.0 g/cm² of CFs for 1, 3, 6, 9, 12, 24, or 48 h (Fig. 2).

**Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from hPDLCs using an RNeasy Mini kit (Qiagen Co., Tokyo, Japan). Aliquots containing equal amounts of mRNA were used to perform real-time PCR. The resultant mRNA was reverse transcribed to cDNA using the Prime Script RT Reagent Kit (Takara Co., Shiga, Japan) as per the manufacturer’s protocol. Real-time PCR amplification was performed using...
the SYBR Premix Ex Taq (Takara Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara). The PCR primers for Wnt5a and GAPDH were purchased from Takara Co. and were designed with reference to their respective cDNA sequences as follows:

Wnt5a
Forward: 5’-TTACCACTGCAACTATTTGCACCTC-3’
Reverse: 5’-CACAATGAACCTTTAGTTTCCAACC-3’
GAPDH
Forward: 5’-GCACCGTCAAGGCTGAGAAG-3’
Reverse: 5’-TGGTGAAGAGCCAGTGGA-3’

Enzyme-linked immunosorbent assay (ELISA)
For ELISA, the culture medium was collected, and Wnt5a levels in the culture supernatants were measured using a human Wnt5a ELISA kit (CUSABIO Co., Houston, TX, USA) as per the manufacturer’s protocol.

Statistical analyses
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 groups with \( P < 0.05 \) and \( P < 0.01 \), which indicated significant differences from the corresponding controls.

Results
- In vivo studies

Body weights during the experimental period
The body weights of the rats in the experiment group transiently decreased on day 1 after the application of orthodontic devices but subsequently recovered. No significant differences were observed between the two groups with regard to body weight (not shown the data).

Histological changes around the periodontal ligament during orthodontic tooth movement (HE staining)
The PDL specimens in the control group at 7 days after tooth movement comprised relatively dense connective tissue fibers and fibroblasts. The alveolar bone surface was relatively smooth, exhibiting only a few mononuclear and multinucleated osteoclasts without resorption lacunae (Fig. 3 A).

In the CF-10 g group, on the root surface, observed few resorption and odontoclasts on day 7. On the other hands, alveolar bone surface, observed mononuclear and osteoclasts on resorption lacunae (Fig. 3 B).

In the CF-50 g group, on the root surface, root resorption lacunae and odontoclasts were identified; these lacunae appeared to invade the dentin from the cementum (Fig. 3 C).

Immunohistochemical findings of TRAP
In the control group, root resorption lacunae was not found on day 7, and multiclear osteoclasts which TRAP positive were not observed (Fig. 3 D).

In the CF-10 g group, on 7 days, alveolar bone surface were several resorption lacunae was observed and multiclear osteoclasts which TRAP positive were observed. Root resorption lacunae were not observed (Fig. 3 E). In contrast, in the CF-50 g group, on day 7, multiclear osteoclasts which TRAP positive were observed along the root resorption lacunae (Fig. 3 F).

Protein expression of Wnt5a and RANKL
In the control group, on day 7, Wnt5a- and RANKL-positive cells were mostly not observed around the periodontal tissue (Fig. 3 G and 3 J).

In the CF-10 g group, on day 7, Wnt5a- and RANKL-positive cells were observed on the alveolar bone surfaces (Fig. 3 H and 3 K).

In the CF-50 g group, on day 7, Wnt5a- and RANKL-positive cells were observed along the root resorption lacunae (Fig. 3 I and 3 L).

Quantitative evaluations of the number of TRAP-, Wnt5a-, and RANKL-positive cells
The number of TRAP-, Wnt5a- and RANKL-positive cells were quantitative evaluations using ImageJ. On day 7, TRAP- and RANKL-group the number of positive cells CF-50 g group were significantly higher than that in the CF-10 g group and control group. Wnt5a group the number of positive cells CF-50 g group and CF-10 g group were significant increase than that in the control group (Fig. 4) (p < 0.05).

