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# Effect RANKL Produced by Periodontal Ligament Cells on Orthodontic Tooth Movement

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## **Abstract**

The bone remodeling process involved in orthodontic tooth movement consists of bone resorption on the compression side and bone formation on the tension side of the teeth. Osteoclasts play an important role in bone remodeling and are necessary for orthodontic tooth movement. Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is essential for osteoclast formation and differentiation. Several cell types have been reported to be capable of producing RANKL. We are interested in whether there is a dominant cell type which RANKL production is critical in generating orthodontic tooth movement. In this study, we used a Cre recombinase mouse model to study the effect of RANKL deletion in periodontal ligament cells on orthodontic tooth movement. We found RANKL deletion in periodontal ligament cells significantly decreased the amount of orthodontic tooth movement and reduced the number of osteoclasts formed on the compression side after subjecting the teeth to orthodontic force. It suggests RANKL production from periodontal ligament cells contributes greatly to orthodontic tooth movement and serves as an important source of RANKL in osteoclastogenesis during orthodontic tooth movement.

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Dana T. Graves, DDS, DMSc

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University of Pennsylvania Dental Medicine

**Effect of RANKL produced by periodontal ligament cells on  
orthodontic tooth movement**

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Presented to the Faculty of Penn Dental Medicine in Fulfillment of the  
Requirements for the Degree of Master of Science in Oral Biology

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Evlambia Hajishengallis

## **Abstract**

The bone remodeling process involved in orthodontic tooth movement consists of bone resorption on the compression side and bone formation on the tension side of the teeth. Osteoclasts play an important role in bone remodeling and are necessary for orthodontic tooth movement. Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is essential for osteoclast formation and differentiation. Several cell types have been reported to be capable of producing RANKL. We are interested in whether there is a dominant cell type which RANKL production is critical in generating orthodontic tooth movement. In this study, we used a Cre recombinase mouse model to study the effect of RANKL deletion in periodontal ligament cells on orthodontic tooth movement. We found RANKL deletion in periodontal ligament cells significantly decreased the amount of orthodontic tooth movement and reduced the number of osteoclasts formed on the compression side after subjecting the teeth to orthodontic force. It suggests RANKL production from periodontal ligament cells contributes greatly to orthodontic tooth movement and serves as an important source of RANKL in osteoclastogenesis during orthodontic tooth movement.

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## **Introduction**

### **Osteoclastogenesis is stimulated by M-CSF, RANKL**

Orthodontic tooth movement involves with a sequential bone remodeling process that consists of bone resorption in the direction of tooth movement and bone formation on the opposite side. Osteoclasts are responsible for bone resorption and are derived from hematopoietic precursor cells of monocyte/macrophage lineage <sup>1</sup>.

Receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF, also known as colony-stimulating factor-1, CSF-1) are essential and sufficient for stimulation of osteoclast formation <sup>2</sup>. RANKL, a TNF-related protein in both soluble and membrane-bound forms <sup>3</sup>, can bind and activate the receptor activator of nuclear factor- $\kappa$ B (RANK), which is a TNF-related

transmembrane protein receptor that is commonly expressed on hematopoietic precursor cells, and further initiate osteoclast formation and differentiation of osteoclast<sup>4</sup>. M-CSF stimulates proliferation and differentiation of mononuclear phagocytes in addition to the dispersion and motility and survival of macrophages<sup>1</sup>. M-CSF binds to its receptor, c-Fms, expressed on myeloid cells of the mononuclear phagocytic lineage and early osteoclast precursors, thereby providing signals necessary for the survival, proliferation and differentiation of these cells<sup>5,6</sup>.

Both RANKL and M-CSF are necessary for the development of mature osteoclasts through inducing the expression of the genes required for proper osteoclast activity, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K, calcitonin receptor and integrin  $\beta_3$ <sup>7</sup>. Mice with RANKL-knockout displayed obvious osteopetrosis at birth<sup>8</sup>. This corroborated the significant role of RANKL in osteoclast formation.

### **Osteoclast formation, differentiation, and function**

Mature osteoclasts derive from the hematopoietic stem cell lineage. The committed myeloid progenitor cells are then influenced by the activation of early

factors such as PU.1 and microphthalmia-associated transcription factor (MITF) and are directed toward a monocyte-macrophage lineage. These cells further express receptors for CSF-1 (CSF-1R), and it follows by the activation of intracellular proteins including c-Fos. The consequent upregulation of the RANK receptors identifies this population of cells as typical osteoclast precursors.

Subsequently, RANKL is the primary stimulus for osteoclast differentiation toward mature osteoclasts with continued resorptive activity through the activation of the principal transcription factors of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1). The bone-resorbing ability of the osteoclasts relies on the formation of a tight attachment to the bone surface, where a cocktail of ions and matrix-degrading enzymes is secreted into the sealed lacunae to further break down the inorganic and organic components of bone<sup>4,9</sup>.

### **RANK-RANKL signaling pathway**

After RANKL binds to RANK, the recruitment of TNF receptor-associated factor 6 (TRAF6) protein leads to the phosphorylation of the inhibitory I $\kappa$ B protein by I $\kappa$ B kinase (IKK). I $\kappa$ B is then degraded by the proteasome, thereby releasing active

NF- $\kappa$ B to translocate to nucleus and induce gene transcription. NF- $\kappa$ B contributes in part to the activation of NFATc1. NFATc1 is a key regulator of osteoclast differentiation. The activation of NFATc1 is regulated by RANKL in two ways: the NF- $\kappa$ B/activator protein 1(AP-1)/c-fos pathway and calcium signaling pathway. NFATc1 collaborates with other transcription factors, such as AP-1, PU.1, cAMP-responsive element-binding protein (CREB) and MITF, to induce the expression of various osteoclast-specific genes. These osteoclast-specific effector genes include TRAP, calcitonin receptor and cathepsin K (CATK), integrin  $\beta_3$ , etc <sup>4,9,10,11</sup>.

### **RANK/RANKL/OPG axis**

Osteoclastogenesis is regulated by RANKL/RANK/OPG axis. RANKL stimulates osteoclast formation and further induces bone resorption by local osteoclasts. As a result of osteoclast activation, bone formation is also stimulated at the same time by neighboring osteoblasts through a process called 'coupling' <sup>12</sup>. Osteoprotegerin (OPG) is a secreted TNFR-related soluble protein, which acts as a decoy receptor that binds to RANKL and competes with RANK, therefore, OPG can inhibit osteoclastogenesis.



OPG has shown to block osteoclast formation *in vitro* and inhibit bone resorption *in vivo* <sup>4</sup>. Overexpression of OPG blocks osteoclast formation, which leads to osteopetrosis in mice <sup>13</sup>; in other animal studies where OPG was deleted in mice resulted in enhanced bone resorption and yielded osteoporosis in those mice <sup>13,14</sup>. The ratio of the level of expression of RANKL and OPG regulate bone remodeling process through controlling the activation status of RANK on osteoclasts <sup>4</sup>.

### **RANKL expression in cells**

Multiple cell types have been reported that are able to express RANKL mRNA or protein, including both T and B lymphocytes, osteoblast precursors, mature osteoblasts, osteocytes, keratinocytes, mammary epithelial cells, vascular endothelial cells, synovial fibroblasts, cells within periodontal tissue, and hypertrophic chondrocytes. In addition, some malignant cell types appeared in prostate cancer and multiple myeloma have also been shown of capability to express RANKL <sup>3</sup>.

Several soluble factors can stimulate RANKL production and further induce osteoclastogenesis. These factors include parathyroid hormone(PTH), parathyroid hormone-related peptide (PTHrP), tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1

(IL-1), IL-6, IL-11, IL-17, thyroid hormone, 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>3,15,10</sup>.

### **Increased RANKL expression after orthodontic force application**

Past study researched on the expression of various cytokines during orthodontic tooth movement in humans, they found higher expression of TNF- $\alpha$ , RANKL, and matrix metalloproteinase-1 (MMP-1) on the compression side; in contrast, the tension side expressed higher levels of IL-10, tissue inhibitor of MMP-I (TIMP-1), type I collagen, OPG, and osteocalcin (OCN). The level of expression of transforming growth factor-beta (TGF- $\beta$ ) was similar on both compression and tension sides<sup>16</sup>.

### **Cre mouse model**

Recent studies have identified osteocytes as the main source of RANKL production required for osteoclastogenesis in bone remodeling under physiological condition<sup>17,18</sup>. However, to our knowledge, there haven't been studies that directly identify the major source of RANKL produced during orthodontic tooth movement. Herein, we generated a transgenic mouse model using CreER recombinase to delete

RANKL genes in periodontal ligament cells and studied the significance of RANKL production of these cells in orthodontic tooth movement.

