Changes in the spatial distribution of sclerostin in the osteocytic lacuno-canalicular system in alveolar bone due to orthodontic forces, as detected on multimodal confocal fluorescence imaging analyses

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Abstract

Objective

Mechanical loading on the bone is sensed by osteocytes. Sclerostin is a molecule secreted by osteocytes that is downregulated by mechanical loading; therefore, its expression level is a potent sensor that indicates the spatial transduction of biomechanical properties in bone. This study applied macroconfocal microscopy to observe the spatial response of alveolar bone to orthodontic forces after immunofluorescence using anti-sclerostin antibodies.

Design

Orthodontic tooth movement with the Ni-Ti closed-coil spring was applied between the upper bilateral incisors and the left first molar of mice. Four days after this application, the animals were subjected to multimodal confocal fluorescence imaging analyses.

Results

Obvious downregulation of sclerostin in the osteocytic lacuna-canalicular system (LCS) was observed specifically in tensile sites of alveolar bone. Confocal-based three-dimensional fluorescence morphometry further quantitatively demonstrated that the distribution and expression of sclerostin in the tensile sites was significantly reduced compared to that observed in the corresponding

control sites. Interestingly, the levels of sclerotin signals in the compression sites were significantly higher than those observed in the control sites, although the distribution of sclerotin was not significantly different.

Conclusions

Our observations suggest that spatial changes in the level and distribution of sclerostin in the alveolar LCS trigger successive bone remodeling due to orthodontic tooth movement. The multimodal confocal imaging analyses applied in this work will enhance comprehensive understanding regarding the spatial regulation of molecules of interest from the tissue to the cellular level.

Key words: bone remodeling, osteocyte, osteoblast, osteoclast, imaging, mechanotransduction,

1. Introduction

Osteocytes are cells buried in the mineralized bone matrix with extending numbers of dendritic cellular process that enable them to make contact with other osteocytes and bone cells on the bone surface, such as osteoblasts and osteoclasts (1). A growing body of evidence obtained within the last decade has revealed the conceptual framework of osteocytes as master orchestrators of bone physiology, in which these cells are responsible for integrating the mechanical and biochemical signals that regulate the differentiation and function of osteoblasts and osteoclasts, thus governing bone modeling and remodeling (2, 3).

Orthodontic tooth movement is achieved via a spatially well-balanced process of alveolar bone remodeling, with bone resorption and formation in compression and tensile sites, respectively (4). Our research group recently documented the essential role of osteocytes in bone resorption during orthodontic tooth movement (5) using osteocyte-ablated mice in which the diphtheria toxin receptor-mediated cell knockout system was applied (6). Orthodontic forces exert two distinct types of mechanical loading forces on alveolar bone: compression and tensile forces, which lead to bone resorption and formation,

respectively. How osteocytes respond to these distinct forces remains to be explored.

Osteocytes are the major source of sclerostin, the protein product of the SOST gene (7-11). Sclerostin antagonizes the canonical Wnt signaling pathway by binding to members of the Wnt coreceptor family, lipoprotein receptor-related proteins (LRP5/6), thereby interfering with Wnt ligands to interact with their receptor, Frizzled (12-14). Since canonical Wnt signaling plays an essential role in early osteoblast differentiation, the sclerostin produced by osteocytes exerts an inhibitory effect on bone formation (14-16). Mechanical loading decreases the expression of sclerostin; therefore, the sclerostin expression in osteocytes is proportional to the mechanical strain on bone (17).

In order to understand how osteocytes respond to the mechanical strain induced by orthodontic forces in a spatial manner at the tissue to cellular levels, we employed multimodal confocal imaging combining macroconfocal microscopy and confocal-based fluorescence morphometry to analyze spatial changes in the sclerostin expression in the osteocytic lacuna-canalicular system.

2. Materials and methods

Experimental animals

Animals and preparation of the skeletal tissues

Mice (laboratory strain C57BL6J) were purchased from a local distributor.

Eight-week-old male mice were used in all experiments. All experimental procedures were approved by the Experimental Animal Committee of Tokyo Medical and Dental University (No. 0090252), and the experiments adhered to ARRIVE guidelines.

