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Osteopontin is Required for Unloading-Induced Osteoclast Recruitment and Modulation of RANKL Expression during Tooth Drift-associated Bone Remodeling, but Not for Super-Eruption

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Abstract

Unloading of teeth results in extensive alveolar bone remodeling, causing teeth to move in both vertical (“super-eruption”) and horizontal direction (“drift”). In order to decipher the molecular mechanisms of unloading-induced bone remodeling during tooth movement, we focused on the role of osteopontin (OPN) in the un-opposed molar model, comparing wild-type (WT) and OPN-null mice. Our data indicated that OPN was not required for the continuous eruption of un-opposed teeth while OPN was necessary for the drift of teeth. OPN expression and osteoclast counts were greatly increased on alveolar bone surfaces facing the direction of the drift in WT mice, while osteoclast counts were diminished in OPN^{-/-} mice. RANKL expression in the distal periodontal ligament of WT molars increased significantly by day 6 following unloading, while overall levels of RANKL expression were decreased in both WT and OPN-null mice. In vitro treatment of MC3T3 cells, WT BMCs and OPN^{-/-} BMCs with recombinant OPN resulted in significantly increased RANKL expression in all three cell types. The PI3K and MEK/ERK pathway inhibitors Ly294002 and U0126 reduced RANKL expression levels *in vitro*. Treatment of BMCs and MC3T3 with OPN also resulted in increased ERK phosphorylation and reduced OPG levels. Together, our studies suggest that increased OPN expression during unloading-induced drifting of teeth enhances localized RANKL expression and osteoclast activity on drift-direction alveolar bone surfaces via extracellular matrix signaling pathways.

Keywords

Osteopontin; RANKL; PI3K; ERK; Bone remodeling; Mechanical Stress

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Introduction

Loading and unloading of bone results in continuous modeling and remodeling of the skeleton throughout life [1–3]. On a molecular level, the mechanical stresses-induced remodeling caused by loading and unloading of bone is mediated by the extracellular matrix phosphoprotein osteopontin (OPN) [4–8]. Osteopontin is an extracellular glycoprotein commonly found in bone that contains an Arg-Gly-Asp integrin binding domain and binds to multiple organic or mineral ligands such as the integrin receptor $\alpha V\beta 3$, CD44, and hydroxyapatite [9–12].

OPN^{-/-} mice subjected to force induced tooth movement have decreased bone resorption and osteoclast counts, demonstrating the vital role of OPN in force initiated remodeling [5,13]. The use of transgenic animals with reporter constructs driven by varying lengths of OPN promoter, demonstrated OPN to have an upstream promoter region that is activated in response to mechanical stress [13]. OPN is also important for bone remodeling that occurs as a result of unloading or disuse. Tail suspension studies with OPN^{-/-} mice have established OPN to be essential for unloading induced enhancement of bone resorption and suppression of bone formation [7,8,14].

OPN expression induced by mechanical loading or unloading is essential for the migration of osteoclasts to remodeling sites [5,14] and facilitates osteoclast chemotaxis, motility, and anchorage to bone through CD44, $\alpha V\beta 3$ integrin, and G-protein coupled receptors [5,11,15–17]. OPN has also been shown to be a suppressor of osteoblastic bone formation during bone remodeling induced by unloading [7,14,18]. Moreover, bone resorption and osteoclast function in OPN deficient animals is greatly reduced in response to orthodontic force [5,13,19]. Furthermore, mechanical stress induces OPN expression via ATP through P2Y1 and through Rho Kinase pathway receptors in human periodontal ligament cells [20,21].

Osteoclast recruitment at bone resorption sites is facilitated by the TNF-related ligand RANKL, which binds to its cellular receptor RANK in order to direct osteoclast differentiation, activation, survival and apoptosis [22–25]. The relationship between osteoclasts and osteoblasts in bone metabolism is greatly affected by the balance between RANKL and osteoprotegerin (OPG), a non-signaling receptor decoy for RANKL [26–29], which modulates the level of bone resorption on bone surfaces.

Movement of teeth within bone, either through changes in biological loads or through mechanical appliances, is one of the premier examples of mechanical stress-induced bone remodeling. Appliance-driven tooth movement models have contributed several key findings related to the function of OPN during bone remodeling [5,13]. During tooth movement, mechanical stress stimulates osteocytes to express OPN to initiate bone remodeling [5,13,19], resulting in bone resorption and bone apposition on opposing sides of the tooth. Supporting the pivotal function of OPN in unloading-induced enhancement of bone resorption and suppression of bone formation during tooth movement, bone resorption and osteoclast function in OPN deficient animals were greatly reduced in response to mechanical force [5,7,13,14,18].

In previous studies we have shown that unloading-induced biological tooth movement in mice is a result of osteoclastic bone resorption on the distal aspect of the alveolar socket combined with alveolar bone and cementum formation on the mesial and apical aspects of the alveolar socket [30,31]. However, the detailed molecular mechanism underlying the contribution of individual molecules on unloading-induced bone turnover is not yet known. Based on the key role of OPN in the mediation of force response in bone, we have now hypothesized that OPN is essential for the remodeling of alveolar bone that takes place during the distal drift of unopposed molars. In order to determine the role of OPN as it relates to key events in the complex

mineralized tissue remodeling processes, we have assayed key bone remodeling factors such as RANKL and OPG and tested their behavior during unloading-induced tooth movement in wild-type and OPN^{-/-} models.

Materials and Methods

Treatment of Mice

Osteopontin (OPN or Spp1) knockout mice (strain B6.129S6(Cg)- Spp1^{tm1Blh}/JB40) and wild-type (WT) C57BL/6J mice were obtained from Jackson Labs (Bar Harbor, Maine, USA). These OPN^{-/-} (Spp1^{-/-}) mice were generated by injecting targeted ES cells into C57BL/6 blastocysts and the resulting chimeric animals were crossed to outbred Black Swiss, maintained on the mixed Black Swiss, 129S6 background and then backcrossed to C57BL/6 for 10 generations (<http://jaxmice.jax.org/strain/004936.html>). In our study, C57BL/6J and OPN^{-/-} mice were obtained from Jackson and cross-bred in our lab, and littermates were used for experiments. Male OPN^{-/-} and WT (40 days of age) were separated into various experimental groups. The presence or absence of OPN was confirmed by PCR amplification of genomic DNA extracted from tail snips using *Taq* polymerase (Clontech, Mountain View, CA) and primer sequences provided by Jackson Labs. All animal experiments and procedures followed the guidelines of the University of Illinois at Chicago Animal Care Committee.