Double staining of RANK-RANKL and Wnt5a-Ror2
We investigated the immunoreactive expression using double-immunofluorescence staining for RANK-RANKL and Wnt5a/Ror2. The double-immunofluorescence analysis for RANK-RANKL and Wnt5a/Ror2 demonstrated the expression in the PDL and cementum. The immunolocalization of RANK-(green: Fig. 5 A and 5 D), RANKL-(red: Fig. 5 B and 5 E), Wnt5a-(green: Fig. 5 G and 5 J), and Ror2-
Fig. 3. Hematoxylin and eosin and immunohistochemical staining for tartrate-resistant acid phosphatase (TRAP) and Wnt5a.

After applying a compression force (CF) for 7 days, alveolar bone resorption was observed in the CF-10 g group, root resorption was observed in the CF-50 g group, and not in the control group (A, B, C). Multinucleated osteoclasts were observed on the alveolar bone surface in the CF-10 g group, and that were noted on the root surface in the CF-50 g group. TRAP immunoreactivity was observed in the multinucleated cells (D, E, F). Immunoreactivity for Wnt5a was observed on the alveolar bone surface and in the periodontal ligament (PDL)/tissues (arrows) in the CF-10 g group, and that observed on the root surface in the CF-50 g group (G, H, I).

Immunoreactivity for RANKL was observed on the alveolar bone surface and in PDL tissue in the CF-10 g group, and that observed on the root surface in the CF-50 g group (J, K, L). AB, alveolar bone; D, dentine; C, cementum; P, periodontal ligament. Original magnification 200×, Bar: 50 μm.
positive cells was observed in PDL tissues from CF-10 g and CF-50 g groups. On day 7 in the CF-10 g group, several RANK-RANKL-(yellow: Fig. 5 C) and Wnt5a/Ror2-(yellow: Fig. 5 I) positive cells appeared in the compression side of PDL tissues. Meanwhile, in the CF-50 g group, more intense immunoreactions for RANK-RANKL (yellow: Fig. 5 F) and Wnt5a/Ror2 (yellow: Fig. 5 L) were noted in the cementum. In the control group, RANK-RANKL-(yellow: Fig. 5 M) and Wnt5a/Ror2-(yellow: Fig. 5 N) positive PDL cells were rarely observed.

In vitro studies

Changes to expression of Wnt5a-mRNA by CF in hPDL cells.

CF was applied 1, 3, 6, 9, 12, and 24 h each group. Used real time PCR we measurement Wnt5a mRNA expression in hPDL cells. In the CF groups (1.0 g and 4.0 g), the mRNA expression of Wnt5a was significantly increased in a time-dependent manner after CF application, which peaked at 12 h after application. Furthermore, the expression of 1.0 g was significantly increased than that of 4.0 g (Fig. 6).

Changes to release of Wnt5a by CF in hPDL cells.

The release of Wnt5a from the hPDL were increased in compression to the corresponding group in a time-dependent manner CF was applied 1 to 48 h. The expression of 1.0 g was significantly increased than that of 4.0 g (Fig. 7).

Changes to expression of Wnt5a-mRNA by CF in HCEMs cells.

The Wnt5a mRNA expressions in the CF-4.0 g group increased at 0-6 h and decreased thereafter. In contrast, those in the CF-1.0 g and control groups showed a downward trend at 0-6 h; moreover, expressions in both groups were significantly lower than those in the CF-4.0 g group (Fig. 8) (p<0.05).
Fig. 5. Double immunoreactivity in the periodontal ligament (PDL) region in rats.