Orthodontic tooth movement

Orthodontic tooth movement with the Ni-Ti closed coil spring was achieved as described previously (5, 18), and was applied continuously as a force of 10 gf between about 0.1 to 5.0 mm. In brief, we inserted this appliance between the upper bilateral incisors and the left first molar of mice. Four mice were treated with this orthodontic appliance; there were no cases of detachment of the appliance during the experiments. Successful bone remodeling was confirmed by the histological observation of Hematoxylin-Eosin staining and TRAP (tartrate-resistant acid phosphatase) staining on sections obtained 12 days after

tooth movement, as described previously (5) (Supplementary Figure 1). The right maxillary molars were used as the controls in each mouse.

Tissue preparation

Four days after the application of the orthodontic tooth movement, the maxillae were dissected from the mice under anesthesia with 0.1% ethylanthranilate, and then fixed in 4% paraformaldehyde at 4°C. The specimens were decalcified in 20% ethylenediaminetetraaceticacid (EDTA) for three to four weeks at 4°C. A total of 50 serial horizontal paraffin-embedded sections of the first molar region were cut from the first molar bifurcation to 300 µm toward the apex of the root (19). The alveolar bone site encasing the distobuccal root of the upper first molar was focused (5) for the following confocal microscopy-based analyses, since this root was vertically enforced without rotation movement throughout the orthodontic tooth movement procedure (5). We histologically evaluated 10 sections at 30-µm intervals in each animal, and analyzed mid-horizontal sections of the whole distobuccal root.

Immunofluorescence studies of the decalcified bone sections

Deparaffinized sections were processed for immunofluorescence with primary anti-SOST/sclerostin antibodies (R&D Systems, Minneapolis, MN) and secondary antibodies of Donkey-Anti Goat IgG AlexaFluor 488 (Invitrogen, Carlsbad, CA) (1:1,000), and then counterstained with propidium iodide (PI) (Invitrogen) to stain the nuclei (20). Specific staining was confirmed by performing negative control staining using non-specific IgG instead of the anti-SOST/sclerostin antibodies (Supplementary Figure 2), thus also confirming that the non-specific background signal did not affect the subsequent fluorescence morphometric analyses.

Macroconfocal imaging

Macroconfocal optical sectioning was performed using an AZ-C1 microscopy system (Nikon, Tokyo, Japan) with a Plan Fluor (12.0x/0.38/15.00 Dry). Two laser lines of 488 nm and 561 nm were used. The frame size of the image was 1061×1061 μm , with 512×512 pixels and a 16-bit color depth. The pixel size was 2.07 μm . Z-series of optical sections were obtained with a 2.0- μm step size for voxel sampling. The profiles of the spatial fluorescence intensity were analyzed

using an image processing software program, the NIS-Elements (Nikon, Tokyo, Japan).

Three-dimensional fluorescence morphometry

After targeting regions of interest using a macroconfocal analysis, we performed immunofluorescence morphometry based on three-dimensional high-resolution imaging, with which the distribution and expression of molecules of interest can be observed at the cellular and subcellular levels and statistically analyzed the results. Confocal optical sectioning was performed using a LSM5 Pascal5 confocal laser scanning (CLS) microscopy system (Carl Zeiss, Oberkochen, Germany) with Plan Fluor objective (63 $\times$, N.A.=1.4) and Plan Neofluoar (40 $\times$, N.A.=0.75), as previously described (20, 21). Two laser lines of 488 nm and 543 nm were used. Confocal images were obtained with a 0.29- or 0.5- μ m step size for voxel sampling, then processed four times with Kalman averaging. 3D immunofluorescence images were constructed from a z-series of CLS images using the IMARIS software program (Bitplane, Zurich, Switzerland), as previously described (20, 21). Osteocytic lacunae around the distobuccal root within a 100 μ m width were analyzed. The morphometric parameters of the

volume, the surface area and the intensity of sclerostin spots and nuclei of osteocytic lacunae was obtained from distinct 3 specimens. For the statistical analyses, fluorescence spots below $50\text{ }\mu\text{m}^3$ of sclerostin and nuclei, which were determined to be partially scanned by optical z-sections, were excluded. In this way, signals from fully scanned lacunae were analyzed. The average surface area and volume per lacuna were calculated by dividing each total value by the number of osteocyte nuclei.