Unloading of the right-side mandibular teeth was accomplished by extraction of the right-side maxillary molars. Functional occlusion of the molars on the left side was maintained as detailed previously [30,31]. Anesthesia for the procedure was accomplished using Ketamine (100 mg/kg) and Xylazine (5 mg/kg). Mice were also given Buprenorphine (0.05 mg/kg) intraperitoneally post procedure to manage any pain.

Skeletonization and Measurement of Movement

In order to measure unloading-induced tooth movement, groups of OPN^{-/-} and WT mice ($n = 3$ each) were maintained in the unopposed state for 12 days. Wild-type and OPN^{-/-} control mice ($n = 3$ each) were maintained in normal occlusion for a period of 12 days after which they were sacrificed. Control and treatment groups contained mice of the same age and were sacrificed together on the same day. Skeletonization of the mandibles was completed atraumatically by *Dermestes maculatus*. Beetles of the *Dermestidae* family are scavengers that feed on animal flesh and hairs. Anatomists and taxidermists take advantage of the dietary preference of dermestid beetles to clean skeletons.

In preparation for morphological analysis, mandibles were photographed at uniform magnification after which distances were scaled and measured using image software (Adobe Systems, San Jose, CA). Molar drifting was measured as the difference of distances from the anterior most point of the molars to the condyles on the right and left sides (Fig 1A.b). Measurements based on these landmarks were highly reproducible [30–32]. The magnitude of drift was graphed using the formula $[(L - R) + 1]$, where L and R are the distances from anterior most point of the first molar to the condyle on the left and right sides respectively. In a similar manner, the magnitude of molar eruption was determined and graphed using the formula $[(L - R) + 1]$, where L and R are the distances from left and right molar cusp tips to the plane connecting the superior borders of the left and right mental foramina (Fig 1A.f).

Tissue Processing

WT and OPN^{-/-} mice were maintained in the unopposed state for periods of 0, 1, 3, and 6 days ($n = 10$ each). The experiments were timed so that all mice subjected to varying treatment lengths were of the same age upon sacrifice. Collected mandibles were fixed in 4% paraformaldehyde for 24 h followed by decalcification for 2 wk with 5% EDTA and 2%

paraformaldehyde. Specimens were dehydrated, embedded in paraffin, and cut in 6µm sagittal sections along the long axis of the molar teeth or in cross section to be used for TRAP staining or immunohistochemistry.

Tartrate resistant acid phosphatase staining and osteoclast counting

Osteoclasts were visualized using a tartrate resistant acid phosphatase (TRAP) staining procedure. Paraffin sections were deparaffinized, rehydrated and incubated in acetate buffered solution containing naphthol AS-MX phosphate, Fast Garnet GBC salt, and tartrate solution (.67 mol/L) (Sigma, St Louis, MO, USA) for 60 min. Sections were counterstained with hematoxylin and Villanueva osteochrome bone stain. Only cross sections of mandibular second molar roots were used for cell counting. These sections were oriented parallel to the occlusal table. For statistical evaluation, three sections each from five mice per group were subjected to morphometry. Areas of $175 \times 700\mu\text{m}$ were defined on the mesial and distal side of mandibular second molar roots (Fig 3D.a). As a reference, “mesial” is the direction that points toward the middle of the front of the jaw while “distal” is the opposite direction. Root heights of sections selected for counting were determined by the thickness of bone in between mesial and distal roots and an absence of cellular cementum. A representation of the root level of the sections is depicted by the lines in figure 3D.b. Multinucleated TRAP positive cells located adjacent to bone tissue in the predefined areas were counted as osteoclasts.

Immunohistochemistry

Anti-rabbit RANKL polyclonal antibody (ab62516) (Abcam) and anti-OPN mouse monoclonal antibody (SC-21742) (Santa Cruz Biotechnology) were used for immunostaining of paraffin sections as previously described [33]. Briefly, sections were deparaffinized, rehydrated and treated with 6% peroxide and methanol followed by a brief incubation in 10 mM sodium citrate buffer with .05% Tween 20 at pH 6.0 for antigen retrieval. Sections were then incubated with 1% bovine serum albumin (BSA) for 30 minutes at room temperature to block nonspecific binding of the antibody. After blocking sections were incubated with anti-OPN at dilution of 1:200, or anti-RANKL at a concentration of 5µg/ml and washed three times in PBS. Antigen localization in tissues was visualized using a Histomouse Broad Spectrum AEC kit (Zymed) containing secondary antibody, streptavidin enzyme conjugate, and an AEC substrate chromogen mixture. Incubation times and washes were according to the manufacturer's instructions and Hematoxylin was used as a counterstain. As a negative control, non-immune rabbit serum was used instead of the primary antibody. For dual TRAP and OPN immunoassayed slides, sections were first stained with anti-OPN as described above, after which they were incubated in a naphthol AS-MX phosphate, Fast Blue Salt (Sigma), and tartrate solution as described above. Dual stained sections were counterstained with acid fast green.

Cell culture and treatment with OPN, P13-Kinase and MEK/ERK Inhibitor

To verify the effect of OPN on RANKL expression we cultured bone marrow stromal cells from WT and OPN^{-/-} mice and cells from the osteoblast precursor cell line MC3T3-E1, subclone 4 (ATCC), in the presence or absence of recombinant osteopontin (rOPN). Bone marrow stromal cells (BMCs) were obtained by dissection of soft tissue free femurs of 40 day-old OPN^{-/-} and WT mice ($n = 15$ each). Cells were seeded at a density of 10^6 cells/cm² onto culture flasks and incubated at 37 °C, 5% CO₂. Nonadherent cells were removed by changing the medium at day 3 and adding fresh complete expansion medium every 4 days for 12 days until cells reached 70% confluency.

