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Inherent physical characteristics and gene expression differences between alveolar and basal bones

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Abstract

Objectives—The objective of this study was to evaluate the baseline differences between alveolar and basal areas of the rat mandible.

Study Design—Rat mandibular alveolar and basal bones were evaluated using histology and micro-computed tomography to compare osteocyte number as well as bone density and architecture and polymerase chain reaction to measure gene expression levels.

Results—Micro-computed tomography data indicated that basal bone is denser and less porous than alveolar bone. Histologic analysis showed that alveolar bone has more osteocytes per unit area compared with basal bone. Real-time polymerase chain reaction results showed higher levels of expression of the following genes in basal bone than in alveolar bone: *SOST, E-11, DMP-1*, and *MEPE*.

Conclusions—Three of these gene products are associated with mature osteocytes, and this suggests that basal bone has more mature osteocyte phenotypes compared with alveolar bone. These findings are suggestive of fewer bone mineralization units and therefore a slower remodeling rate.

Several studies in the 1970s and 1980s showed that as a result of various factors, alveolar bone shows signs of bone resorption and deposition earlier compared with other bone types.[1,2] The mandible is constantly remodeling because of several factors, including mechanical stress, tooth extraction, orthodontic compression, tooth loss, and periodontitis.[3] The mandible is made up of two bone types—alveolar and basal—and it is not clear if these bone types significantly differ. For example, in Klemetti's report of his 1993 research, he

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Part of these data was presented at the following meetings: American Association of Orthodontist Annual Meeting, Hawaii, in May 2012 (“Alveolar and basal bone microstructure and osteogenic gene profile”); and The American Association for Dental Research Abstract, Tampa, FL, in March 2012 (“Microstructure and osteogenic gene profile variation between alveolar/basal bone”).

stated that bone resorption begins at the alveolar part of the mandible, whereas the basal region of the mandible remains unchanged. He further explained that factors such as osteoporosis do not change the lower part of the mandible.[4]

Much of the research on bone and bone cells has concentrated on cells from long bones, but less is known about mandibular basal and alveolar bones.[5] The majority of studies on dental bone have concentrated on the alveolar type. Alveolar and basal bones have significantly different resorption rates.[6,7] Because of the knowledge gap in this area, dental professionals have fewer treatment options for patients.

To better understand alveolar bone resorption and the limited resorption in basal bone, it is important to know the specifics about the mandible and how it differs from other bone types. Assumptions cannot be made about mandibular bone resorption based on other bone types or their corresponding bone cells. The main function of the osteocyte is to signal both osteoblasts and osteoclasts to maintain the structure and mass of bone.[8] As mechanosensors, osteocytes are important for studying bone resorption among bone types. However, differences between osteocytes within the types of bone must be understood.

In this study, the physical and gene expression profiles of the alveolar and basal bones of a rat mandible were investigated. There is a lack of significant knowledge regarding ways to prevent or reverse overall bone loss in patients. Without bone grafting, many dental procedures are difficult to perform. Understanding the differences in physical and molecular properties between alveolar and basal bones is essential for better dental treatment outcomes. Therefore, our overall hypothesis is that there will be significant differences in the physical properties and gene expression of bone regulatory proteins between alveolar and basal bones.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee approved the use of six 6-month-old Sprague-Dawley male rats, which had been used as untreated controls in another study. The committee approved use of these animals after euthanization as cadaver tissue sources.

Tissue preparation and processing for micro-computed tomography

Bone preparation for micro-computed tomography (micro-CT) began by cutting portions of the incisor and leaving only the molar area of the rat mandibles. The collected bone samples were fixed in formalin for 48 hours, transferred to 70% ethanol, and stored at −20°C. The mandible samples were then placed in phosphate-buffered saline solution and scanned in 70% alcohol.

Micro-CT

Following harvest, the mandibles were frozen until the time of scanning and then were placed in a small saline-filled tube. For bone mineral density (BMD) measurement and three-dimensional (3-D) morphometric analysis, 4% paraformaldehyde-fixed mandibles were scanned by using Skyscan 1172 (Skyscan, Aartlesaar, Belgium). The mandibles were

placed in a container filled with phosphate-buffered saline and scanned, using a 0.25-mm aluminum filter, at an image pixel size of 36.65 μm, 0.5° rotation step, and frame averaging of 3. Reconstruction of the scanned images was done with a Skyscan Nrecon program (Skyscan, Aartlesaar, Belgium). The reconstructed data sets were loaded into Skyscan CT-analyzer software (Skyscan, Aartlesaar, Belgium) for measurement of BMD and 3-D morphometric parameters. Four regions of interest were selected in alveolar and basal bones. BMD was measured in each region of interest after calibration with 0.25 and 0.75 density hydroxyl apatite phantoms. The average density of the alveolar and basal regions of interest was calculated.