Receptor activator of nuclear factor-κ-B (RANK)- and Wnt5a-immunoreactive cells appeared green along the alveolar bone in the compression force (CF)-10 g group (A, G) or along the root in the CF-50 g group (D, J). Receptor activator of nuclear factor-κ-B ligand (RANKL)- and Frizzled and receptor tyrosine kinase orphan receptors 2 (Ror2)- immunoreactive cells appeared red along the alveolar bone in the CF-10 g group (B, H) or along the root in the CF-50 g group (E, K). Colocalization of RANKL (red) and RANK (green) or Ror2 (red) and Wnt5a (green) was observed in the alveolar bone in the CF-10 g group (C, I) or in the root to the CF-50 g group (F, L); double-stained cells are indicated in yellow. Control group: colocalization of RANKL (red) and RANK (green) (M) or Ror2 (red) and Wnt5a (green) (N) was observed in the PDL lesion; double-stained cells were yellow. AB, alveolar bone; C, cementum; D, dentine; P, periodontal ligament; Original magnification 200×, bar: 50 μm.
Fig. 6. Effects of compression force (CF) (1.0 and 4.0 g/cm²) on the mRNA expression of Wnt5a by human periodontal ligament cells. The mRNA expression of Wnt5a was determined using real-time polymerase chain reaction after 1, 3, 6, 9, 12, and 24 h. The mRNA expression of Wnt5a was significantly higher when subjected to 1.0 g/cm² of CF than when subjected to 4.0 g/cm² of CF after 12 h (*P < 0.05, **P < 0.01 from the Mann-Whitney U-test, indicating a significant difference from the corresponding control). Data are expressed as means ± S.D. of the four independent experiments.

Fig. 7. Effects of compression force (CF) (1.0 and 4.0 g/cm²) on Wnt5a release by human periodontal ligament cells. The Wnt5a level was determined using an enzyme-linked immunosorbent assay (ELISA) after 1, 3, 6, 9, 12, 24, and 48 h. Wnt5a levels were significantly higher in both CF groups than in the control group (*P < 0.01 from the Mann-Whitney U-test, indicating a significant difference from the corresponding control). Data are expressed as means ± S.D. of the four independent experiments.
Discussion

Wnt signaling has been reported potent bone stimulating signaling pathway. Kim et al. (19). Additionally, Baron et al. (8) demonstrated Wnt signaling is important for regulator of bone mass. Furthermore, a study by Takada et al. (20) demonstrated that the missing of a single allele in the Wnt5a gene of mice (Wnt5a +/- mice) resulted in low-bone-mass phenotypes that additionally demonstrated increased adipogenesis. Lim et al. (12) reported that the normal osteogenic nature of the PDL depends on Wnt signaling. The upregulation of Wnt signaling induces the expression of osteogenic markers (21). Isogai et al. (22) demonstrated that during OTM, Wnt5a expression was observed in the compression side of the periodontal ligament tissue in the CF-10 g. This indicates that Wnt5a is involved in PDL metabolism and alveolar bone formation during OTM.

To investigate whether Wnt5a is involved in root resorption during OTM, we observed the teeth movement by application of a heavy CF in an animal model. RANKL and Wnt5a immunoreactivity was admitted in rat PDL tissues subjected to an optimum force. (Figs. 3 and 4). Meanwhile, RANKL and Wnt5a expressions were detected in the cementum following the application of a heavy CF. This phenomenon indicated that Wnt5a stimulated not only bone formation but also bone and root resorption. Furthermore, different forces induce the root resorption at different positions.

To determine the mechanisms underlying root and bone resorption due to Wnt5a induced by heavy or optimum orthodontic force during OTM, we evaluated the Wnt5a expression in hPDL cells/cementoblasts subjected to CF using the catabolic in vitro model as per the method indicated by Asano et al. (15). Applying CF in the PDL cells, the mRNA of Wnt5a significantly increased after 12 h in a time dependent manner. The protein expressions of Wnt5a significantly increased after 24 h in a time dependent manner. In addition, Wnt5a expression at 1.0 g of CF was higher than that at 4.0 g of CF. (Figs. 6 and 7). Conversely, mRNA levels of Wnt5a were reversed in cementoblasts (Fig. 8).