BMCs and MC-3T3 cells ($3 \times 10^4/\text{cm}^2$) were cultured for 12 hours in the presence or absence of 1µg/ml OPN (R&D Systems, Minneapolis, MN) and pathway inhibitors. The MEK/ERK inhibitor, UO126 (Sigma), and the PI3K inhibitor, LY294002 (Sigma), [38] were used at 10

μM to test for potential mechanisms for OPN induced RANKL expression. All experiments were conducted in triplicate.

Analysis of gene expression by real-time quantitative PCR

Apical tissues of unloaded molars from OPN^{-/-} and WT mice treated for periods of 0 (control), 1, 3, and 6 days ($n = 5$ per time point), were carefully removed as described previously [30]. RNA was extracted from tissues and cultured cells using TRIZOL LS Reagent (Invitrogen) according to the manufacturer's instructions. The expression of RANKL, OPN, and OPG in the periodontal tissues was assessed by real-time quantitative PCR. RNAs underwent RT-PCR using Sprint RT Complete kit (Clontech). Real-time quantitative PCR was conducted using Taqman Fast Universal PCR Master Mix (Applied Biosystems) with DLUX fluorogenic primers. Commercially available primers RANKL (MLUX3313140), OPN (MLUX3312179), OPG (MLUX3309083) were designed and synthesized by Invitrogen (FAM labeled LUX Designer primers, sequences maintained by Invitrogen, Carlsbad, CA, USA). Samples were normalized using ribosome 18s RNA (JOE labeled LUX primer set, Invitrogen, Carlsbad, CA, USA). Reaction condition were as follows: 2 min at 50 °C (one cycle), 10 min at 95 °C (one cycle), and 15 s at 95 °C and 1 min at 60 °C (40 cycles). PCR products were continuously monitored with an ABI PRISM 7900 detection system (Applied Biosystems). Relative expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (34) and values were graphed as the mean expression level $\pm$ standard deviation.

Western blot analysis

After 12 hrs of culture, cells were lysed and proteins extracted as described [32]. Proteins were then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, mouse molar root tissues at day 0, day 3, day 6 in our unloading model and control, OPN^{-/-} and OPN^{-/-} unloaded mouse molars were homogenized and lysed in SDS-PAGE sample buffer, and total proteins extracted. Identical amounts of protein extracts from all regenerates were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane in a semi-dry blotting apparatus containing transfer buffer (25mM tris, 40mM glycine, 10% methanol) for 45 minutes at 15V. The PVDF membrane was then blocked with 2.5% BSA for 1 hour at room temperature and the blot was incubated with 1:1000 dilution of ERK1/2 (Millipore, Billerica, MA), ERK1/2-Phospho (Millipore), 1 $\mu\text{g/mL}$ RANKL, (Abcam) or GAPDH (Abcam) antibodies for 2 hours, washed with TBST 3 times and incubated with 1:2500 dilution of HRP conjugated anti-rabbit or anti-mouse secondary antibody respectively (Zymed, South San Francisco, CA) for 1 hour, and further washed 3 times with TBST. HRP detection was performed using a chemiluminescent substrate (Supersignal West Pico Chemiluminescent Substrate, Pierce). Histograms were calculated from $n=3$ samples and analyzed in triplicate.

Statistical Analysis

Significance of the tooth movement data was calculated using Student's T-test. Osteoclast counts on mesial and distal surfaces at 0, 1, 3, and 6 days were analyzed for significance by 1-way analysis of variance (ANOVA) followed by a Tukey-Kramer pairwise test of significance at $\alpha = .05$. Statistical analysis of Real-time RT-PCR data was performed using 1-way analysis of variance (ANOVA) followed by a Tukey-Kramer pairwise test of significance at $\alpha = .05$. Graphic visualization of RT-PCR values, tooth movement studies, TRAP number count, and Western blots displays data as mean $\pm$ standard deviation.

Results

OPN is required for tooth drift as documented in OPN^{-/-} mice

In order to determine the role of OPN in unloading-induced drifting of teeth, WT and OPN^{-/-} mice were maintained in the unopposed state for 12 days and compared to mice with intact occlusion (Fig. 1). Unloading of teeth by unilateral maxillary molar extraction resulted in significant distal drifting of mandibular molars in WT mice after 12 days compared to WT controls ($P < 0.01$). In contrast, OPN^{-/-} mice teeth did not display significant distal drifting compared to OPN^{-/-} controls ($P > 0.4$) or WT controls ($P > 0.3$), demonstrating that OPN is essential for unloading-induced distal tooth drift.

OPN is not required for tooth super-eruption in OPN^{-/-} mice

To determine if OPN has a function in tooth eruption, the level of vertical tooth displacement was analyzed in WT and OPN^{-/-} mice (Fig. 1). Unloading of WT mouse molars resulted in significant molar eruption ($P < 0.001$) compared to controls with opposing teeth. Similar to WT mice, OPN^{-/-} mice also displayed significant eruption of unloaded molar teeth after 12 days ($P < 0.001$) compared to OPN^{-/-} controls (Fig. 1C), indicating that OPN is not required for tooth super-eruption.

During tooth drift in WT mice, increased localized OPN expression on distal alveolar surfaces was associated with osteoclast recruitment and increased RANKL expression in the distal periodontal ligament, while overall OPG levels were decreased or not affected

In order to determine the role of OPN in unloading-induced tooth drift, real-time quantitative PCR and immunohistochemistry were used to detect and localize changes in OPN expression. Analysis of gene expression by quantitative real-time PCR revealed that OPN expression increased significantly at 1, 3, and 6 days compared to controls (Fig. 2A). OPN was visualized by immunostaining on alveolar bone surfaces in control mice and at days 1, 3, and 6 of unloading (Fig. 2B). In the day 0 control, extracellular osteopontin was localized on alveolar bone surfaces mesial and distal to tooth roots, but not immediately apical to root apices. By day 6 of unloading, OPN was localized almost exclusively on alveolar bone distal to root surfaces ("distal alveolar bone"). Dual TRAP and OPN immunostained sections revealed osteoclast mediated bone resorption in areas of OPN expression. Although osteopontin was localized in cementum, where it reportedly has a role in mineralization (9), no change in cementum OPN expression was detected during unloading.