Cre (cyclization recombination) is a site-specific recombinase that allows for conditional gene ablation in specific target cells utilizing Cre/loxP system. Cre/loxP system works through delivering Cre to specific cells or tissues by a particular promoter, the essential exon of the gene of interest which is floxed with two loxP sites will be excised by the Cre recombinase, thus generating a null allele in all cells where Cre is active <sup>19</sup>.

In this study, we used CreER recombinase (ER stands for estrogen receptor) to delete the floxed RANKL genes (encoded by *Tnfrsf11* gene) in cells expressing COL1A1 gene in transgenic mice. CreER recombinase is a ligand-dependent chimeric Cre recombinase. It is composed of Cre fused to mutated hormone-binding domains of the estrogen receptor. This mutated estrogen receptor does not bind to natural ligand at physiological concentrations but will bind to the synthetic estrogen receptor ligand, 4-hydroxytamoxifen (OHT). Therefore, CreER recombinase is initially inactive but can be activated by tamoxifen, which later metabolized to OHT. Without administering tamoxifen to the transgenic mice, the CreER will not be activated,

therefore the target genes in specific cells will not be deleted. The tamoxifen-dependent activity and tissue-specific expression of CreER recombinase allow conditional gene ablation be controlled both spatially and temporally<sup>19</sup>.

A proposed method to examine Cre recombination efficiency and tissue-specific distribution of Cre transgenic mouse line with a specific promoter is the use of Cre reporter mice. The most common reporter mouse line is the R26R line which expressed ROSA26 reporter genes. Through crossbreeding CreER mice with R26R reporter mice, we can generate CreER.ROSA26 mice that have CreER recombinase with a loxP-floxed STOP cassette inserted in a broadly-expressed ROSA26 locus. After administering tamoxifen to activate CreER, the floxed STOP cassette will be excised and the cells will produce  $\beta$ -galactosidase, which gene is located in the downstream of the STOP cassette. By detecting  $\beta$ -galactosidase activity with either X-gal staining or immunofluorescence staining targeting  $\beta$ -galactosidase in the tissues, we can examine the effectiveness of Cre activation, and identify the tissue-specific distribution of CreER recombinase with a specific promoter<sup>19</sup>.

### **Promoter introduction- 3.2 kb Col1 $\alpha$ 1 promoter**

Type I collagen has a triple helix structure that comprises two alpha 1 chains and one alpha 2 chain. It is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. The genes coding for the two kinds of type I collagen chains, COL1A1 and COL1A2 genes respectively, are active selectively in osteoblasts, odontoblasts, fibroblasts, and some mesenchymal cells<sup>20</sup>.

In this study, we crossbred 3.2 kb Col1 $\alpha$ 1 promoter- CreER recombinase transgenic mice with mice with RANKL gene floxed with loxP to generate Col1 $\alpha$ 1-CreER<sup>+/-</sup>.RANKL<sup>f/f</sup> transgenic mice. Rossert et al found that 3.2 kb Col1 $\alpha$ 1 promoter was expressed at high levels in osteoblasts and odontoblasts, as well as in tendon and fascia fibroblasts, and at low levels in skin<sup>20</sup>. Ouyang et al further identified that 3.2 kb Col1 $\alpha$ 1 promoter was active in early-differentiated osteoblasts. They referred those osteoblasts as committed osteoblasts because they were immature osteogenic progenitor cells that lack chondrogenic capacity<sup>21</sup>. We referred this sort of immature osteogenic progenitor cells as immature osteoblasts in the following texts.

In order to further identify the tissue-specific expression of 3.2 kb Col1 $\alpha$ 1 promoter in dental areas, we crossbred 3.2 kb Col1 $\alpha$ 1-CreER mice with the reporter mouse line R26R to generate Col1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 mice. We use

Coll1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 mice to identify the tissue-specific expression of 3.2 kb Coll1 $\alpha$ 1 promoter and to test the effectiveness of CreER recombinase in temporal and spatial activation. We divided Coll1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 into two groups: we administered tamoxifen in one group and vehicles without tamoxifen in the other group. The group of mice that received tamoxifen would activate CreER and express  $\beta$ -galactosidase. We also gave tamoxifen to the third group consists of wild-type C57BL/6J mice. The course of tamoxifen administration for CreER activation for the Coll1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 transgenic mice was the same as the CreER mice used in the present study, which was illustrated later in the Material and Methods. The expression of activated CreER recombinase was examined by immunofluorescence staining for  $\beta$ -galactosidase. The results showed that from the Coll1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 mice which received tamoxifen, there was positive staining in the periodontal ligament (Figure 1).

Combining our findings with the past studies which identified expression of 3.2 kb Coll1 $\alpha$ 1 promoter was in the immature osteoblasts on the periosteum, it suggests that the expression of Coll1A1 gene in the dental region using 3.2 kb Coll1 $\alpha$ 1 promoter is localized in periodontal ligament cells, including a population of immature

osteoblasts, fibroblasts and other possible cells. We further examined if there's significant contribution of RANKL produced from these cells to orthodontic tooth movement.

## **Materials and Methods**

### **Experimental design**

This animal study was approved by Institutional Animal Care and Use Committee in University of Pennsylvania. All procedures were performed in accordance with approved guidelines of the Institutional Animal Care and Use Committee.

All the animals were closely monitored for their health condition and were weighed trice a week throughout the experimental period. They were fed powdered food, diet gel and water ad libitum.

### **Generating Transgenic Mice**

We bred mice harboring a conditional RANKL allele (RANKL<sup>fl/fl</sup>, RANKL floxed with loxP) with mice harboring a transgene consisting of 3.2 kb Col1 $\alpha$ 1 promoter

inserted upstream from CreER coding sequence (Coll $\alpha$ 1-CreER transgenic mice) and generated Coll $\alpha$ 1-CreER.RANKL<sup>f/f</sup> transgenic mice.

A total of thirty-two Coll $\alpha$ 1-CreER.RANKL<sup>f/f</sup> transgenic mice were used in this study. Sixteen Coll $\alpha$ 1-CreER<sup>+</sup>.RANKL<sup>f/f</sup> mice (later in the text referred as Cre<sup>+</sup> mice) and sixteen Coll $\alpha$ 1-CreER<sup>-</sup>.RANKL<sup>f/f</sup> mice (later referred as Cre<sup>-</sup> mice) were each randomly distributed into two different time points: 5 days (n=16 mice: 8 Cre<sup>+</sup> mice and 8 Cre<sup>-</sup> mice) and 12 days (n=16 mice: 8 Cre<sup>+</sup> mice and 8 Cre<sup>-</sup> mice).

All animals were given 50  $\mu$ l tamoxifen/day through a oral gastrogavage tube for a total of five days. One week after the last day of tamoxifen application, orthodontic appliance was delivered to the mice. This course of tamoxifen application allowed activation of CreER recombinase in the target cells. All mice were 14 week-old when the orthodontic appliances were placed. A nickel-titanium(NiT<sub>i</sub>) closed coil spring was used to generate mesializing tooth movement at the maxillary right first molar for a duration of either 5 days or 12 days.

### **Application of Orthodontic Force**



Mice were anaesthetized by intraperitoneal injection of ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg). The maxillary and mandibular incisors were retracted with a customized mouth-opening device fabricated with 0.030-inch stainless steel(SS) wire. A 4.5 mm NiTi closed coil spring (Ultimate arch wires, Bristol, Connecticut, USA) was fixated between the maxillary right first molar and both of the maxillary incisors with 0.004-inch SS ligature wires (Fort Wayne Metals, Fort Wayne, Indiana, USA) connecting the coil spring to the teeth (Figure 2). Two segments of 0.004-inch SS ligature wires were tied at the ends of the nickel-titanium coil spring. Ligature wire on the posterior end was threaded under the proximal contact between the maxillary right first and second molars and fixed around the cemento-enamel junction (CEJ) of the maxillary right first molar. An activation of 2mm of the NiTi coil spring in each animal would deliver approximately 10 grams of force, confirmed with a force gauge. Ligature wire on the anterior end was fixated around the most cervical portion of both of the maxillary incisors (Figure 2). Self-etching primer (Transbond Plus Self Etching Primer, 3M Unitek, Monrovia, CA, USA) and composite resin (Transbond XT Light Cure Adhesive Paste, 3M Unitek, Monrovia, CA, USA) were

used to secure the fixation of the ligature wires at the maxillary incisors due to the regular function of the animals.

No reactivation of the NiTi coil spring was performed during the entire experimental period. The left side of the maxilla (without an orthodontic appliance) was used as a control that was without external mechanical force.

### **Euthanasia and Tissue Harvest**

The transgenic mice were euthanized at two different time points, 5 days and 12 days, after orthodontic force application.