Statistics

The two-sample t-test and Kolmogorov-Smirnov-test were used as tests of statistical significance. These were applied for the datasets of the morphometric parameters, the volume, the surface area and the intensity of sclerostin and nuclei, between control and OTM samples. Differences were considered to be significant for values of $p < 0.05$. These analyses were carried out using MATLAB software program (The Mathworks, Inc.) with the “ttest2” and “kstest2” functions. The mean intensity of the 95% confidence interval was calculated using MATLAB software with the “normfit” function.

3. Results

In order to obtain a comprehensive view of the sclerostin expression in the osteocytic lacuno-canalicular system (LCS) in alveolar bone at the tissue level, we first employed macroconfocal microscopy, with which high-contrast fluorescence images with a wide range of a few mm in order can be acquired (Figure 1). Four days after the application of the orthodontic force on the left upper jaw, horizontal alveolar bone sections were processed for immunofluorescence using specific antibodies against sclerostin. The sclerostin expression was distributed in almost the entire LCS of alveolar bone on the control side, exhibiting a “sclerostin network,” as previously described (20) (Figure 1A, upper panel). However, on the orthodontic force side, a portion of alveolar bone distal to the distobuccal root of the left first molar exhibited reduced sclerostin signals (highlighted by the broken-lined squares in Figure 1A, lower panel) compared to that observed in the corresponding sites on the control side. The intensity profile of the sclerostin signals along the mesial-distal line (shown in white solid lines in each broken-lined square) demonstrated lower expression levels of sclerostin in the portion distal to the distobuccal root at

which the orthodontic force was applied compared to that observed in the corresponding control region (Figure 1B, indicated by the arrow).

Next, we analyzed the spatial distribution pattern of sclerostin focusing on the region of alveolar bone adjacent to the distobuccal root and compared the control side with the orthodontic force side using three-dimensional confocal microscopy (Figure 2). On the control side, sclerostin fluorescence signals were predominantly detected in the LCS throughout the alveolar bone, as observed on the macroconfocal imaging findings described above (Figure 2, upper panel). Additionally, sclerostin signals were detected in the periodontal ligament surrounding the tooth root. On the orthodontic force side, the sclerostin signals exhibited a “sclerostin network,” as observed throughout the entire portion of the control side, although the network was uniquely detected in the mesial portion of the tooth root (Figure 2, lower panel). In the distal portion, sclerostin signals were detected only in some of the osteocytic lacunae, with few signals in the canaliculi. Notably, sclerostin signals were detected in the periodontal ligament without a graded pattern, as observed in the corresponding tissue on the control side.

In order to quantitatively assess the changes in the sclerostin expression pattern

induced by the application of orthodontic forces, we performed three-dimensional immunofluorescence morphometry (20) after obtaining three-dimensional confocal images at a higher magnification (Figure 3, upper panels A-D). Surface rendering of the sclerostin fluorescence spots in the osteocytic lacunae and nuclei of the osteocytes was conducted using an image processing software program (Figure 3, lower panels E-H). We measured the average volume and surface area of the sclerostin spots and nuclei. The relative intensity of each sclerostin spot to the intensity of the nuclei was also measured. The morphometric values were then compared between the mesial and distal sides of the distobuccal roots on the control and orthodontic force sides (Figure 4). The average volume and surface area of the sclerostin spots in the mesial (compression) sites of the control and orthodontic force sides were not significantly different (volume; $134.6 \pm 38.8 \mu\text{m}^3$ and $103.6 \pm 19.6 \mu\text{m}^3$, area; $143.1 \pm 25.8 \mu\text{m}^2$ and $113.5 \pm 20.9 \mu\text{m}^2$, respectively) (Figures 4A and B). The same values of nuclei in the same sites of the control and orthodontic force sides also did not show any significant differences (volume, $94.1 \pm 6.1 \mu\text{m}^3$ and $117.1 \pm 6.2 \mu\text{m}^3$; area, $114.6 \pm 5.9 \mu\text{m}^2$ and $130.0 \pm 4.7 \mu\text{m}^2$, respectively) (Figures 4A and B). However, in the distal (tensile) sites, the average volume

and surface area of the sclerostin spots were significantly reduced on the orthodontic force side (volume, $120.4 \pm 11.9 \mu\text{m}^3$; area, $127.2 \pm 10.2 \mu\text{m}^2$) compared to that observed on the control side (volume, $146.0 \pm 9.7 \mu\text{m}^3$; area, $153.4 \pm 8.1 \mu\text{m}^2$), while these values of the nuclei of the control and orthodontic force sides showed no significant differences (volume, $90.1 \pm 7.5 \mu\text{m}^3$ and $94.9 \pm 7.6 \mu\text{m}^3$; area, $111.5 \pm 7.1 \mu\text{m}^2$ and $114.2 \pm 3.0 \mu\text{m}^2$; respectively) (Figures 4A and B).