To further clarify the role of osteopontin in osteoclast response and bone resorption during distal drifting of unloaded teeth, TRAP stained osteoclasts were counted on molar teeth sections at days 0, 1, 3 and 6 (Fig. 3A–D). Osteoclast counts on bone surfaces distal to tooth roots gradually increased through day 6, demonstrating a 5-fold increase compared to the control (Fig. 3B). In contrast, no significant increase in osteoclasts was detected on alveolar bone mesial to tooth roots ("mesial alveolar bone") (Fig. 3C).

In force-induced orthodontic tooth movement, increased osteoclast counts are well correlated with increases in RANKL expression [28,29,39]. In order to test the effect of OPN on osteoclastogenesis and bone remodeling in the un-opposed molar model, RANKL expression during unloading-induced drifting of teeth was analyzed by quantitative real-time PCR (Fig. 4). Due to the large 5-fold increases in osteoclasts, we predicted a large increase in RANKL expression. Unexpectedly, there was a 6-fold decrease in RANKL expression in the WT group at day 1, followed by similarly significant decreases at days 3, and 6 compared to the control. In order to address this discrepancy, we analyzed the regional distribution of RANKL expression by immunohistochemistry. Stained sections of WT mice at day 0 (control) revealed uniform RANKL distribution throughout the periodontal ligament. However, at day six of

unloading, RANKL expression was markedly localized to areas distal of tooth roots compared to the mesial areas, which displayed reduced signal compared to the day 0 control. Hence, as a consequence of unloading, overall RANKL expression decreased in the periodontal tissues, except in cells lining alveolar bone distal to tooth roots.

Osteoclast formation and activity can be modulated by the RANKL/OPG expression ratio [28,29,39]. For this reason, overall OPG expression in periodontal tissues was examined by quantitative real-time PCR. Changes in OPG expression during unloading were not significant except for a brief decrease at day 1 ($P < 0.05$). Together, these data indicate that tooth drift in WT mice was associated with increased RANKL and OPG expression and osteoclast recruitment at distal alveolar surfaces, while OPG levels were decreased or not affected.

Following unloading of OPN^{-/-} mouse molars, osteoclast counts increased less than in WT counterparts, while OPG expression was increased and RANKL was little affected

To determine the effect of OPN expression during unloading-induced drifting of teeth, osteoclast counts, RANKL, and OPG expression were compared between OPN^{-/-} and WT mice. Osteoclast numbers on bone surfaces distal to tooth roots in OPN^{-/-} mice at day 6 were significantly less than in the WT group ($P < 0.001$) at day 6, highlighting the importance of OPN in bone remodeling during unloading-induced distal drifting. However, the greatly reduced osteoclast response in OPN^{-/-} mice at day 6 still constituted a significant increase ($P < 0.05$) relative to the OPN^{-/-} controls. Similar to the WT mice, no significant increases in osteoclast counts were recorded on the alveolar bone surfaces mesial to tooth roots in OPN^{-/-} mice (Fig. 3). Also similar to WT mice, unloading of teeth in OPN^{-/-} mice resulted in an overall decrease in RANKL expression in the periodontal tissues. Real-time quantitative PCR analysis revealed a significant decrease in RANKL expression in OPN^{-/-} mice at days 1 and 6 relative to OPN^{-/-} controls. However, RANKL expression in OPN^{-/-} mice was dramatically reduced in the day 0 control ($P < 0.003$) and during unloading at days 3 ($P < 0.02$) and 6 ($P < 0.001$) compared to WT mice of corresponding treatment length (Fig. 4C).

RANKL expression detected by immunohistochemistry in OPN^{-/-} mice at day 0, revealed a homogenous distribution of RANKL similar to day 0 WT mice, although less intense. Unlike the WT group, sections from OPN^{-/-} mice after 6 days of unloading did not display discernable differences in RANKL signal localization or intensity distal to tooth roots (Fig. 4B). Moreover, while WT mice exhibited a decrease or little change in OPG expression during unloading-induced drift, OPG expression in OPN^{-/-} mice increased from day 0 to 6, reaching significant levels at day 6 ($P < 0.05$). Thus, in OPN^{-/-} mice compared to WT mice, RANKL and osteoclast accumulation at distal alveolar bone surfaces was reduced or no longer detectable, while overall OPG expression was increased during tooth unloading.

In cell culture, lack of OPN in OPN null BMCs corresponded with a decrease in RANKL expression, which was partially ameliorated by adding exogenous OPN

To test whether there was a potential relationship between OPN and RANKL expression, WT BMCs, OPN^{-/-} BMCs, and pre-osteoblast MC3T3-E1 cells with or without recombinant OPN (rOPN) were cultured and subjected to real-time quantitative PCR to measure RANKL gene expression (Fig. 5A). The addition of rOPN significantly increased RANKL expression in WT BMCs ($P < 0.001$), OPN^{-/-} BMCs ($P < 0.002$) and MC3T3 cells ($P < 0.003$) compared to rOPN absent controls (Fig. 5A). WT BMCs displayed the greatest base level of RANKL expression (Fig. 5A). Furthermore, addition of rOPN to the OPN^{-/-} BMCs increased RANKL expression 3.6 fold in WT BMCs, 2.3 fold in OPN^{-/-} BMCs, and 2.9 fold in MC3T3 cells (Fig. 5A). Thus, the decrease in RANKL expression observed in cultured OPN^{-/-} BMCs compared to the WT corresponded to a lack of OPN, an effect that was partially ameliorated by addition of exogenous OPN. In another set of quantitative real-time RT-PCR experiments, the effect of

OPN on OPG expression was analyzed, both in MC3T3 cells and in BMCs. This dataset revealed that OPG expression was reduced following addition of recombinant OPN. The OPN induced reduction of OPG was significant in BMCs ($P < 0.05$), while the change was not significant in MC3T3 cells (Fig. 5B).