The maxilla was harvested in each animal after euthanasia and tissue was fixed under 10% paraformaldehyde at 4°C for 24 hours. Afterwards, the samples were washed by distilled water and preserved in 0.01M PBS. And then, the samples were scanned with micro-CT.

### **Measurement of Orthodontic Tooth Movement**

After the animals were euthanized, the amount of orthodontic tooth movement was measured by micro-CT (MicroCT35; SCANCO Medical, Bassersdorf,

Switzerland) with section thickness of 20  $\mu\text{m}$ . Orthodontic tooth movement was measured as the closest distance between the most distal point of the crown of maxillary right first molar and the most mesial point of the crown of maxillary right second molar in all sagittal sections with the axis orientation according to the alignment of the maxillary molars. Distance between the crowns of the contralateral first and second molars was also measured. The amount of orthodontic tooth movement was defined as the value obtained from subtracting the value on the left side (control side) from the value on right side (experimental side), assuming both maxillary first molars started with same proximal relationship with the neighboring second molars.

### **Measurement of the Width of Periodontal Ligament (PDL) from micro-CT images**

Measurement of the widths of periodontal ligament (PDL) mesial and distal to distobuccal roots of both maxillary right and left first molars was also preformed using the micro-CT images. The sagittal section selected for average PDL width measurement was the radiographic image with both of the root canals of the buccal

roots of the maxillary first molar most thoroughly visible. The average PDL width was measured from the coronal portion to the apical portion of the PDL. Another measurement aiming to measure the PDL width at the mid-root portion was also performed. The coronal section 400µm below the topmost point of furcation was selected for mid-root PDL width measurement in all samples. The mesial and distal PDL widths of distobuccal root were measured along the axis line passing through both of the root canals of the buccal roots of the maxillary first molar in that particular coronal section.

Osirix software was used for all the 3D image reconstruction and the measurement of orthodontic tooth movement. Image Pro Plus software was used for PDL width measurement.

### **Tissue preparation**

After micro-CT scans, the maxillae were hemi-sectioned, and the samples were placed in 10% ethylenediamine tetraacetic acid (EDTA) for slow decalcification for 5 weeks. After decalcification, the samples were processed for paraffin embedding.

### **Histology section and TRAP staining**

Serial sagittal paraffin sections (4 µm) were obtained with microtome with the orientation of both of the root canals of the mesiobuccal and distobuccal roots of the maxillary first molars.

The histology sections were stained for tartrate-resistant acid phosphatase (TRAP) activity.

#### ***TRAP Staining***

Naphthol AS-MX phosphate (30 mg, Sigma, St. Louis, MO) was dissolved in 0.2 ml of N,N-dimethylformamide (Sigma). 5 mg of fast red violet LB salt (Sigma) and 50 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate were added to the mixture (TRAP-staining solution). After rehydration, sample slides were added to the mixture (TRAP-staining solution). After rehydration, sample slides were incubated with the TRAP-staining solution for 15 min in water bath at 37°C. The slides were counterstained with hematoxylin afterwards.

The osteoclasts were identified as positive staining, multinucleated cells. Only the osteoclasts attached to the bone surface on the alveolar bone side of the PDL were counted for analysis. Both the compression and tension side of the distobuccal roots were analyzed. Due to some periodontal inflammation at the furcation areas of the

maxillary right first molars was observed in some of the animals. The topmost 100  $\mu\text{m}$  of the alveolar bone lining at the compression side of the distobuccal root of the maxillary right first molars were excluded for osteoclast count in all animals to preclude the possible influence from the adjacent periodontal bone destruction at the furcation area on osteoclastogenesis induced by orthodontic force.

Images of TRAP-stained sections were captured with a Nikon Eclipse 90i microscope (Nikon, Melville, NY, USA). NIS Elements-AR software (Nikon) was used for analysis.

### **Measurement of the Width of Periodontal Ligament (PDL) from histology sections**

The widths of periodontal ligament (PDL) mesial and distal to distobuccal roots of both maxillary right and left first molars were measured from the image of histology sections captured by Nikon microscope. The average PDL width and mid-root PDL width were recorded. Mid-root PDL width measurement was measured at the level of a horizontal line perpendicular to occlusal plane drawn 400 $\mu\text{m}$  below the furcation.

Image Pro Plus software was used for all PDL width measurement.

## Statistic Analysis

Two-sided unpaired Student's t-test was used for the comparison of Cre<sup>+</sup> and Cre<sup>-</sup> mice within the same time point. The significance level was set at P< 0.05.

All values were represented as the means± SEM.

## Results

**RANKL gene deletion in periodontal ligament cells reduced the amount of orthodontic tooth movement on day 12** (Figure 3).

As measured by micro-CT images, on day 5, the average orthodontic tooth movement of Cre<sup>-</sup> mice was 13.64± 3.02 μm; whereas in Cre<sup>+</sup> mice, the average orthodontic tooth movement was 7.29± 1.75 μm. The amount of orthodontic tooth movement was 1.9 times greater in Cre<sup>-</sup> mice compared to Cre<sup>+</sup> mice, which was not significant (P>0.05).

On day 12, the average orthodontic tooth movement of Cre<sup>-</sup> mice was 51.23± 10.06 μm; whereas in Cre<sup>+</sup> mice, the average orthodontic tooth movement was 18.16± 6.10 μm. The amount of orthodontic tooth movement was 2.8 times greater in Cre<sup>-</sup> mice compared to Cre<sup>+</sup> mice, which was significant (P<0.05).

**More compressed PDL at the mesial side of distobuccal roots in mice with RANKL gene deletion in periodontal ligament cells on day 12.**

The PDL width mesial and distal to distobuccal roots of both maxillary first molars were measured with micro-CT images in both coronal sections and sagittal sections in both 12-day groups (Figure 4).

In sagittal sections of micro-CT images, the average PDL width of the compression side of distobuccal root of the subjected tooth (maxillary right first molar) in  $Cre^{-}$  mice was  $35.74 \pm 3.11 \mu\text{m}$ ; whereas in  $Cre^{+}$  mice, the average PDL width of the compression side was  $15.82 \pm 2.42 \mu\text{m}$ . The average PDL width on the compression side in  $Cre^{-}$  mice was 2.3 times wider than in  $Cre^{+}$  mice, which was statistically significant ( $P < 0.01$ ).  $Cre^{+}$  mice showed significantly narrower PDL on the compression side of the tooth comparing to  $Cre^{-}$  mice. This results in accordance with the significant difference in the orthodontic tooth movement measured at the crown level on micro-CT images between the groups within 12-day time point.



When measured on the selected coronal sections of micro-CT images, the average mid-root PDL width on the compression side of distobuccal root of the subjected tooth in Cre<sup>-</sup> mice was 36.81± 4.52 μm; whereas in Cre<sup>+</sup> mice, the average mid-root PDL width on the compression side was 13 ± 3.87 μm. The compressed PDL width at mid-root level in Cre<sup>-</sup> mice was 2.8 times wider than in Cre<sup>+</sup> mice, which was also statistically significant (P<0.01).

We also measured the PDL width around distobuccal root of maxillary first molars on the histology sections of the 12-day groups, the average PDL width on the compression side of distobuccal root of the subjected tooth in Cre<sup>-</sup> mice was 59.95± 5.40 μm; whereas in Cre<sup>+</sup> mice, the average PDL width on the compression side was 37.81± 4.35 μm. The average PDL width on the compression side was 1.6 times wider in Cre<sup>-</sup> mice comparing to Cre<sup>+</sup> mice. This result was statistically significant (P<0.01).

Corresponding to the measurement of mid-root PDL width in coronal sections of micro-CT, we measured the mid-root PDL width mesial and distal to distobuccal root on histology sagittal sections at the level of 400μm below the furcation. The mid-root PDL width on the compression side in Cre<sup>-</sup> mice was 72.01 ± 8.13 μm; whereas in

Cre<sup>+</sup> mice, the mid-root PDL width on the compression side was 35.41± 4.48 μm. The difference was also statistically significant. The width of the compressed PDL at mid-root level in Cre<sup>-</sup> mice was 2 times wider than in Cre<sup>+</sup> mice, which was also statistically significant (P<0.01). All four methods of measuring PDL width yielded same conclusion that Cre<sup>+</sup> mice showed more constricted PDL on the compression side on day 12.

**RANKL gene deletion in periodontal ligament cells decreased osteoclast formation after orthodontic force application in both 5-day and 12-day groups.**

After orthodontic force application, there was an increase in osteoclasts at the subjected tooth comparing to the contralateral control tooth (Figure 5). On day 5, the average number of osteoclasts on the compression side of distobuccal root in Cre<sup>-</sup> mice was 9.4± 1.8; whereas in Cre<sup>+</sup> mice, the average number of osteoclasts was 2.2± 0.6. The number of osteoclasts on the compression side was 4.3 times greater in Cre<sup>-</sup> mice comparing with Cre<sup>+</sup> mice on day 5, which was statistically significant (P<0.01).