The Skyscan 1174 Micro CT analyzer (Micro-photonics, Allentown, PA) has the ability to study up to 29 parameters. Micro-CT analysis calculated the following 14 parameters: tissue volume (TV), bone volume (BV), percent bone volume (%BV), tissue surface (TS), bone surface (BS), bone surface/volume ratio (BS/V), mean total cross-sectional bone area (A), mean total cross-sectional bone perimeter (P), trabecular thickness (plate model, TbTh), trabecular diameter (rod model, TD), trabecular number (rod model, TN), closed porosity (percent) (Po), mean fractal dimension (MFD), and total intersection surface (S). To help clarify the results, we will discuss three parameters: bone surface/bone volume (BS/BV), trabecular thickness (TbTh), and porosity (Po).

BV is the sum of voxels above threshold, with an additional dilation—erosion step to fill in occasional small voids in the cortical wall. BS is measured in square millimeters. A lower bone surface is connected with increased bone strength and solidity. The micro-CT analyzer measures the surface based on the faceted surface of the “marching cubes volume model.”[9] From these calculations, the BS/BV can be derived—a measurement of bone surface per given BV. This parameter was used as an indicator of bone strength.

TbTh has been standardized and is considered one of the descriptors of trabecular bone architecture.[10] Through a combination of several formulas, the TbTh of the object is calculated.

Po is a key parameter that can determine the performance of bone. The micro-CT analyzer measures the Po of bone as a percentage of the total area of binarized objects contained in fully enclosed spaces.[9]

Statistical analysis included analysis of variance (ANOVA), pairwise Student's *t* tests, with a level of significance chosen at $\alpha < 0.05$.

Tissue preparation and processing for histologic analysis

Mandibles were fixed, decalcified, and embedded in paraffin for histologic analysis. Five-micron sections were stained with hematoxylin and eosin, and the number of osteocytes per field area of each bone was counted. Other samples were stained with Podoplanin (E-11; Abbiotec, San Diego, CA) avidin-biotin complex by the Augusta University Histology Core facility in Augusta, Georgia. The blood vessel area and bone marrow space were eliminated to provide a final osteocyte number per unit area.

Tissue preparation and processing for RNA isolation and real-time polymerase chain reaction (PCR)

Bone samples were flash-frozen in liquid nitrogen, wrapped in foil, and crushed by using a steel ball mill. The crushed bone powder from each alveolar or basal bone sample underwent RNA isolation by Trizol isolation and alcohol precipitation. RNA purity was assessed by using 260/280 nm absorbance ratio (Thermo Scientific NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA). Purified total RNA (1-2 μg/reaction) was reverse transcribed by using the High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All PCR amplifications were carried out by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The preformulated assay (20× mix) primers used in this study are from the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA).

The genes were chosen either because they are expressed differentially between osteocytes or osteoblasts or because they are important for bone remodeling. The *DMP-1, E-11*, and *MEPE* genes are grouped as genes that are highly expressed by osteocytes.[11] *DMP-1* and *MEPE* are expressed during mineralization,[12,13] and *E-11* is key to the elongation of the dendritic processes of the osteocyte.[9] *SPP1* or *OPN* is important for bone turnover,[14] serving as a potent chemotaxant for osteoclasts.[15] *SOST* codes for sclerostin, which is a negative regulator of bone formation, and *PGE2S* is stimulated during bone loss or orthodontic tooth movement.[16-18] *iNOS* and *RANKL* are both released in response to mechanical stress. *RANKL* assists in the bone resorption process, but *OPG* neutralizes *RANKL* and prevents bone resorption. *OPN* mediates osteoclast attachment to bone. *eNOS* is involved in early mechanical response and promotes osteoclastogenesis, inhibits the production of prostaglandin E2, and disrupts gap junctions.[19,20] *FGF-23* helps regulate phosphate levels and, thus, calcium levels in the body.[21] *GAPDH* was chosen as the housekeeping gene because it is consistently highly expressed in most tissues and cells in the body[22] (Table I[23-29]).