In parallel with the differences in the average volume and surface area, the relative signal intensity of the sclerostin spots in the distal sites on the orthodontic side was significantly reduced (relative intensity; 1.62 ± 0.23) compared to that observed on the control side (3.05 ± 0.31) (Figure 4C and Table 1). Interestingly, the relative intensity of sclerostin in the mesial sites on the orthodontic force side was significantly higher (relative intensity, 3.36 ± 0.49) than that observed on the control side (1.58 ± 0.16) (Figure 4C and Table 1), in contrast to the lack of significant differences noted in the average volume and surface area, suggesting that there was enhanced expression of sclerostin per lacuna.

4. Discussion

By applying multimodal confocal imaging analyses of macroconfocal microscopy and three-dimensional immunofluorescence morphometry, we observed spatial changes in the distribution and level of the sclerostin expression in the osteocytic lacuno-canalicular system (LCS) in orthodontic force-applied maxillary bone. The distribution and expression of sclerostin were significantly reduced in the tensile sites. In contrast, in the compression sites, the level of sclerostin was enhanced, while the distribution of sclerostin was not significantly different.

It is well accepted that the skeletal system responds to mechanical stimuli of loading and unloading in a site-specific and local manner, thereby exhibiting functional adaptation to habitual activity (22-25). Orthodontic tooth movement is the application of this physiological property of bone that occurs in response to mechanical stimuli.

Robling et al. demonstrated that the application of mechanical loading to the rodent ulna reduced the osteocytic expression of the SOST gene and the subsequent production of sclerostin, while the application of unloading using hind limb suspension enhances the SOST/sclerostin levels (17). Lin reported that, in SOST-deficient mice, hind-limb unloading did not cause significant bone

loss and is not associated with alterations in Wnt signaling (26). Since SOST/sclerostin is predominantly expressed in osteocytes, and osteocytes are mechanosensing cells in bone (6, 8, 11), it is now well accepted that SOST/sclerostin is an effector molecule under the control of mechanoregulation in bone, coordinating site-specific and local bone formation primarily by regulating canonical Wnt signaling in the osteoblast lineage.

Our observations in this work demonstrate that the regulatory expression of sclerostin in alveolar bone is involved in the initial phase of orthodontic tooth movement prior to apparent bone remodeling (Supplementary Figure 1). One possible interpretation of this observation is that, in tensile sites, the contraction of collagen fibers in periodontal ligaments induces mechanical loading, effectively downregulating the sclerostin levels in osteocytes in these local regions, whereas the loss of physiological contraction in compression sites in periodontal ligaments causes an unloading effect on osteocytes, thus enhancing the sclerostin expression in these cells. This observation is also compatible with the detection of successive bone formation and bone resorption in tensile and compression sites, respectively. In tensile sites, the downregulation of sclerostin associated with its loss from the LCS favors osteogenesis on the bone surface

facing the periodontal ligament. An enhanced expression of sclerostin in compression sites may serve as a prerequisite for successive bone resorption by inhibiting osteoblast differentiation at these bone sites, although no obvious roles of sclerostin in the process of osteoclastogenesis have been reported (27, 28). The possible contribution of the RANKL expressed by osteocytes, particularly at these bone sites, is likely, as previously discussed (5, 28, 29), since Nakashima et al. identified RANKL to be a mechanical loading-induced molecule produced by the osteocyte enriched fraction.

Our confocal-based immunofluorescence observations demonstrated that sclerostin proteins are present in the LCS at a detectable level. The spatial distribution pattern of sclerostin in the LCS that was numerically analyzed by the surface volume and area of the fluorescence signal in this study appeared to be associated with the production of sclerostin in the lacunae (Figure 3), as was previously observed in developing bone sites (20). It is not well elucidated how macromolecules, such as sclerostin, FGF23 and RANKL, travel or flow in the LCS. Mechanical loading may induce a convective fluid flow that enables the effective transport of these molecules in the LCS (2).