On day 12, the average number of osteoclasts on the compression side in Cre<sup>-</sup> mice was 12.8± 2.2; whereas in Cre<sup>+</sup> mice, the average number of osteoclasts was

6.2± 1.2. The number of osteoclasts on the compression side in Cre<sup>-</sup> mice was 2.1 times greater comparing with Cre<sup>+</sup> mice on day 12, which was also statistically significant (P<0.05) (Figure 6). Based on the significant decreased osteoclasts in both 5-day and 12-day group in Cre<sup>+</sup> mice, it showed prolonged inhibition in osteoclast formation in mice with RANKL deleted in periodontal ligament cells.

## **Discussion**

Most of the past animal studies that researched on orthodontic tooth movement were performed on rats because the larger size of rats facilitates easier placement of the orthodontic appliance comparing to mice. However, using mice for studying orthodontic tooth movement provides valuable opportunities to use transgenic animals with gene manipulation to discover the cellular or molecular mechanism involved in orthodontic tooth movement. Therefore, in recent years, various researchers have utilized different mouse models for studying of the biologic mechanism behind orthodontic tooth movement.

In some of the more previous studies, researchers utilized the Waldo's model, which involved with placing an elastic band between the maxillary right first and second molars in mice. The force magnitude of this model was unknown<sup>22,23</sup>.

More recently, researchers have developed different methods to place coil springs to generate orthodontic tooth movement in mice. Silva et al proposed a mouse model using a NiTi open coil spring bonded on the occlusal surface of maxillary first molar to the maxillary incisors with exact orthodontic force of 0.35 N, measured by a force gauge<sup>24</sup>. The advantage of this model is that the devise they developed specially for this mouse model provides standardization for the amount of orthodontic force applied to all the mice in the experiment; the disadvantage of this model is that bonding on the occlusal surface may cause occlusal interference that might induce occlusal trauma or increase the risk of possible dislodgement of orthodontic appliance. Nanda et al used a NiTi closed coil spring with 0.004-inch SS ligature wires fixed at the CEJ of maxillary right first molar and the maxillary incisors. They applied approximately 10 gm of force to the subjected tooth<sup>25</sup>. The advantage of this model is that there isn't occlusal interference from the orthodontic appliance directly on the subjected tooth comparing to the model presented by Silva et al; the disadvantage of

this model is that the ligature wire tying around the CEJ could cause localized periodontal inflammation, therefore it adds a complicating factor from the potential localized periodontal inflammation with or without bony destruction to the objective of studying the pure effect of orthodontic force application. Yoshimatu et al utilized a NiTi closed coil spring fixed between maxillary left first molar and maxillary incisors with 0.1-mm SS wires around the teeth using a dental adhesive agent. They applied approximately 10 gm of orthodontic force upon activation of the appliance <sup>26</sup>. This mouse orthodontic model was similar to Nanda et al. Olsen et al applied 20gm of orthodontic force with a Elgiloy coil spring bonded to the occlusal surface of maxillary left first molar to maxillary incisors <sup>27</sup>. Yan et al bonded a NiTi coil spring from the maxillary right molar to maxillary incisors under a force of 30-35 gm <sup>28</sup>. Non-exhaustively, these were just some of the mice models used in the studies published in recent years.

In addition to different methodology of the delivery of orthodontic appliance from the previously published literatures studying orthodontic tooth movement using various mouse models, the force magnitude also differed between studies. Furthermore, the age of the mice in those studies also varied from a span of

7-week-old up to 12-week-old. Lastly, the time points of euthanizing the animals varied among the past studies, ranging from 3 days up to 14 days. All these variants make comparison of the results between the past studies difficult. In our study, we used 14 week-old mice as our age of choice because mice of this age resemble young adults in humans. Young adults are a growing population in current orthodontic treatment, and thus far no data have been published using mice of this age group yet, therefore, we chose to use mice of this age group for the present study.

In order to investigate the site-specific contribution of RANKL production to orthodontic tooth movement, we used mice harboring Cre recombinase to first examine the RANKL production from periodontal ligament cells in orthodontic tooth movement.

Our results showed that when RANKL genes were deleted in periodontal ligament cells in mice, the amount of orthodontic tooth movement significantly decreased comparing to the control mice after 12 days of orthodontic force application. It suggests the significance of RANKL production from periodontal ligament cells in orthodontic tooth movement. We also found there were significantly less osteoclasts formed after orthodontic force application in mice which RANKL gene were

conditionally ablated in periodontal ligament cells comparing to the control mice on both day 5 and day 12. It suggests periodontal ligament cells are a critical source of RANKL in stimulating osteoclast formation after orthodontic force applied. The past studies using rats orthodontic model showed that peak osteoclast formation in murine orthodontic tooth movement occurs around the 3rd day after orthodontic force application, and then the number osteoclasts tapered down due to homeostasis<sup>29,30</sup>. In this study, the difference in the osteoclast number between two groups of mice on day 5 was expected after seeing difference in orthodontic tooth movement from micro-CT. Interestingly, the difference in the osteoclast number between mice with or without RANKL deletion in periodontal ligament cells on day 12 was still significant. The averaged number of osteoclasts on the compression side on day 12 was slightly higher than the averaged number of osteoclasts on day 5 (by 1.36 fold). This is similar with the findings of Moin et al using a similar mouse orthodontic model, where they found increased osteoclasts on day 7 comparing to day 3<sup>31</sup>. Sakai et al also studied the change in osteoclast number on the compression side of tooth in mice with a time course of 1, 3, 7, 14, 21 days. However, they used a different mouse orthodontic model applying compressive force on the maxillary right molar toward the palatal

side with a customized Y-spring. They found significant increase in osteoclast on day 3, and peak osteoclast formation occurred on day 7, and then osteoclasts decreased with time<sup>32</sup>. The time points we chose in our study were day 5 and day 12. We could not confirm if we also found the osteoclasts reached the peak formation on day 7 comparing to day 5 and day 12. Based on the data we have, there were more osteoclasts present on day 12 than day 5 in both groups of mice. The difference of the number of osteoclasts within the group between day 5 and day 12 was not statistically significant in the Cre<sup>-</sup> control mice (P>0.01), but it was statistically significant in Cre<sup>+</sup> mice with RANKL deletion in periodontal ligament cells (P<0.01). Combining the results of the inter-groups and intra-group comparisons between the two time points, it implies a sustained suppressive effect on osteoclastogenesis under orthodontic force in mice with RANKL deletion in periodontal ligament cells, where this suppressive effect was stronger on day 5 comparing to day 12.

From our previous experiment examining cell-specific expression of the Cre recombinase using 3.2 kb Col1 $\alpha$ 1 promoter by crossbreeding with ROSA26 reporter mice, the positive cells stained of  $\beta$ -galactosidase were concentrated in the periodontal ligament area. With the spindle shapes of those positive-stained cells, we



identified those as periodontal ligament fibroblasts (Figure 1). Therefore, we considered Cre recombinases with 3.2 kb *Coll1 $\alpha$ 1* promoter are highly-expressed in periodontal ligament fibroblasts in addition to immature osteoblasts.

Periodontal ligament (PDL), located between the root cementum and alveolar bone, acts as a cushion to withstand mechanical stress that is applied to teeth, either from normal occlusal force physiologically or from pathological occlusal trauma or from orthodontic force <sup>33</sup>. PDL can receive the mechanical forces and respond to these external forces by remodeling of surrounding tissues. It is assumed that forces such as mastication, and those from orthodontic treatment can stimulate PDL cells to produce local factors and cytokines that trigger not only the maintenance and remodeling of the ligament itself but also the bone remodeling process of adjacent alveolar bone <sup>34</sup>. The signaling mechanism of how mechanical stress initiates such remodeling processes of periodontal tissues through PDL cells is currently unclear.

Periodontal ligament contains a complex collection of cells, including osteoblasts, osteoclasts, fibroblasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells, neural elements, and endothelial cells <sup>35</sup>. Fibroblast are the most abundant cells in the periodontal ligament and are thought to

be derived from undifferentiated mesenchymal cells <sup>36</sup>. Osteoblasts are also derived from mesenchymal stem cells <sup>37</sup>. It's highly possible that 3.2 kb Col1 $\alpha$ 1 promoter is highly active in immature osteoblasts and PDL fibroblasts within periodontal ligament since they are derived from the same lineage and are both capable of expressing type I collagen <sup>20</sup>.