Results

The BMD of alveolar bone (1.30 ± 0.02 g/cm^2) was significantly lower than that of basal bone (1.45 ± 0.06 g/cm^2; Table II). More than 90% osteocytes within each field were positively stained for E-11 (podoplanin). The number of osteocytes of each type of bone was quantified. The blood vessel area and bone marrow space were eliminated when counting the osteocytes. After removing the blood vessel and marrow areas from the analysis, the average number of osteocytes per bone section area was 224 (106.16/mm^2) for alveolar bone and 196 osteocytes per bone section area (98.54/mm^2; see Table II) for basal bone. Representative micrographs (Figure 1) demonstrate positively stained osteocytes at ×10 magnification.

The micro-CT analysis calculated 14 parameters. The majority of the parameters calculated did not show a significant difference between the rat alveolar and basal bone samples. Three parameters were significantly different and are considered indicators of bone strength and density: BS/BV, TbTh, and Po. Because each parameter is measured in different units, the results were first normalized to the alveolar bone value, as illustrated in Figure 2. Basal bone

showed higher trabecular thickness, whereas alveolar bone showed higher BS/BV or Po. The parameters indicate that basal bone is denser and less porous than the alveolar bone. Figure 3 is a micro-CT scan of the mandible.

The delta delta CT values converted to fold regulation change of the 11 genes studied with real-time PCR are shown in Figure 4. The following genes were expressed at a higher level in the basal bone: *MEPE*, *SOST*, *E-11*, *OPG*, *RANKL*, *DMP-1*, *SSP1*, *PGES2*, and *FGF23*. *eNOS* and *iNOS* were expressed at higher levels in alveolar bone. Four genes showed a significant difference in expression between alveolar and basal bones: *MEPE*, *SOST*, *E-11*, and *DMP-1*.

Discussion

The data revealed that basal bone is significantly different from alveolar bone in properties related to strength. Micro-CT analysis of rat alveolar and basal bones revealed that compared with alveolar bone, basal bone samples had lower BS/BV as well as much lower Po, suggesting that basal bone is denser and stronger than alveolar bone. The trabecular thickness measurement changes to the strain on the mandible resulting from mandibular remodeling, bone loss, and a reduction in both cortical and trabecular bones.[30] The results showed that basal bone had a higher trabecular thickness compared with alveolar bone. High porosity is considered an identifier for weaker bone, as in cases of osteoarthritis and osteoporosis, and in this study, alveolar bone had a significantly higher percentage of bone porosity compared with alveolar bone. The higher BMD of basal bone also supports the hypothesis that alveolar and basal bone has differences in bone structure. Studies on the mandible since the 1960s have shown that in people over the age of 50 years, the increase in the porosity of the cortical layer results in a decrease in bone mass.[1] Measurements of BMD and the use of biochemical markers of bone turnover are useful in diagnosing bone diseases, such as osteoporosis. A lower BMD can be an indication of higher bone resorption. BMD measurement is one of the best indicators of bone strength. Furthermore, in our study, the number of osteocytes per area was less in basal bone, suggesting a more mature, denser bone type. These data indicate that basal bone is denser, stronger, and less porous.

Four genes are expressed significantly higher in basal bone than in alveolar bone: *SOST*, *E-11*, *DMP-1*, and *MEPE*. Three of the proteins (SOST, MEPE, and DMP-1) are associated with mature osteocytes, indicating that basal bone has more mature osteocyte phenotypes because the immature osteocyte converts to a mature osteocyte as a result of bone mineralization.[23,31] The maturation of osteocytes is not necessarily related to elapsed time or the distance from the bone surface.[31] Bone mineralization is essential for the hardness and strength of bone; therefore, a denser bone will have more mature osteocyte phenotypes.[31]

E-11 was the fourth gene that was expressed at a significantly higher level in basal bone. Less is known about *E-11* in relation to mineralized tissue. In cortical bone, such as basal bone, *E-11* expression is mainly located in the osteocytes near the bone surface, and expression decreases with cells deeper within the mineralized matrix.[9] More studies, including the introduction of mechanical stress on basal bone, may be able to provide more

insight as to why basal bone has such a high expression of *E-11* compared with alveolar bone.