In the present study, sclerostin was also detectable in periodontal ligament cells

particularly on high-resolution imaging (Figure 2 and 3). However, the level of sclerostin expression did not exhibit any apparent differences between the compression and tensile sites, thus establishing a clear contrast to adjacent alveolar osteocytes, as demonstrated in Figures 3B and C. These findings prompted us to focus on osteocytes rather than on periodontal ligament cells. In our orthodontic tooth movement system, we occasionally observed acellular periodontal ligament space, especially in the compression sites in which the sclerostin fluorescence signal was detectable (Figures 2B and 3C). It is possible that the sclerostin secreted from periodontal ligament cells and osteocytes through their canaliculi diffuses throughout the periodontal space. In contrast to these observations, *periostin* was specifically expressed in the periodontal ligament cells, and its level was enhanced in compression sites compared to that observed in tensile sites (30). Therefore, our observations suggest that the regulatory mechanisms of sclerostin stimulated in response to mechanical stimuli may be specific to osteocytes.

As recently described (31), we observed that there was sclerostin production in cementoblasts (data not shown). Since the sclerostin level in cementoblasts appeared to be lower than that in alveolar osteocytes, we did not analyze the

cementoblasts. Nevertheless, it is interesting to consider that cementoblasts may play a role in alveolar bone remodeling through mechanosensing activities and sclerostin production. Further analyses on cementoblasts would be useful for further elucidating the mechanisms of bone remodeling.

Macroconfocal microscopy enables the researcher to view a wide field of specimens at the millimeter to centimeter level with unprecedented high resolution, thus providing spatial information at the tissue level. We applied macroconfocal microscopy to acquire wide and high-resolution views of the sclerostin expression in bone at the entire tissue level, which successfully demarcated specific bone sites at which sclerostin was downregulated. Successive three-dimensional confocal microscopy and image processing enabled us to quantitatively analyze local changes in the distribution and expression of sclerostin. The multimodal application of confocal-based imaging makes it possible to clarify the spatial regulation of molecules of interest in the tissue to the subcellular level in a comprehensive manner, thus providing benefits in the realm of bone biology and medicine.

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Figure legends

Figure 1

Macroconfocal images of immunofluorescence of sclerostin in alveolar bone. (A) Horizontal sections of alveolar bone were processed for immunofluorescence

using anti-sclerostin antibodies (in green) with nuclear counterstaining (in red). Optical slices obtained with macroconfocal microscopy on the control side (upper panel) and orthodontic force side (lower panel), respectively, are shown. The proximal-distal and palatal-buccal sides are indicated by horizontal and vertical black arrows, respectively. The red horizontal arrow indicates the direction of the orthodontic force. The white broken-lined rectangles highlight the distobuccal roots of the first molar. Scale bars, 200 μ m DB, DP and M indicate distobuccal, distopalatal and mesial roots of upper first molar, respectively. (B) The spatial fluorescence intensity profiles of the proximal-distal line (shown in white solid line) in the highlighted areas on the control side (left graph) and orthodontic force side (right panel) are shown. Fluorescence images were obtained with a 16-bit color depth, and were analyzed by an image processing software program, NIS-Elements (Nikon, Tokyo, Japan). The white vertical arrow indicates obvious downregulation of sclerostin signals in a portion of alveolar bone distal to the distobuccal root on the orthodontic force side compared to that observed on the control side. The white solid lines indicate the tooth roots, while the white dotted lines demarcate the portions of alveolar bone proximal and distal to the tooth roots. A.B., alveolar bone

Figure 2

Confocal projection images of the distinct spatial distribution patterns of the sclerostin expression induced by the orthodontic force. Distinct another specimen from that shown Figure 1 is shown. Immunofluorescence signals of sclerostin (in green) and nuclear counterstaining (in red) on the control side and orthodontic side are shown in the upper and lower panels, respectively. Scale bars, 50 μm

Figure 3

Obvious reductions in the distribution of sclerostin at the distal (tensile) sites of the osteocytic lacuno-canalicular system induced by the orthodontic force. (A-D) Enlarged confocal projection images demonstrate the sclerostin expression (green) in the mesial (compression) (A) and distal (tensile) (B) sites on the control side and the corresponding sites (C and D) on the orthodontic force side. (E-F) Surface rendering images reconstructed by IMARIS software program (Bitplane, Zurich, Switzerland) (E, F, G and H) corresponding to A, B, C and D, respectively, are shown. Scale bars, 20 μm . AB, alveolar bone. PDL, periodontal ligament. TR, tooth root.