Stromal/osteoblastic cells are capable of producing RANKL <sup>38</sup>. Osteoblast lineage cells and bone marrow stromal cells are thought to be the major cell types that express RANKL in the process of osteoclastogenesis <sup>39</sup>. Osteoblast lineage cells contains osteoblasts, osteocytes, and bone-lining cells. The majority of osteoblasts will eventually undergo apoptosis. The rest of them are then converted into either quiescent bone-lining cells or osteocytes after embedded in the mineralized bone matrix <sup>37</sup>. Previous evidence has shown that 3.2 kb Col1 $\alpha$ 1 promoter is expressed in immature and early-differentiated osteoblasts <sup>20,21</sup>. Among the previous studies researching on the effect of RANKL produced by osteoblasts on osteoclastogenesis, there was a variation of the differentiation status of the osteoblastic cells the researchers used in the studies. Regarding whether there's a difference between the amount of RANKL mRNA expression among different stages of differentiation of

osteoblasts, past *in vitro* studies have shown conflicting results. Huang et al studied the regulation of PTH on RANKL and OPG level in early-differentiated osteoblasts and mature osteoblasts, and their results suggested that PTH could stimulate osteoclast formation primarily by suppressing OPG gene expression in early-differentiated osteoblasts and stimulating RANKL gene expression in mature osteoblasts<sup>40</sup>. This suggests elevation of RANKL gene in mature osteoblasts through differentiation. Gori et al, on the other hand, showed a decrease of RANKL mRNA expression by 5-fold and an increase of OPG mRNA expression by 7-fold during osteoblast differentiation using a immortal human marrow stromal cell line<sup>41</sup>.

Previous *in-vivo* study provided evidence that the number of osteoclasts, bone resorption, or the expression level of RANKL in bone tissue were not affected in mice with targeted ablation of committed osteoblasts<sup>42</sup>. The authors concluded that mature osteoblasts are not essential for osteoclastogenesis during regular bone remodeling. They postulated that osteocytes or osteoblast precursors may play more of an important role in the initiation of osteoclast formation. Various other mouse models showed that after increasing the number of osteoblasts, mice didn't exhibit with

increased osteoclast number<sup>38</sup>. These results suggest that osteoblasts are less likely to be an important source for simulating osteoclastogenesis in bone remodeling.

Past studies examined the change of cytokines secreted by osteoblastic cells under mechanical forces are mainly conducted *in vitro*. RANKL mRNA was upregulated in osteoblasts after subjecting to compressive force<sup>43,44</sup> and in hydrostatic pressure apparatus<sup>45</sup> *in vitro*. Under mechanical stress, different *in-vitro* studies have shown conflicting results whether OPG gene expression was upregulated<sup>46</sup> or down-regulated<sup>44</sup>. The contribution of osteoblasts-produced RANKL in orthodontic tooth movement is still unclear.

PDL fibroblasts is different from other fibroblasts because of their additional specialized functions in the formation and maintenance of the PDL in addition to their ability to differentiate in order to contribute to repair, remodeling and regeneration of the adjacent alveolar bone and cementum<sup>33</sup>. PDL fibroblasts also exhibit characteristics of osteoblasts by showing high basal alkaline phosphatase (ALP) activity<sup>47,48</sup>. PDL fibroblasts are considered to be mechanosensitive cells of the PDL that are able to transduce mechanical strain into intracellular signals<sup>49</sup>. PDL fibroblasts can transmit mechanical strain to surrounding cells and initiate remodeling

or periodontal tissue, including mineralized and non-mineralized tissue <sup>49</sup>. However, the transduction mechanism from sensing the mechanical loading signals from PDL fibroblasts to the initiation of bone remodeling is yet clarified.

After receiving orthodontic force, PDL cells release cytokines that can recruit osteoclast precursors from the bone marrow to the periodontal ligament space via blood stream. They can also further differentiate locally into mature osteoclasts. On the other hand, osteoblasts derive from local PDL cell proliferation and differentiation. Cytokines secreted from PDL cells further activate mature osteoclasts and initiate bone resorption <sup>50</sup>. Ankylosed teeth cannot be moved by orthodontic treatment clinically due to lack of PDL <sup>51</sup>. This indicates PDL cells play a vital role in osteoclast formation during orthodontic tooth movement.

Past studies regarding the response of PDL cells to mechanical force were mainly conducted *in vitro*, and some studies have shown the capability of PDL cells producing RANKL and OPG under compressive or tensile force <sup>50,52,53</sup>. Human PDL cells are found to express osteoblastic phenotypes under intermittent mechanical force loading by up-regulating alkaline phosphatase mRNA, decreasing OPG expression, and inducing osteocalcin (OCN) mRNA expression in the 24-hours

loading cycle. It has been identified *in vitro* that static compressive force up-regulated the expression of ephrin-A2 (inhibitor of osteogenesis) in PDL fibroblasts and down-regulated the expression of ephrin-B2 (stimulator of osteogenesis)<sup>54</sup>. While most of the *in-vitro* studies focused on the relationship between PDL fibroblasts and the initiation of osteogenesis under mechanical force<sup>54,55,56</sup>, few *in-vitro* studies researched about the mechanism between PDL fibroblasts and stimulation of osteoclastogenesis.

Kanzaki et al found RANKL mRNA expression on PDL cells when co-cultured with peripheral blood mononuclear cells (PBMCs) and postulated that RANKL expressed by PDL cells advocates osteoclastogenesis through cell-to-cell contact with osteoclast precursors, and the RANKL expressed by PDL cells further regulates the differentiation of osteoclasts<sup>50</sup>. PDL cells not only can activate osteoclasts for bone resorption in the final stage of osteoclast differentiation through RANK-RANKL signaling pathway, but they also can inhibit osteoclastogenesis in the early stage of osteoclast differentiation via OPG secretion<sup>50</sup>. Li et al showed that RANKL mRNA was significantly up-regulated in the PDL cell after applying 6 hours and 24 hours of compressive force *in vitro*, along with an increased expression of other potential

osteoclastogenic inducers, including PTHrP, IL-11, IL-8, and FGF-2 both *in vitro* and *in vivo*<sup>57</sup>. Garlet et al conducted an *in-vivo* study and found higher expression of TNF- $\alpha$ , RANKL, and MMP-1 on the compression side of the PDL from extracted human teeth post-orthodontic force application<sup>16</sup>.

The limitation of past studies investigating on the response of PDL cells to orthodontic force is that most studies were *in-vitro* studies. Those *in-vivo* studies where the mRNA expression of various cytokines was measured from the gingival crevicular fluid (GCF) obtained in humans<sup>58</sup> were indirect evidence to the possible underlying molecular mechanism, and there is difficulty in relating certain cytokines to specific cell-type in the general PDL cell population. The *in-vivo* studies examined the mRNA expression of cytokines from extracted human teeth are considered more direct evidence<sup>16</sup>; however, we still couldn't identify which cell types are responsible for the secretion of each of those cytokines in the PDL from those studies. The 3.2-kb *Coll1 $\alpha$ 1CreER* mouse orthodontic model presented in our study can be useful in researching on PDL cells-specific cellular mechanism after subjected to orthodontic force and can provide direct *in-vivo* evidence. Future experiment needs to be done to confirm whether the specific cells that express 3.2 kb *Coll1 $\alpha$ 1* promoter in periodontal

ligament region are limited to periodontal fibroblasts by using double immunofluorescence staining of  $\beta$ -galactosidase along with other sort of fibroblast-specific markers. If 3.2 kb *Coll1a1* promoter is proved to have high specificity in expression in PDL fibroblasts, it could have higher applicational value in the field of future research.

In this study, after RANKL gene deletion in periodontal ligament cells, the mice showed significantly decreased orthodontic tooth movement and osteoclast formation under orthodontic force. This suggests that periodontal ligament cells serve as an important source of RANKL production in stimulating osteoclastogenesis after subjected to orthodontic force.

In all our mice, we rarely found obvious root resorption on the experimental side under orthodontic force. If any root resorption was present, it was localized at either the mesio-cervical part of distobuccal roots or at the apical area of mesiobuccal roots. In the few samples with root resorption on the compression side of distobuccal root near cervical region, we analyzed the average PDL width of the compression side by connecting the dots that were immediately in proximity to the resorptive pits of the root structure to stimulate the intact root surfaces.