PI3K/Akt and MEK/ERK pathway inhibitors blocked OPN induced RANKL expression and enhanced ERK phosphorylation

Investigation of potential pathways involved in OPN induced RANKL expression was conducted using various pathway inhibitors in the presence or absence of recombinant OPN. Addition of OPN alone resulted in a more than two-fold increase in RANKL expression (Fig. 5C) and also enhanced ERK phosphorylation in BMCs and MC3T3 cells (Fig. 5E). The PI3K/Akt pathway inhibitor Ly294002 completely blocked the effect of OPN on RANKL expression and the MEK/ERK pathway inhibitor U0126 resulted in a partial block (Fig. 5D and F). Together, these data suggest that OPN mediates tissue-specific RANKL expression through the PI3K and MEK/ERK pathways.

Discussion

In the present study we have used the un-opposed (unloaded) molar model system to decipher the role of OPN in horizontal and vertical components of physiological tooth movement. Previous studies had demonstrated that unloading mandibular mouse molars by removing opposing teeth resulted in rapid super-eruption and drift in the direction of overall tooth axis orientation [30,31]. In this model system, two types of controls are commonly employed, (i) teeth from the contralateral side and (ii) teeth from untreated mice. Vital dye stains from earlier studies indicated a lack of mesio-distal drift on the contralateral side after 12 days [30], supporting the overall concept of our paper that detectable degrees of drift only occurred in un-opposed molars. Thus, untreated mice were used as standard controls because contra-lateral molars showed slight remodeling due to changed loading. In the present study we have used the un-opposed molar system to detect the function of OPN and its effect on RANKL expression, bone resorption, and rapid distal drift induced by unloading of the teeth.

Eruption and distal drift induced by unloading are a result of distal bone resorption and mesial/apical bone apposition [31]. In the present study we demonstrated that unloading of teeth in OPN^{-/-} mice resulted in tooth eruption without distal drift. Previous studies using OPN^{-/-} mice have indicated that osteopontin is essential for the enhancement of bone resorption and inhibition of bone formation that occurs as a result of unloading [14]. Thus, the lack of distal drift demonstrated by OPN^{-/-} mice suggests that OPN is essential for the enhancement of distal bone resorption during unloading. We therefore conclude that OPN is involved in the transduction of mechanical force signals that mediate unloading-induced distal resorption of alveolar bone, but not alveolar bone and cementum apposition.

Previous studies have reported that OPN is expressed on the surface of alveolar bone and in osteocytes in response to mechanical stress [5,19]. Here we have demonstrated that OPN was localized on bone surfaces mesial and distal to tooth roots in normal occluding controls, while increased OPN expression was found exclusively distal to tooth roots during unloading. The expression of OPN on mesial and distal bone surfaces during normal occlusion is corroborated by previous findings from our laboratory indicating that OPN expression is localized on alveolar bone that provides primary support against occlusal force [33]. However, distal inclinations of mouse molar teeth cause greater functional stress to alveolar bone distal to root surfaces than to the mesial aspects [31,40]. We propose that the greater distal forces of occlusion may account for the exclusively distal expression of OPN when unloaded, especially since previous studies have indicated that OPN is rapidly upregulated in osteocytes deprived of mechanical loading [18]. We therefore suggest that the increased mechanical stress on

alveolar bone distal to tooth roots induces increased OPN expression on distal alveolar bone surfaces following unloading (Fig. 6).

As mentioned previously, OPN modulates bone remodeling by inhibiting bone formation while increasing osteoclast resorption activity, attachment to bone, and chemotaxis [11,14,17,41]. Here we demonstrated significant increases in unloading induced osteoclast counts in areas of OPN expression in WT mice compared to OPN null mice. We interpret these findings to indicate that the absence of distal drift in OPN^{-/-} mice in concert with decreased osteoclast numbers implicates that OPN is necessary for osteoclast activation and function during unloading-induced movement. Continued bone apposition and super-eruption in tandem with inhibited distal alveolar resorption and drift in OPN^{-/-} mice also suggests that distal alveolar resorption is a necessary first step and a requirement for mesial alveolar bone apposition and drift of teeth. In the context of the continuous distal “drift” in rodents [32], we argue that distal bone resorption causes “unloading” of the mesial alveolar walls, which in turn results in new bone formation at mesial alveolar walls (Fig. 6). The concept of distal alveolar bone resorption preceding bone apposition at mesial alveolar walls is further supported by the Takahashi/Frost theorem postulating that bone resorption precedes apposition [42].

RANKL has been shown to be an important regulator of osteoclast activation during orthodontic force-induced tooth movement [28,29]. Remarkably little is known about the relationship between RANKL and OPN, even though it has been shown that OPN up-regulated RANKL and down-regulated OPG in arthritis cell culture models [35]. Our study revealed specific increases in RANKL expression in cells adjacent to the alveolar bone distal to tooth roots of WT mice, areas that also displayed increased OPN expression and osteoclast counts in response to unloading. In contrast, differences in RANKL signal localization were not detected mesial or distal in OPN^{-/-} mice in response to unloading, indicating that OPN may facilitate localized increases in RANKL expression stimulating bone resorption in WT mice. Moreover, previous studies of gene expression in bone during unloading reported activation of NF-κB pathway factors in WT mice that were not seen in the absence of OPN [43]. Other studies have shown that OPN^{-/-} mice have a reduced odontoclastic response and less root resorption during orthodontic tooth movement [44]. Together, these data suggest that OPN expression during unloading may stimulate bone lining cells or PDL derived effector cells to produce RANKL and subsequently activate osteoclasts and facilitate bone resorption.

Osteoprotegerin (OPG) is a soluble RANKL decoy receptor that inhibits the binding of RANKL to its receptor [26,27]. Consequently, increases in OPG expression relative to RANKL result in inhibition of osteoclastogenesis during tooth movement [29]. High ratios of RANKL to OPG expression have also been closely correlated with increased root resorption [45,46]. Our studies support the concept of a key role of OPN in controlling the classic RANKL/OPG antagonism. Absence of OPN in OPN^{-/-} mice resulted in increased OPG during unloading compared to WT counterparts as well as an abolishment of the unloading induced increase in RANKL. In contrast, increased OPN reduced OPG in BMC culture, while exogenous OPN was able to ameliorate a decrease of RANKL in OPN^{-/-} cells, suggesting that bone lining or PDL cells increase RANKL expression levels in response to OPN. Together, our studies suggest that unloading-induced OPN expression offsets the RANKL/OPG balance at distal alveolar wall bone resorption sites in favor of RANKL, resulting in osteoclast recruitment, bone resorption, and subsequent drift.