Nakashima et al. (23) considering relationship between mechanical stress and Wnt signaling in bone resorption, showed that the upregulation of RANK expression by Wnt5a it might be play a important role in osteoclastogene-
sis in response to RANKL secreted from osteoblast-lineage cells, including osteocytes. Reported that Wnt5a/ROR2/ JNK crosstalk signaling between osteoblasts and osteoclast precursors enhances osteoclast differentiation and bone resorption in mice, suggesting promotion of osteoclastogenesis by Wnt5a/ROR2 signaling. Maeda et al (14). These findings by Maeda et al suggest that Wnt5a secreted from the synovial tissue is involved in bone destruction in rheumatoid arthritis (14, 9). Fig. 5 shows that RANKL and Wnt5a/Ror2 expression was detected in bone resorption following optimum CF application and in root resorption following heavy CF application. In summary, these results and findings suggest that osteoclastogenesis is activated by the increased Wnt5a expression observed in hPDL cells treated with an orthodontic force.

Considering to the relationship between root resorption and cementum, Huynh et al. demonstrated that cementoblasts have the capacity to induce osteoclastogenesis.(24) Moreover, Diercke et al. showed an IL-1beta-dependent induction of RANKL by human cementoblasts, but this was apparent only under compressive force mimicking orthodontic tooth movement (25). These findings support our results that loaded heavy force during OTM induces Wnt5a expression in HCEMs, eventually causing root resorption.

Interestingly, Wnt5a expressions showed different responses in cementoblasts and PDL cells subjected to different mechanical forces. PDL cells reacts to an optimum force, and stimulates alveolar bone resorption with tooth movement. On the other hand, cementoblasts responds to a heavy force, and stimulates root resorption. Therefore, these results suggest that in Wnt5a signaling, PDL cells may be involved in osteoclastogenesis and cementoblasts may be involved in cementoclastogenesis. The discrepant findings between the previous studies and the present study can be attributed to differences in the cell types used and the CF applied to the cells. Further studies investigating the role of Wnt signaling in PDL cells and cementoblasts are warranted.

In conclusion, our present findings suggest that Wnt5a, which is involved in Wnt signaling, plays an important role in bone remodeling during OTM by an optimum CF application. Furthermore, the Wnt5a response to heavy orthodontic force may stimulate root resorption by HCEMs.

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References
1. Shimizu M, Yamaguchi M, Fujita S, Utsunomiya T, Yamamoto H, Kasai K: Interleukin-17/T-helper 17 cells in an atopic dermatitis mouse model aggravate orthodontic root resorption in dental pulp. Eur J Oral Sci, 121: 101-110, 2001.
2. Brezniaik N, Wasserstein A: Orthodontically induced inflammatory root resorption. Part II: The clinical aspects. Angle Orthod, 72: 180-184, 2002.
3. Levander E, Malmgren O, Stenback K: Apical root resorption during orthodontic treatment of patients with multiple aplasia: a study of maxillary incisors. Eur J Orthod, 20: 427-434, 1998.
4. Yamaguchi M, Yao-Umezawa E, Tanimoto Y, Shimizu M, Kikuta J, Hikida T, Takahashi-Hikida M, Horihata S, Suzuki K, Kasai K: Individual variations in the hardness and elastic modulus of the human cementum. Journal of Hard Tissue Biology, 25: 345-350, 2016.
5. Chan E, Darendeliler MA: Physical properties of root cementum: Part 5. Volumetric analysis of root resorption craters after application of light and heavy orthodontic forces. Am J Orthod Dentofacial Orthop, 127: 186-195, 2005.
6. Krishnan V, Davidovitch Z: Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop, 129: 469.e1-532, 2006.
7. Wang Y, Gao S, Jiang H, Lin P, Bao X, Zhang Z, Hu M: Lithium chloride attenuates root resorption during orthodontic tooth movement in rats. Exp Ther Med, 7: 468-472, 2014.
8. Baron R, Kneissel M: WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med, 19: 179-192, 2013.
9. Maeda K, Takahashi N, Kobayashi Y: Roles of Wnt signals in bone resorption during physiological and pathological states. J Mol Med, 91: 15–23, 2013.
10. Dale TC: Signal transduction by the Wnt family of ligands. Biochem J, 329: 209-223, 1998.
11. Tamura M, Nemoto E, Sato MM, Nakashima A, Shimauchi H: Role of the Wnt signaling pathway in bone and tooth. Front Biosci (Elite Ed), 2: 1405-1413, 2010.
12. Lim WH, Liu B, Cheng D, Williams BO, Mah SJ, Helms JA: Wnt signaling regulates homeostasis of the periodontal ligament. J Periodontol Res, 49: 751-759, 2014.
13. Yang T, Zhang J, Cao Y, Zhang M, Jing L, Jiao K, Yu S, Chang W, Chen D, Wang M: Wnt5a/Ror2 mediates temporomandibular joint subchondral bone remodeling. J Dent Res, 94: 803-812, 2015.
14. Maeda K, Kobayashi Y, Udagawa N, Uehara S, Ishihara A, Mizoguchi T, Kikuchi Y, Takada I, Kato S, Kani S, Nishita M,
Marumo K, Martin TJ, Minami Y, Takahashi N: Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. Nat Med, 18: 405-412, 2012.