Conclusions

Based on biochemical markers and measurements of bone strength, our study results showed that basal bone is different from alveolar bone. Micro-CT data and BMD indicated that alveolar bone is more porous and less dense compared with basal bone. PCR data showed statistically different expressions of three genes with mature osteocytes. This study can be used as the foundation for further investigation into the possible gene expression and protein profile changes resulting from alterations in mechanical stress and how these alterations relate to bone loss. Better understanding of the differences in bone types can lead to more effective treatments, especially for patients who are more at risk for significant bone loss. Further studies will include remodeling rate and gene expressions in alveolar and basal bones following extraction of teeth.

Acknowledgments

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Statement of Clinical Relevance

If fundamental differences in the gene expression of dental alveolar and basal bone could be identified, therapeutic targets may be identified to help reduce or totally eliminate the loss of alveolar dental bone caused by resorption.

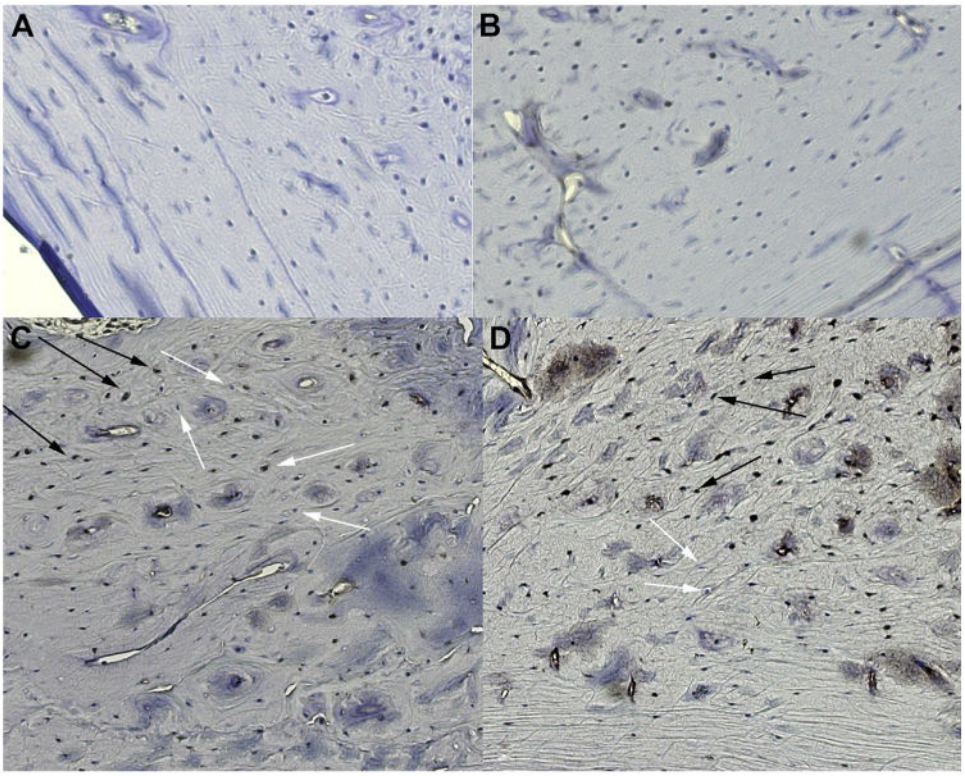

Fig. 1.

Representative micrographs of alveolar and basal bone from rats (×10 magnification). **A**, Alveolar bone—negative control (no anti-podoplanin antibody). **B**, Basal bone—negative control (no anti-podoplanin antibody). **C**, Alvolar bone—immunohistochemical stained with anti-podoplanin. **D**, Basal bone—immunohistochemical stained with anti-podoplanin. Black arrows indicate examples of cells stained positively for podoplanin. White arrows indicate examples of cells not stained positively for podoplanin.

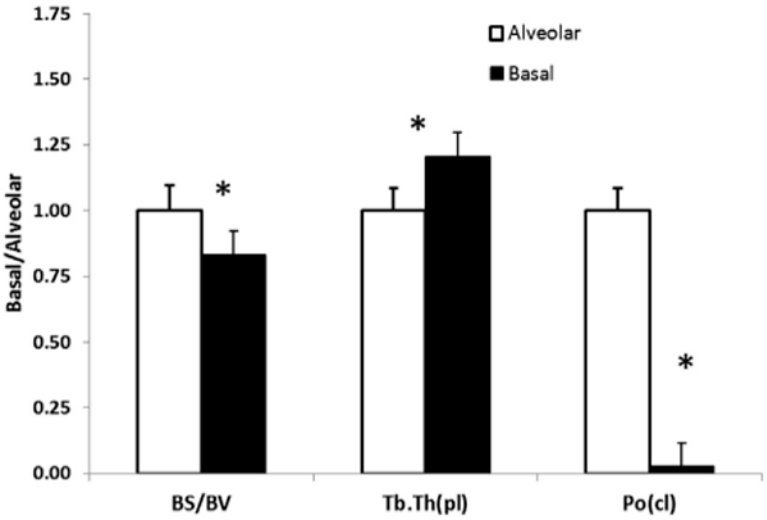

Fig. 2.