We identified the PI3K and MEK/ERK pathways as potential intermediary pathways to explain OPN-mediated RANKL induction during unloading-induced bone remodeling. The PI3K and MEK/ERK signaling pathways plays an important role in the regulation of various cellular processes such as proliferation, differentiation, and development [47,48]. Here our data indicate that increased ERK expression and phosphorylation might contribute to the effects of

OPN on bone remodeling. We propose that members of the PI3K and MEK/ERK signaling pathways provide a molecular interface between rising extracellular levels of OPN as a response to mechanical stress and increased levels of RANKL expression as direct effectors related to bone remodeling.

In summary, we have characterized OPN expression during unloading-induced movement of teeth and demonstrated that OPN is essential for unloading-induced distal drifting, but not eruption of mouse teeth. While tooth eruption continued in unloaded OPN^{-/-} teeth similar to WT controls, tooth drift was entirely abolished in OPN^{-/-} mice. We suggest that OPN mediates osteoclast activity, RANKL expression and bone resorption at un-loaded alveolar bone walls using a PI3K and ERK-dependent mechanism. We propose that the site-directed skeletal tissue resorption at distal alveolar walls facilitates bone apposition at mesial alveolar walls, explaining the distal drift of teeth as an osteopontin-dependent process (Fig. 6).

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References

1. Wolff J. Über die innere Architectur der Knochen und ihre Bedeutung für die Frage vom Knochenwachstum. Virchows Arch Pathol Anat Physiol Klin Med 1870;50:389–463.
2. Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999;45:1353–8. [PubMed: 10430818]
3. Frost HM. Bone's mechanostat: a 2003 update. Anat Rec A Discov Mol Cell Evol Biol 2003;275:1081–101. [PubMed: 14613308]
4. Storey E, Feik SA. Remodelling of bone and bones: effects of altered mechanical stress on caudal vertebrae. J Anat 1985;140 (Pt 1):37–48. [PubMed: 4066469]
5. Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, Kawahata H, Inaguma N, Kitamura Y, Nomura S. Role of osteopontin in bone remodeling caused by mechanical stress. J Bone Miner Res 1999;14:839–49. [PubMed: 10352091]
6. Nomura S, Takano-Yamamoto T. Molecular events caused by mechanical stress in bone. Matrix Biol 2000;19:91–6. [PubMed: 10842092]
7. Ishijima M, Tsuji K, Rittling SR, Yamashita T, Kurosawa H, Denhardt DT, Nifuji A, Noda M. Resistance to unloading-induced three-dimensional bone loss in osteopontin-deficient mice. J Bone Miner Res 2002;17:661–7. [PubMed: 11918223]
8. Ishijima M, Tsuji K, Rittling SR, Yamashita T, Kurosawa H, Denhardt DT, Nifuji A, Ezura Y, Noda M. Osteopontin is required for mechanical stress-dependent signals to bone marrow cells. J Endocrinol 2007;193:235–43. [PubMed: 17470514]
9. Sodek J, Ganss B, McKee MD. Osteopontin. Crit Rev Oral Biol Med 2000;11:279–303. [PubMed: 11021631]
10. Kazanecki CC, Uzwiak DJ, Denhardt DT. Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. J Cell Biochem 2007;102:912–24. [PubMed: 17910028]
11. Zhu B, Suzuki K, Goldberg HA, Rittling SR, Denhardt DT, McCulloch CA, Sodek J. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of osteopontin. J Cell Physiol 2004;198:155–67. [PubMed: 14584055]
12. Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, Sodek J. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol 2000;184:118–30. [PubMed: 10825241]

13. Fujihara S, Yokozeki M, Oba Y, Higashibata Y, Nomura S, Moriyama K. Function and regulation of osteopontin in response to mechanical stress. *J Bone Miner Res* 2006;21:956–64. [PubMed: 16753026]
14. Ishijima M, Rittling SR, Yamashita T, Tsuji K, Kurosawa H, Nifuji A, Denhardt DT, Noda M. Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin. *J Exp Med* 2001;193:399–404. [PubMed: 11157060]
15. Weber GF, Ashkar S, Glimcher MJ, Cantor H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 1996;271:509–12. [PubMed: 8560266]
16. Chellaiiah MA, Hruska KA. The integrin $\alpha(v)\beta(3)$ and CD44 regulate the actions of osteopontin on osteoclast motility. *Calcif Tissue Int* 2003;72:197–205. [PubMed: 12469249]
17. Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, Sodek J. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. *J Bone Miner Res* 2002;17:1486–97. [PubMed: 12162503]
18. Gross TS, King KA, Rabaia NA, Pathare P, Srinivasan S. Upregulation of osteopontin by osteocytes deprived of mechanical loading or oxygen. *J Bone Miner Res* 2005;20:250–6. [PubMed: 15647819]
19. Kuroda S, Balam TA, Sakai Y, Tamamura N, Takano-Yamamoto T. Expression of osteopontin mRNA in odontoclasts revealed by in situ hybridization during experimental tooth movement in mice. *J Bone Miner Metab* 2005;23:110–13. [PubMed: 15750688]
20. Wongkhantee S, Yongchaitrakul T, Pavasant P. Mechanical stress induces osteopontin expression in human periodontal ligament cells through rho kinase. *J Periodontol* 2007;78:1113–9. [PubMed: 17539726]
21. Wongkhantee S, Yongchaitrakul T, Pavasant P. Mechanical stress induces osteopontin via ATP/P2Y1 in periodontal cells. *J Dent Res* 2008;87:564–8. [PubMed: 18502966]
22. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;423:337–42. [PubMed: 12748652]
23. Teitelbaum SL. Bone resorption by osteoclasts. *Science* 2000;289:1504–8. [PubMed: 10968780]
24. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys* 2008;473:139–46. [PubMed: 18395508]
25. Wu X, Pan G, McKenna MA, Zayzafoon M, Xiong WC, McDonald JM. RANKL regulates Fas expression and Fas-mediated apoptosis in osteoclasts. *J Bone Miner Res* 2005;20:107–16. [PubMed: 15619676]
26. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165–76. [PubMed: 9568710]
27. Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S, Lacey DL. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 1999;145:527–38. [PubMed: 10225954]
28. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;80:887–91. [PubMed: 11379890]
29. Kanzaki H, Chiba M, Takahashi I, Haruyama N, Nishimura M, Mitani H. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. *J Dent Res* 2004;83:920–25. [PubMed: 15557398]
30. Holliday S, Schneider B, Galang MT, Fukui T, Yamane A, Luan X, Diekwisch TG. Bones, teeth, and genes: a genomic homage to Harry Sicher's "Axial Movement of Teeth". *World J Orthod* 2005;6:61–70. [PubMed: 15794043]
31. Luan X, Ito Y, Holliday S, Walker C, Daniel J, Galang TM, Fukui T, Yamane A, Begole E, Evans C, Diekwisch TG. Extracellular matrix-mediated tissue remodeling following axial movement of teeth. *J Histochem Cytochem* 2007;55:127–40. [PubMed: 17015623]
32. Luan X, Diekwisch TG. Vienna-Chicago: the cultural transformation of the model system of the unopposed molar. *Bioessays* 2007;29:819–30. [PubMed: 17621674]