15. Asano M, Yamaguchi M, Nakajima R, Fujita S, Utsunomiya T, Yamamoto H, Kasai K: IL-8 and MCP-1 induced by excessive orthodontic force mediates odontoclastogenesis in periodontal tissues. Oral Dis, 17: 489-498, 2011.

16. Hirate Y, Yamaguchi M, Utsunomiya T, Yamamoto H, Kasai K: Effects of relaxin on relapse after experimental tooth movement in rats. Connective Tissue Research, 53: 207-219, 2012.

17. Somerman MJ, Archer SY, Imm GR, Foster RA: A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. J Dent Res, 67: 66-70, 1988.

18. Nakajima R, Yamaguchi M, Kojima T, Takano M, Kasai K: Effects of compression force on fibroblast growth factor-2 and receptor activator of nuclear factor kappa B ligand production by periodontal ligament cells in vitro. J Periodontal Res, 43: 168-173, 2008.

19. Kim JB, Leucht P, Lam K, Luppen C, Ten Berge D, Nusse R, Helms JA: Bone regeneration is regulated by wnt signaling. J Bone Miner Res, 22: 1913-1923, 2007.

20. Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, Youn MY, Takeyama K, Nakamura T, Mezaki Y, Takezawa S, Yogiashi Y, Kitagawa H, Yamada G, Takada S, Minami Y, Shibuya H, Matsumoto K, Kato S: A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat Cell Biol, 9: 1273-1285, 2007.

21. Zhong Z, Zylstra-Diegel CR, Schumacher CA, Baker JJ, Carpenter AC, Rao S, Yao W, Guan M, Helms JA, Lane NE, Lang RA, Williams BO: Wntless functions in mature osteoblasts to regulate bone mass. Proc Natl Acad Sci U S A, 109: E2197-2204, 2012.

22. Isogai N, Yamaguchi M, Kikuta J, Shimizu M, Yoshino T, Hikida T, Takahashi M, Goseki T, Kasai K: Wnt5a stimulates the bone formation in tension side during orthodontic tooth movement. Int J Oral Med Sci, 13: 120-127, 2015.

23. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H: Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med, 17: 1231-1234, 2011.

24. Huynh NC, Everts V, Pavasant P, Ampornpandemveth RS: Interleukin-1β induces human cementoblasts to support osteoclastogenesis. Int J Oral Sci, 9: 1-8, 2017.

25. Diercke K, Kohl A, Lux CJ, Erber R: IL-1β and compressive forces lead to a significant induction of RANKL-expression in primary human cementoblasts. J Orofac Orthop. 73: 397-412, 2012.