On day 12, the PDL widths on the compression side of distobuccal root measured on both micro-CT images and histology sections in either averaged width or mid-root width measurements showed compatible results with similar magnitude of difference between two groups of mice. The results showed that the width of the compressed PDL in mice with RANKL deletion in periodontal ligament cells was around 2 times more constricted than control mice without any gene deletion in all four measurements. This was probably due to limited bone resorption on the compression side in mice with RANKL deletion in periodontal ligament cells, so that the tooth under orthodontic force couldn't move further mesially, therefore the external orthodontic force subsequently compressed the PDL even more against the alveolar bone in the mesial direction. This result was in agreement with the decreased orthodontic tooth movement observed on micro-CT images and the decreased osteoclast number in mice with RANKL gene deleted in periodontal ligament cells. The decreased osteoclastogenesis on the compression side in mice with RANKL gene deleted in periodontal ligament cells was most likely due to reduction in RANKL production. This requires further immunofluorescence staining on RANKL to confirm this speculation.

In this study, we use CreER mouse model to investigate the effect of RANKL production from periodontal ligament cells on orthodontic tooth movement. The benefit of using CreER recombinase to activate gene deletion in specific cells using tamoxifen is that we minimize the possible effects of early gene deletion in those cells on growth and development in the transgenic mice comparing to mice with gene deletion at embryonic stage, so our experimental mice and control mice would be similar in their physical conditions. This precise temporal activation of CreER recombinase by tamoxifen administration allowed us to obtain more accurate results from the pure effect of the cell-specific gene deletion that we would like to study. Our results revealed that periodontal ligament cells are a critical source of RANKL contributing to osteoclastogenesis in orthodontic tooth movement. Our results suggest that the transduction mechanism for periodontal ligament cells under orthodontic force might involve with significant RANKL production from these cells, which further leads to osteoclast formation that is essential for orthodontic tooth movement.

Here we showed that RANKL produced by periodontal ligament cells plays a vital role in osteoclastogenesis under orthodontic force application. Other possible sources

of RANKL production that may be important to osteoclast formation in orthodontic tooth movement might be osteocytes.

Osteocytes are mechanosensory cells that are well-equipped to facilitate bone remodeling to mechanical loading. Osteocytes have been identified to play an essential role in physiologic bone remodeling <sup>59</sup>. Nakashima et al compared the RANKL expression levels between osteocytes and osteoblasts *in vitro*, they found that purified osteocytes expressed a much higher amount of RANKL than osteoblasts and bone marrow stromal cells. And when co-culturing with osteoclast precursors in the presence of 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> and PGE<sub>2</sub>, osteocytes presented a higher capability in inducing osteoclastogenesis than osteoblasts and bone marrow stromal cells *in vitro*. They also found a severe osteopetrotic phenotype in mice with RANKL deletion specifically in osteocytes. They concluded that osteocytes are the major source of RANKL in bone remodeling under physiological condition comparing to osteoblasts <sup>60</sup>. Furthermore, another *in-vitro* study showed *Tnfsf11*, the gene coding for RANKL, expression was induced by mechanical stress inflicted on MLO-Y4 cells, an osteocyte cell line <sup>60</sup>. A more recent study demonstrated that in transgenic mice with specific ablation of osteocytes, there was significant less orthodontic tooth

movement and significantly decreased osteoclast formation on the compression side<sup>61</sup>.

This suggests that osteocytes play a vital role in initiating osteoclast formation under orthodontic force. However, whether the effect from osteocytes in orthodontic tooth movement comes from RANKL production or secretion of other cytokines or expression of specific transcription factors hasn't been clarified yet.

Activated T cells are also capable of producing RANKL. Past studies have showed non-consistent results regarding how activated T cells affect osteoclastogenesis<sup>62</sup>. It appears that the effect of activated T cells on osteoclastogenesis depends on how they are activated<sup>63</sup>. And most likely, activated T cells affect osteoclastogenesis through the regulation of IFN- $\gamma$  in inflammatory states. IFN- $\gamma$ , a modulatory factor produced by activated T cells, interferes with TRAF6 and therefore strongly inhibits the RANKL-induced activation of NF- $\kappa$ B and c-Jun N-terminal kinase (JNK) *in vitro*. Resting T cells were found to exert no effect on osteoclastogenesis<sup>62</sup>. One study using CD4-Cre mice for RANKL deletion specifically in T-cell. They found homozygous mutant mice manifested a markedly reduced expression of RANKL mRNA in the thymus and spleen, but not in bone or lymph nodes comparing with control mice<sup>64</sup>. This suggests that T cells-produced

RANKL is not involved in physiologic bone remodeling. Another recent study demonstrated a significant decrease in the amount of orthodontic tooth movement and the number of osteoclasts on the compression side in T cell-deficient immunocompromised mice comparing to wild-type mice<sup>28</sup>. The author attributed the observed results to decreased TNF- $\alpha$  and IFN- $\gamma$  found in T cell-deficient immunocompromised mice in the study. However, in that study, they didn't measure the difference between the level of RANKL expression in T cell-deficient transgenic mice and wild-type mice. Therefore, there lies the possibility that the decreased osteoclast formation might be due to the reduced RANKL expression from T cells. It requires further experiment to clarify the effect of T cell-produced RANKL on orthodontic tooth movement.

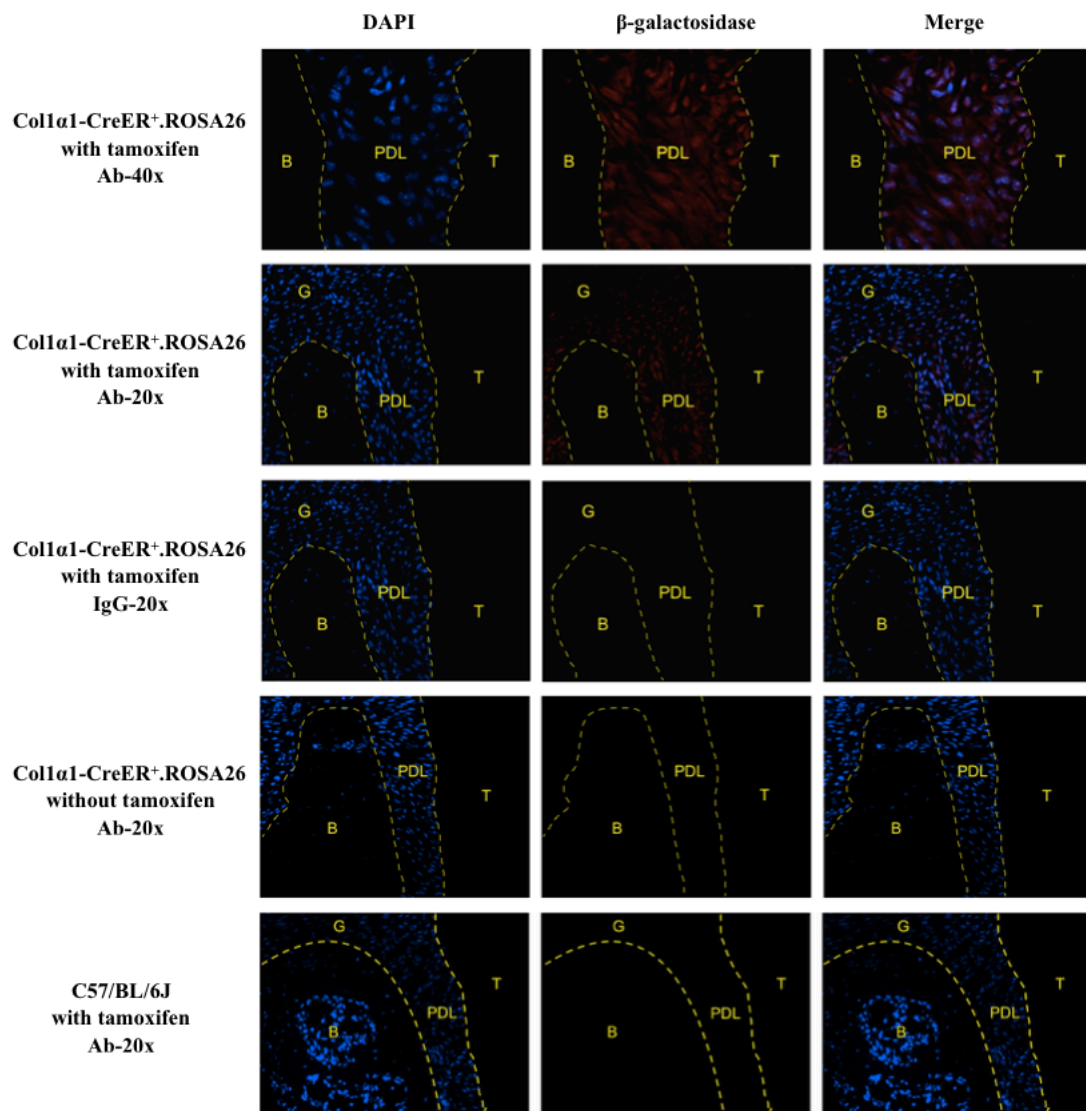
Based on the evidence from past studies, other potential significant sources of RANKL production during orthodontic tooth movement might be osteocytes or activated T cells. In order to identify the contribution in RANKL production from these other potential cell types in orthodontic tooth movement, Cre mice targeting specific genetically-defined cell populations for conditional RANKL deletion could serve as a useful tool.