33. Walker CG, Ito Y, Dangaria S, Luan X, Diekwisch TG. RANKL, osteopontin, and osteoclast homeostasis in a hyperocclusion mouse model. *Eur J Oral Sci* 2008;116:312–8. [PubMed: 18705798]
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8. [PubMed: 11846609]
35. Ishii T, Ohshima S, Ishida T, Mima T, Tabunoki Y, Kobayashi H, Maeda M, Uede T, Liaw L, Kinoshita N, Kawase I, Saeki Y. Osteopontin as a positive regulator in the osteoclastogenesis of arthritis. *Biochem Biophys Res Commun* 2004;316:809–15. [PubMed: 15033472]
36. Bourguignon LY, Peyrollier K, Gilad E, Brightman A. Hyaluronan-CD44 interaction with neural Wiskott-Aldrich syndrome protein (N-WASP) promotes actin polymerization and ErbB2 activation leading to beta-catenin nuclear translocation, transcriptional up-regulation, and cell migration in ovarian tumor cells. *J Biol Chem* 2007;282:1265–80. [PubMed: 17092940]
37. Cao JJ, Singleton PA, Majumdar S, Boudignon B, Burghardt A, Kurimoto P, Wronski TJ, Bourguignon LY, Halloran BP. Hyaluronan increases RANKL expression in bone marrow stromal cells through CD44. *J Bone Miner Res* 2005;20:30–40. [PubMed: 15619667]
38. Mori K, Blanchard F, Charrier C, Battaglia S, Ando K, Duplomb L, Shultz LD, Redini F, Heymann D. Conditioned media from mouse osteosarcoma cells promote MC3T3-E1 cell proliferation using JAKs and PI3-K/Akt signal crosstalk. *Cancer Sci* 2008;99:2170–6. [PubMed: 18957057]
39. Oshiro T, Shiotani A, Shibasaki Y, Sasaki T. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. *Anat Rec* 2002;266:218–25. [PubMed: 11920384]
40. Schneider BJ, Meyer J. Experimental Studies on the Interrelations of Condylar Growth and Alveolar Bone Formation. *Angle Orthod* 1965;35:187–99. [PubMed: 14331019]
41. Yao Z, Xing L, Qin C, Schwarz EM, Boyce BF. Osteoclast precursor interaction with bone matrix induces osteoclast formation directly by an interleukin-1-mediated autocrine mechanism. *J Biol Chem* 2008;283:9917–24. [PubMed: 18250170]
42. Takahashi H, Epker B, Frost HM. Resorption Precedes Formative Activity. *Surg Forum* 1964;15:437–8. [PubMed: 14189452]
43. Ishijima M, Ezura Y, Tsuji K, Rittling SR, Kurosawa H, Denhardt DT, Emi M, Nifuji A, Noda M. Osteopontin is associated with nuclear factor kappaB gene expression during tail-suspension-induced bone loss. *Exp Cell Res* 2006;312:3075–83. [PubMed: 16889770]
44. Chung CJ, Soma K, Rittling SR, Denhardt DT, Hayata T, Nakashima K, Ezura Y, Noda M. OPN deficiency suppresses appearance of odontoclastic cells and resorption of the tooth root induced by experimental force application. *J Cell Physiol* 2008;214:614–20. [PubMed: 17894420]
45. George A, Evans C. Detection of root resorption using dentin and bone markers. *Orthod Craniofac Res* 2009;12:229–35. [PubMed: 19627525]
46. Yamaguchi M, Aihara N, Kojima T, Kasai K. RANKL increase in compressed periodontal ligament cells from root resorption. *J Dent Res* 2006;85:751–6. [PubMed: 16861294]
47. Shaul YD, Seger R. The MEK/ERK cascade: from signaling specificity to diverse functions. *BBA* 2007;1773:1213–26. [PubMed: 17112607]
48. Zehorai E, Yao Z, Plotnikov A, Seger R. The subcellular localization of MEK and ERK – A novel nuclear translocation signal (NTS) paves a way to the nucleus. *Mol Cell Endocrinol* 2010;314:213–20. [PubMed: 19406201]

Fig. 1. OPN is required for unloading-induced distal tooth drift but not tooth eruption

(A) Distal drifting and super-eruption in wild-type (WT - a,b,e,f) and osteopontin null mice (OPN^{-/-} - c,d,g,h). Letters (L) and (R) represent measurements used to determine the magnitude of (b) drift and (f) eruption on the left and right sides, respectively. (B) illustrates differences in distal drift in unloaded and control WT and OPN^{-/-} mice and (C) demonstrates the average magnitude of eruption in unloaded and control WT and OPN^{-/-} mice. All measurements are in mm after 12 days of unloading. While super-eruption in OPN^{-/-} mice was similar to WT controls, there was no distal drift in OPN^{-/-} mice following unloading. MF, mental foramen; M1, M2, M3, first, second and third molars, respectively; ** $P < 0.01$, *** $P < 0.001$; Bars: (A.a-d) = 1mm; (A.e-h) = 1mm.