Micro-computed tomography (CT) results of bone surface/bone volume (BS/BV; measured in mm^2 mm^{-3}), trabecular thickness (TbTh; measured in mm), and closed porosity (Po; measured as a percentage)–normalized basal/alveolar bone. *Denotes significant difference of $\alpha = 0.05$.

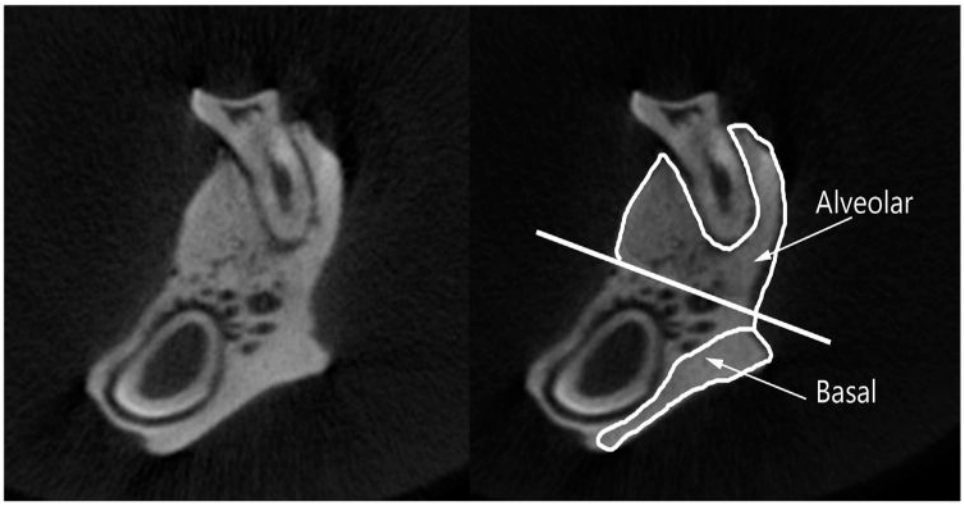

Fig. 3.

Micro-computed tomography (CT) image of bone showing alveolar bone surrounding molar and basal bones below the central incisor (*left*) and alveolar and basal areas indicated (*right*).

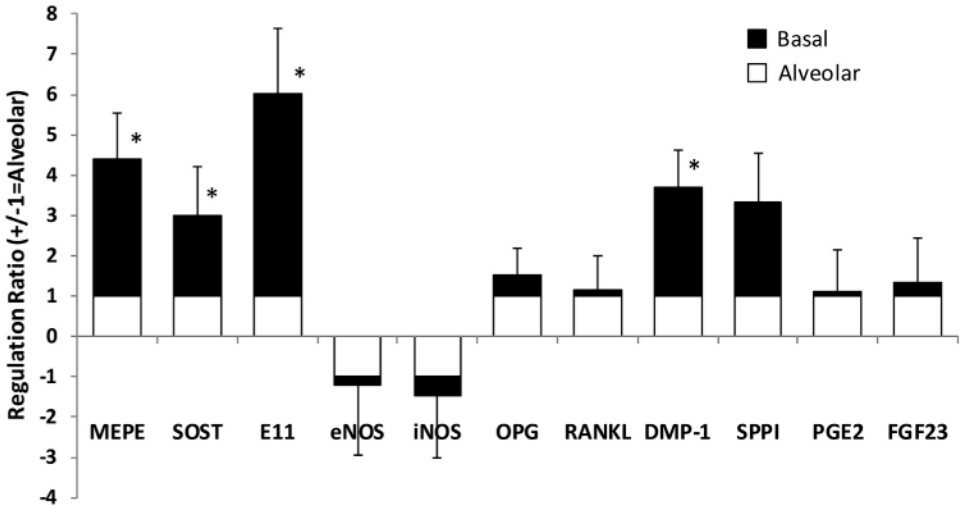

Fig. 4.