A better understanding of the molecular mechanisms involved in orthodontic tooth movement might provide future insights for possible development of molecular therapy to boost orthodontic tooth movement, or in contrary, prevent tooth movement from relapsed after orthodontic treatment is finished.

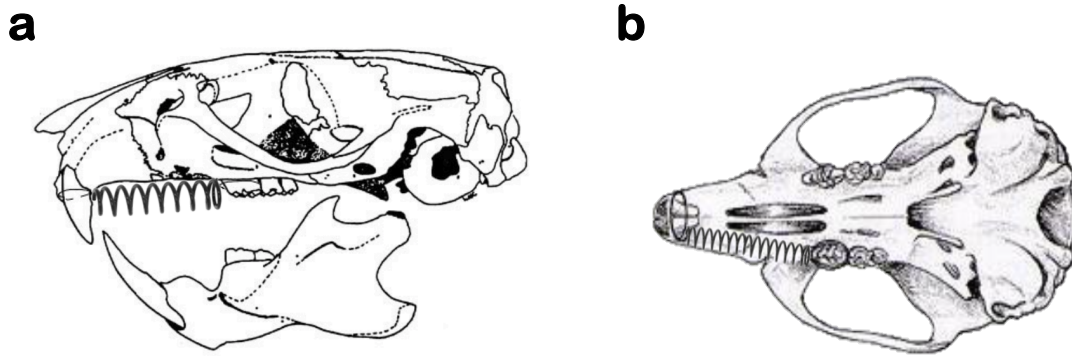
## **Conclusion**

In the present study, we utilized a CreER mouse orthodontic model to examine the effect of RANKL production from periodontal ligament cells in orthodontic tooth movement. We found there was significant reduction in the amount of orthodontic tooth movement and osteoclast formation on the compression side of the subjected tooth in the transgenic mice which RANKL genes were deleted in periodontal ligament cells (Col1 $\alpha$ 1-CreER<sup>+</sup>.RANKL<sup>f/f</sup> mice). These results suggest periodontal ligament cells contribute greatly to osteoclastogenesis in orthodontic tooth movement by acting as an important source of RANKL production.

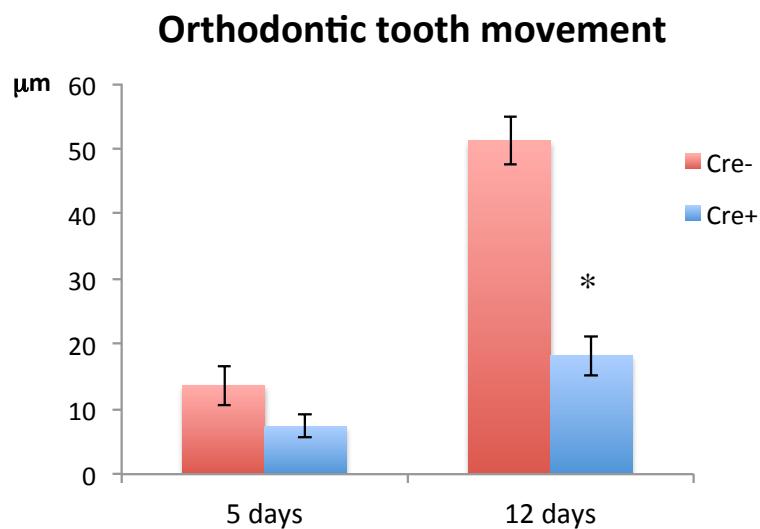
## Figures and Graphs



**Figure 1. Cell distribution of  $\beta$ -galactosidase fluorescence for examining Cre recombinase activity in Col1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 transgenic mice.** Cells expressing 3.2 kb Col1 $\alpha$ 1 promoter gene were examined by immunofluorescence co-staining  $\beta$ -galactosidase(red) and DAPI(blue) for 3.2 kb Col1 $\alpha$ 1-CreER<sup>+</sup>.ROSA26 mice. Positive red-staining cells indicating the target cells for Cre mice using 3.2 kb Col1 $\alpha$ 1 promoter were identified to be located in periodontal ligament. Some positive-stained spindle-shaped cells between bone and tooth structure were more visibly-identifiable as periodontal ligament fibroblast under 40x. G: gingiva, B: alveolar bone, T: tooth, P: periodontal ligament (PDL).

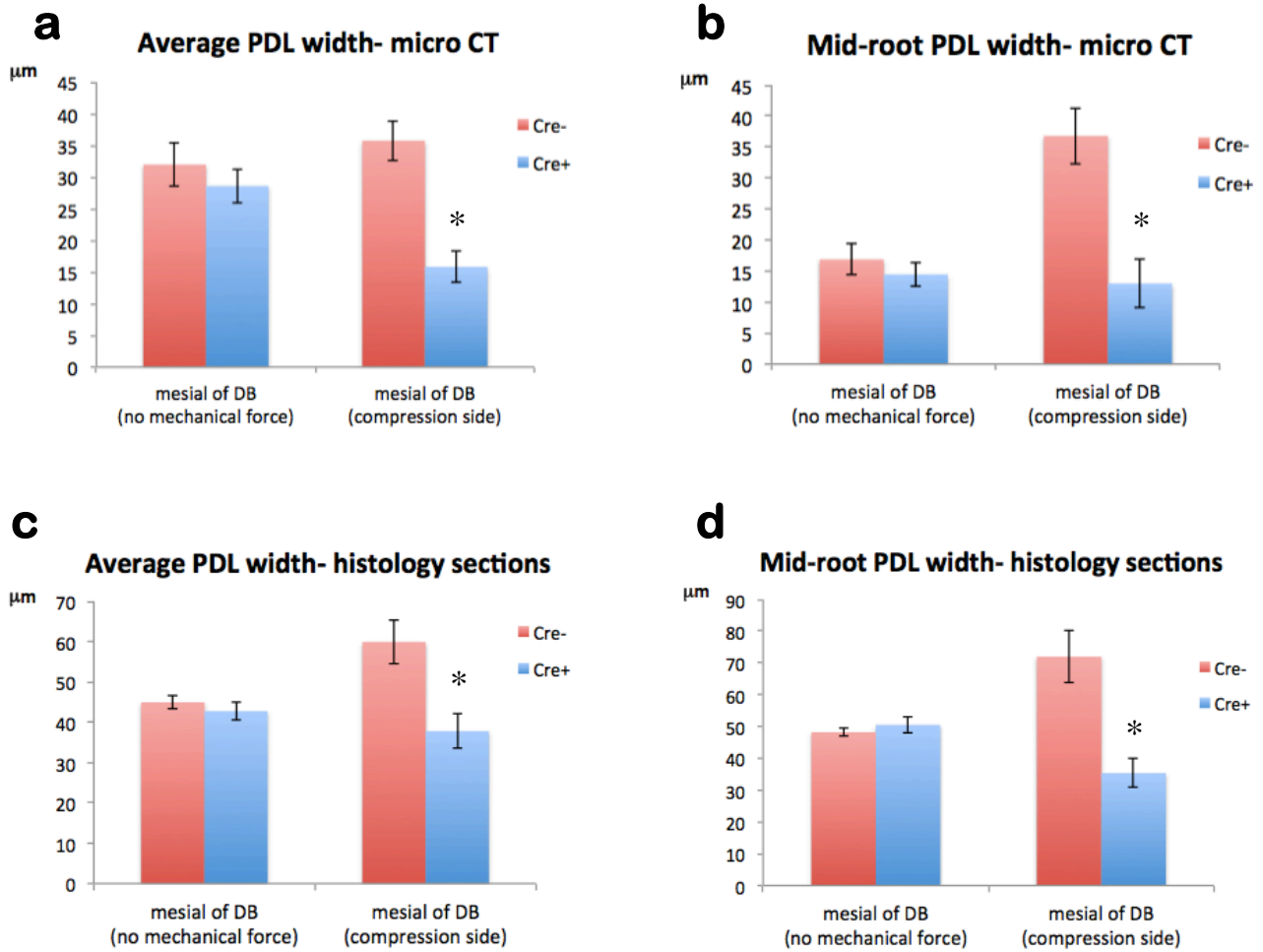


**Figure 2. Mice orthodontic model.** A NiTi closed coil was tied around the cementoenamel junction (CEJ) of maxillary right first molar and both of the maxillary incisors with 0.004-inch stainless steel ligature wire. (a) sagittal view (b) occlusal view.