Fig. 2. Unloading of teeth induces OPN localization on distal alveolar bone surfaces
 (A) Expression of OPN in periodontal tissues during unloading, analyzed by real-time quantitative PCR (* $P < 0.05$). (B) OPN immunohistochemistry on day 0 (a,c) and after 6 days of unloading (b,d). In (a,b) sections were immunolabeled for OPN and in (c,d) sections were subjected to both OPN immunostaining and TRAP labeling. Note the strong OPN signal and TRAP positive staining on distal alveolar bone surfaces. Bars: a, b = 100μm c, d = 100 μm

Fig. 3. Osteoclast-mediated resorption during unloading-induced drift is OPN-dependent (A–D) Number and distribution of TRAP positive cells during unloading in WT and OPN^{-/-} mice. (A) TRAP staining of horizontal (a–d) and vertical sagittal (e–h) representative paraffin sections. (a,b,e,f) are from WT controls, (c,d,g,h) are from OPN^{-/-} mice, (a,c,e,g) are on day 0 of the experiment, and (b,d,f,h) are after 6 days of unloading. Arrows point to osteoclasts. R = tooth root, AB = alveolar bone; PDL = periodontal ligament. (B) and (C) are graphic representations of TRAP positive cell counts at distal (B) and mesial (C) alveolar walls in WT and OPN^{-/-} mice on days 0, 1, 3, and 6 of our unloading experiment. Note the prominent osteoclast response and resorption of distal alveolar bone surfaces in WT mice compared to OPN^{-/-} mice. * $P < 0.05$, *** $P < 0.001$. (D) Osteoclast counting. Rectangles (a) ($175 \times 700 \mu\text{m}$) outline the defined areas used for osteoclast counting and lines (b) indicate the approximate root heights at which sections were taken for osteoclast counting. Bars: a–d = $100 \mu\text{m}$ e–h = $100 \mu\text{m}$.

Fig. 4. Modulation of RANKL expression in periodontal ligament tissues as a result of occlusal unloading and OPN expression

(A) and (B) are quantitative real-time PCR data for RANKL (A) and OPG (B) expression in WT and OPN^{-/-} mice subjected to unloading. Occlusal unloading resulted in significant decreases in RANKL expression in WT and OPN^{-/-} mice as a result of decreased PDL compression (A) while OPG expression in OPN^{-/-} mice significantly increased during unloading and OPG levels in the WT decreased or remained unchanged (B). Note significantly reduced RANKL expression levels in OPN^{-/-} mice compared to the WT controls. * $P < 0.05$; *** $P < 0.001$. (C) and (D) are immunostained sections demonstrating RANKL signals in WT (C) and OPN^{-/-} (D) mice on day 0 (a,c) of the experiment and after 6 days of unloading (b,d). (c,d) are higher magnification micrographs of the boxed area in (a,b). Note intense RANKL immunolabeling in WT PDL cells adjacent to distal alveolar bone surfaces after 6 days compared to evenly stained PDL at day 0 (Cd vs. Cc), while RANKL was reduced in OPN^{-/-} mice (D). Bars A,a,b, B,a,b = 100 μ m A,c,d, B,c,d = 75 μ m.

Fig. 5. OPN affects RANKL expression through the PI3K and MEK/ERK extracellular matrix pathways

(A) Quantitative real-time PCR detection of RANKL gene expression levels following administration of recombinant osteopontin (rOPN) to MC3T3-E1 cells, WT BMCs, and OPN $-/-$ BMCs. Expression levels were calculated relative to beta-actin (control). (B) Effect of OPN administration on OPG expression in BMCs and MC3T3 cells. The significant reduction in BMCs was labeled with an asterisk ($P < 0.05$). (C,E) Effect of OPN addition on RANKL expression and ERK phosphorylation as demonstrated by Western blot. In Fig. C, the left panel represents BMC protein extracts and the right panel MC3T3 cell protein extracts. Fig. E illustrates the relative change in phospho-ERK between OPN treated and non-treated BMC and MC3T3 cells. (D,F) Effect of PI3K (LY294002) and MEK/ERK (U0126) pathway inhibitors on RANKL expression in BMCs and MC3T3 cells based on Western blot data. The upper panel in Fig. D shows BMC and the lower panel shows MC3T3 cell data. Fig. F illustrates the effect of LY294002 and U0126 PI3K and MEK/ERK pathway inhibitors on RANKL protein expression in the presence of OPN based on densitometry. * $P < 0.05$; ** $P < 0.01$.

Fig. 6. Physiological drift of teeth as a result of OPN mediated resorption at the site of unloading
 Our findings suggest that unloading of teeth results in increased OPN expression in stressed areas of alveolar bone immediately distal to tooth roots. (A) Due to tooth axis orientation of upper rodent molars, distally oriented occlusal force stresses distal alveolar bone, resulting in unloading-induced remodeling facilitated by OPN on distal alveolar bone surfaces. (B) Our data suggest that as a result of a localized, unloading-induced OPN expression, osteoclast precursors migrate to OPN expression sites on alveolar bone surfaces and attach in an integrin-dependent manner. Further we have shown that OPN induces localized RANKL expression, which is known to induce osteoclast activation and survival. (C) We propose that as the distal PDL space enlarges as a result of alveolar bone resorption, apposition of bone and cementum in non-resorption areas results in vertical and distal displacement of teeth. (D) The lack of drift in OPN^{-/-} mice is due to a lack of distal remodeling in OPN^{-/-} mice. In the OPN^{-/-} model, teeth were only displaced vertically. (E) Proposed relationship of molecular events in unloading-induced tooth movement based on the present study. Unloading of teeth results in OPN independent events (tooth eruption) and OPN dependent events (distal drift). Among these, tooth eruption is an OPN independent effect of unloading that features a decrease in RANKL and an increase in OPG and is facilitated by apical apposition of bone and cementum. In contrast, distal drift is a RANKL dependent result of unloading, featuring RANKL increase, osteoclast activation, and bone resorption at distal alveolar walls.