Real-time polymerase chain reaction (PCR) results—average fold change values with respect to alveolar bone. *Denotes significant difference of $\alpha < 0.05$.

Table I

Location, function, stimulation, regulation levels, and effected cells for the genes and proteins of interest

	Location	Function	Stimulation	Regulation levels	Affected cell
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Most tissues and cells in the body	Break down glucose; activation and initiation of apoptosis		Used as loading control for real time polymerase chain reaction (PCR)	
DMP-1 (Dentin matrix acidic phosphoprotein)	Osteocyte (surface)	Key regulator of odontoblast differentiation and also promotes mineralization of bone and dentin[23]	Highly expressed during the mineralization of the bone matrix	Deficiencies include flawed dentin development, hypomineralization	Osteoclast
MEPE/OF 45 (Extracellular phosphoglycoprotein/ Osteocyte/Osteoblast Factor-45)	Osteocyte (surface and inside)	Regulate biomineralization and mineral metabolism	Highly expressed during the mineralization of the bone matrix[23]	High levels can adversely affect bone mineralization Downregulated in osteoporosis bone[24]	Osteoclast
SOST (Sclerostin)	Mature osteocyte (inside cell)	Controls rapid bone formation[23]	Bone formation—as a negative regulator	Patients with mutations in the *SOST* gene can have overgrowth of the skeleton Mechanical loading may also reduce sclerostin expression[25]	Osteoblast (negative)
E-11 (Podoplanin)	Osteocytes (surface)	Promotes formation and elongation of osteocyte dendritic processes and is important for osteocyte cell activity	May control osteocyte morphology	Upregulated during fracture repair	Osteocyte
iNOS (Nitric oxide synthase inducible)	Osteocytes		Produced in response to infection or inflammatory cytokines Osteocytes respond to mechanical stimulation by expressing nitric oxide (NO)[25]	Released in response to mechanical strain or fluid-flow shear stress Increased on tension side during orthodontic tooth movement[26]	Osteoclast
eNOS (Nitric oxide synthase, endothelial cells)	Osteocytes		An early mechanical response gene that promotes osteoclastogenesis, inhibits the production of prostaglandin E2, and disrupts gap junctions Osteocytes respond to mechanical stimulation by expressing NO[25]	Released in response to mechanical strain or fluid-flow shear stress Increased on tension side during orthodontic tooth movement[26]	Osteoblast
OPG (Osteoprotegerin)	Both osteoblasts and osteocytes (surface)	Neutralizes RANKL and prevents the *RANKL* gene from binding to RANK	In competition with RANK; blocks bone resorption[27]	A key regulator of osteoclastogenesis in the periodontal ligament during tooth movement[28]	Osteoclast
RANKL (Receptor activator for nuclear factor κ B ligand)	Osteoblasts and osteocytes (surface)	Encourages bone resorption and helps osteoclast differentiation during the bone remodeling process	Produced in abundance during orthodontic tooth movement and periodontitis[29]	RANKL activates RANK and the two bind together to initiate osteoclast activity; regulator of osteoclastogenesis	Apoptotic osteocytes recruit osteoclasts

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	Location	Function	Stimulation	Regulation levels	Affected cell
SPP1/OPN (Secreted phosphoprotein 1/ Osteopontin)	Both osteoblasts and osteocytes (surface and inside)	Mediates the osteoclast attachment to bone	Bone turnover, wound healing, and inflammatory diseases Has been shown to be elevated in a number of tumor types, and its downregulation reduces tumorigenicity	Required for stress-induced bone remodeling because it is necessary for efficient osteoclast action[15]	Osteoclast
PGES2 (Prostaglandin E synthase 2)	Osteocyte	Helps convert prostaglandin H2 to a more stable prostaglandin E2 (lipid)	Bone loss and during orthodontic tooth movement	Released by osteocyte in response to mechanical strain or fluid-flow shear stress	Osteoclast

Table II

Bone mineral density and osteocyte count for alveolar and basal bone

	Alveolar	Basal
Bone mineral density (g/cm^2)	1.30 ± 0.02 *	1.45 ± 0.06 *
Osteocytes ($No./mm^2$)	106.16 ± 0.11 *	98.54 ± 0.14 *

* Denotes significant difference of $\alpha = 0.05$.