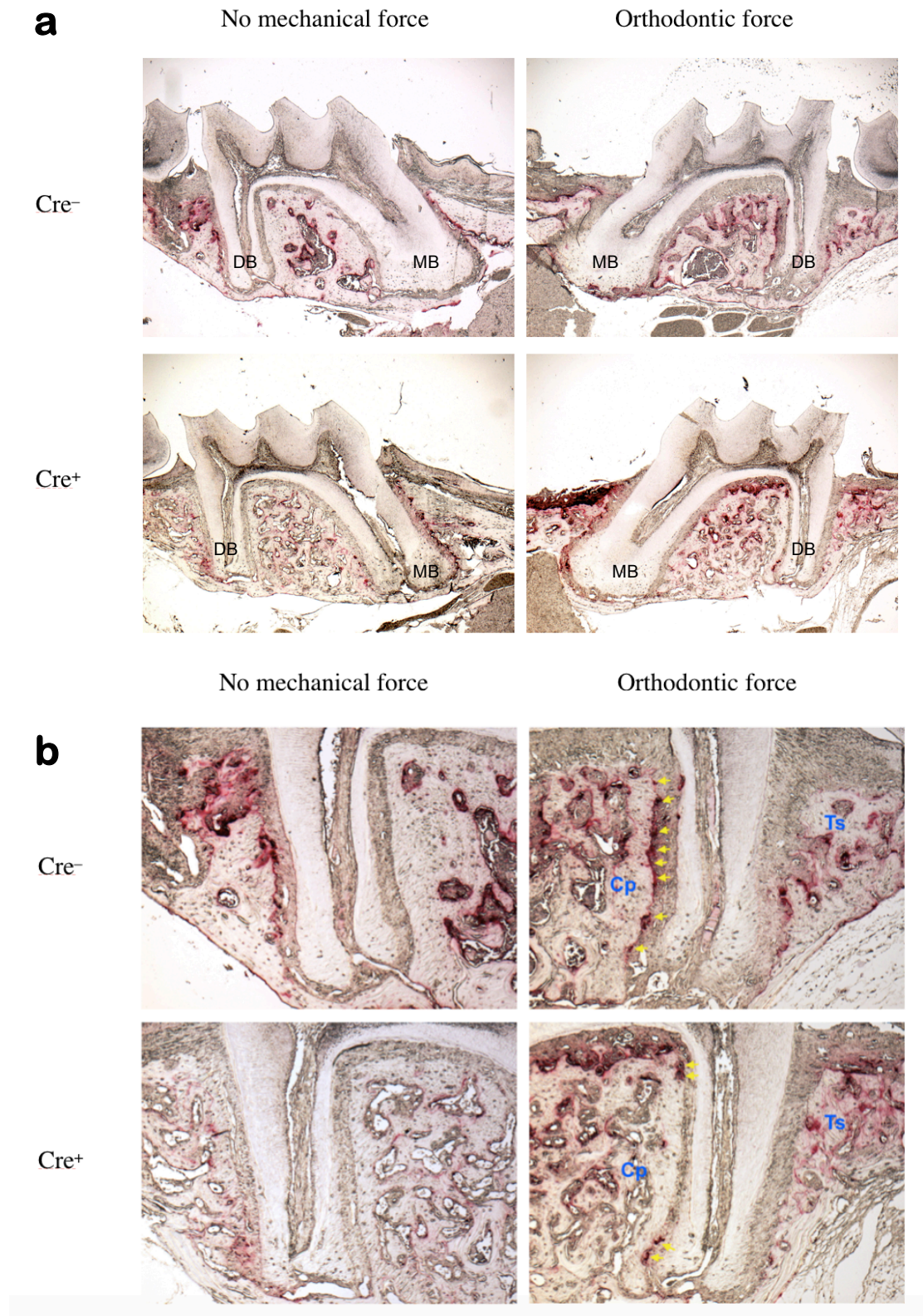


**Figure 3. RANKL gene deletion in periodontal ligament cells decreases orthodontic tooth movement.** The amount of orthodontic tooth movement measured on micro-CT showed Cre<sup>+</sup> mice had significantly reduced orthodontic tooth movement on the 12th day but not on the 5th day after orthodontic appliance insertion. \*significantly different in Cre<sup>+</sup> mice comparing to Cre<sup>-</sup> mice (P<0.05).



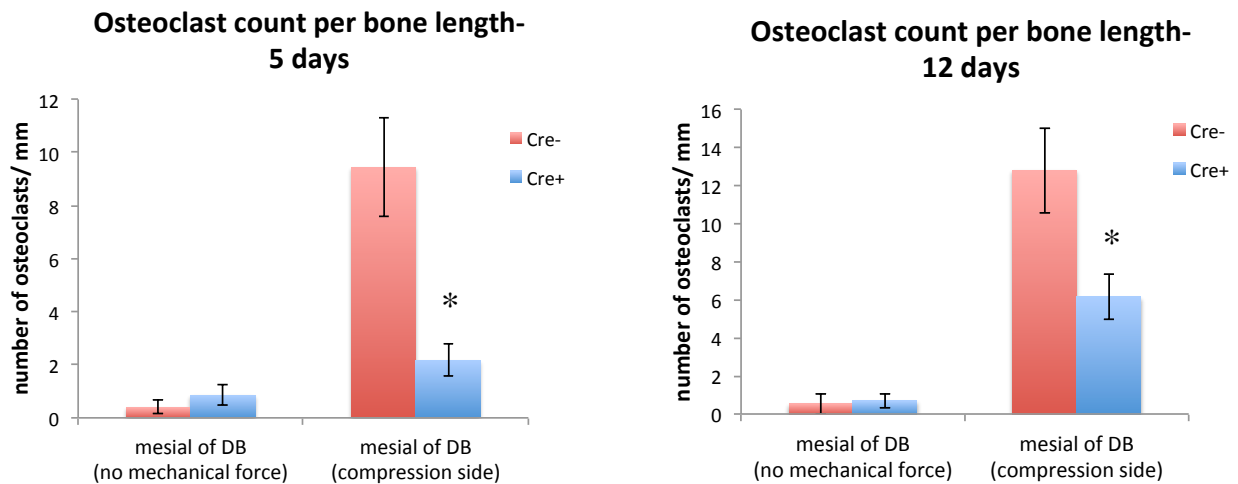


**Figure 4. PDL width measurement on the mesial side of distobuccal roots of maxillary first molars with both micro CT and histology sections.** (a-b) PDL width measurement with micro CT. Average PDL widths were measured with sagittal section on micro CT, and mid-root PDL widths were measured with the coronal sections 400μm below the top of furcation. The width of the compressed PDL mesial to distobuccal roots were significantly reduced in Cre<sup>+</sup> mice. (c-d) PDL width measurement with histology sagittal paraffin sections. Mid-root PDL widths were measured at the level of 400μm below the top of furcation. The results were consistent with micro CT results: significantly narrower PDL on the compression side of the distobuccal root of maxillary right first molars in Cre<sup>+</sup> mice comparing with Cre<sup>-</sup> mice. \*significantly different in Cre<sup>+</sup> mice comparing to Cre<sup>-</sup> mice (P<0.01).



**Figure 5. Histology sections of maxillary first molars with TRAP staining.** (a) 4X view: 4  $\mu$ m sagittal paraffin-embedded sections were oriented toward both of the root canals of mesiobuccal and distobuccal roots of maxillary first molars. Increased osteoclast formation on the compression side of the distobuccal roots on the side with orthodontic force application comparing with the no mechanical force contralateral

side. (B) 10X view of distobuccal root: Significantly decreased osteoclast formation on the compression side of distobuccal roots in Cre<sup>+</sup> mice comparing with Cre<sup>-</sup> mice. Narrower PDL on the compression side of distobuccal roots in Cre<sup>+</sup> mice comparing with Cre<sup>-</sup> mice were also noted. DB: distobuccal root; MB: mesiobuccal root; Cp: compression side; Ts: tension side. Yellow arrows indicate osteoclasts.



**Figure 6. RANKL gene deletion in periodontal ligament cells decreased osteoclast formation on the compression side of the orthodontic force.** Osteoclasts were counted on the mesial side of distobuccal roots of maxillary first molars against the alveolar bone lining. After orthodontic force application, both Cre<sup>-</sup> and Cre<sup>+</sup> mice showed increased osteoclasts comparing to the contralateral molars which weren't subjected to orthodontic force. In both 5-days and 12-days group, there was significant reduction in osteoclast formation in Cre<sup>+</sup> mice comparing to Cre<sup>-</sup> mice, indicating the significance of RANKL production from periodontal ligament cells to osteoclastogenesis in orthodontic tooth movement. \*significantly different in Cre<sup>+</sup> mice comparing to Cre<sup>-</sup> mice (P<0.05).

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