Figure 4

Significant reductions in the distribution and expression of sclerostin specifically in the distal (tensile) sites induced by the orthodontic force. The morphometric values of the average volume (A) and surface area (B) of the sclerostin distribution per osteocytic lacunae and the relative intensity of sclerostin signals to that observed in the osteocytic nuclei in each (C) indicated site were analyzed and statistically compared. These morphometric values were measured from surface rendering images, as shown in Figure 3, by the IMARIS software program (Bitplane, Zurich, Switzerland). Three distinct images from distinct specimens were measured and statistically analyzed (see Method). **, P<0.01. *, P<0.05. (n=4 specimens, >100 osteocytic lacunae) The bars indicate the mean $\pm$ S.E.

References

1. Bonewald LF. The amazing osteocyte. J Bone Miner Res. 2011 Feb;26(2):229-38. PubMed PMID: 21254230. Epub 2011/01/22. eng.

2. Schaffler MB, Cheung WY, Majeska R, Kennedy O. Osteocytes: Master Orchestrators of Bone. *Calcif Tissue Int.* 2013 Sep 17. PubMed PMID: 24042263. Epub 2013/09/18. Eng.
3. Bellido T. Osteocyte-Driven Bone Remodeling. *Calcif Tissue Int.* 2013 Sep 4. PubMed PMID: 24002178. Epub 2013/09/05. Eng.
4. Waldo CM, Rothblatt JM. Histologic response to tooth movement in the laboratory rat: procedure and preliminary observations. *J Dent Res.* 1954 Aug;33(4):481-6. PubMed PMID: 13184036. Epub 1954/08/01. eng.
5. Matsumoto T, Iimura T, Ogura K, Moriyama K, Yamaguchi A. The role of osteocytes in bone resorption during orthodontic tooth movement. *J Dent Res.* 2013 Apr;92(4):340-5. PubMed PMID: 23358677. Epub 2013/01/30. eng.
6. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.* 2007 Jun;5(6):464-75. PubMed PMID: 17550781. Epub 2007/06/07. eng.
7. van Bezooijen RL, Deruiter MC, Vilain N, Monteiro RM, Visser A, van der Wee-Pals L, et al. SOST expression is restricted to the great arteries during embryonic and neonatal cardiovascular development. *Dev Dyn.* 2007 Feb;236(2):606-12. PubMed PMID: 17195180. Epub 2006/12/30. eng.
8. van Bezooijen RL, ten Dijke P, Papapoulos SE, Lowik CW. SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev.* 2005 Jun;16(3):319-27. PubMed PMID: 15869900. Epub 2005/05/05. eng.
9. van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, et al. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med.* 2004 Mar 15;199(6):805-14. PubMed PMID: 15024046. Epub 2004/03/17. eng.
10. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J.* 2003 Dec 1;22(23):6267-76. PubMed PMID: 14633986. Epub 2003/11/25. eng.
11. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW, et al. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J.* 2005 Nov;19(13):1842-4. PubMed PMID: 16123173. Epub 2005/08/27. eng.
12. Semenov M, Tamai K, He X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J Biol Chem.* 2005 Jul 22;280(29):26770-5. PubMed PMID: 15908424. Epub 2005/05/24. eng.
13. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, et al. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem.* 2005 May 20;280(20):19883-7. PubMed

PMID: 15778503. Epub 2005/03/22. eng.

14. ten Dijke P, Krause C, de Gorter DJ, Lowik CW, van Bezooijen RL. Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. *J Bone Joint Surg Am.* 2008 Feb;90 Suppl 1:31-5. PubMed PMID: 18292354. Epub 2008/03/20. eng.

15. Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development.* 2005 Jan;132(1):49-60. PubMed PMID: 15576404. Epub 2004/12/04. eng.

16. Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development.* 2006 Aug;133(16):3231-44. PubMed PMID: 16854976. Epub 2006/07/21. eng.

17. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, et al. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *J Biol Chem.* 2008 Feb 29;283(9):5866-75. PubMed PMID: 18089564. Epub 2007/12/20. eng.

18. Yoshimatsu M, Shibata Y, Kitaura H, Chang X, Moriishi T, Hashimoto F, et al. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. *J Bone Miner Metab.* 2006;24(1):20-7. PubMed PMID: 16369894. Epub 2005/12/22. eng.

19. Kobayashi Y, Hashimoto F, Miyamoto H, Kanaoka K, Miyazaki-Kawashita Y, Nakashima T, et al. Force-induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor beta and osteoprotegerin expression. *J Bone Miner Res.* 2000 Oct;15(10):1924-34. PubMed PMID: 11028444. Epub 2000/10/12. eng.

20. Watanabe T, Tamamura Y, Hoshino A, Makino Y, Kamioka H, Amagasa T, et al. Increasing participation of sclerostin in postnatal bone development, revealed by three-dimensional immunofluorescence morphometry. *Bone.* 2012 Sep;51(3):447-58. PubMed PMID: 22766096. Epub 2012/07/07. eng.

21. Himeno-Ando A, Izumi Y, Yamaguchi A, Iimura T. Structural differences in the osteocyte network between the calvaria and long bone revealed by three-dimensional fluorescence morphometry, possibly reflecting distinct mechano-adaptations and sensitivities. *Biochem Biophys Res Commun.* 2012 Jan 13;417(2):765-70. PubMed PMID: 22198435. Epub 2011/12/27. eng.

22. Hattner R, Epker BN, Frost HM. Suggested sequential mode of control of changes in cell behaviour in adult bone remodelling. *Nature.* 1965 May 1;206(983):489-90. PubMed PMID: 5319106. Epub 1965/05/01. eng.

23. Suva LJ, Gaddy D, Perrien DS, Thomas RL, Findlay DM. Regulation of bone mass by mechanical loading: microarchitecture and genetics. *Curr Osteoporos Rep.* 2005

Jun;3(2):46-51. PubMed PMID: 16036101. Epub 2005/07/23. eng.

24. Frost HM. Bone "mass" and the "mechanostat": a proposal. *Anat Rec.* 1987 Sep;219(1):1-9. PubMed PMID: 3688455. Epub 1987/09/01. eng.

25. Frost HM. The mechanostat: a proposed pathogenic mechanism of osteoporoses and the bone mass effects of mechanical and nonmechanical agents. *Bone Miner.* 1987 Apr;2(2):73-85. PubMed PMID: 3333019. Epub 1987/04/01. eng.

26. Lin C, Jiang X, Dai Z, Guo X, Weng T, Wang J, et al. Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. *J Bone Miner Res.* 2009 Oct;24(10):1651-61. PubMed PMID: 19419300. Epub 2009/05/08. eng.

27. Kamiya N, Ye L, Kobayashi T, Mochida Y, Yamauchi M, Kronenberg HM, et al. BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. *Development.* 2008 Nov;135(22):3801-11. PubMed PMID: 18927151. Epub 2008/10/18. eng.

28. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med.* 2011 Oct;17(10):1235-41. PubMed PMID: 21909103. Epub 2011/09/13. eng.

29. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med.* 2011;17(10):1231-4. PubMed PMID: 21909105. Epub 2011/09/13. eng.

30. Wilde J, Yokozeki M, Terai K, Kudo A, Moriyama K. The divergent expression of periostin mRNA in the periodontal ligament during experimental tooth movement. *Cell Tissue Res.* 2003 Jun;312(3):345-51. PubMed PMID: 12761672. Epub 2003/05/23. eng.

31. Bao X, Liu Y, Han G, Zuo Z, Hu M. The effect on proliferation and differentiation of cementoblast by using sclerostin as inhibitor. *International journal of molecular sciences.* 2013;14(10):21140-52. PubMed PMID: 24152444. Pubmed Central PMCID: 3821662.

Table 1

A Mesial (Compression) site

Relative intensity of Sclerostin to Nucleus

	Control	OTM
Mean	1.58	3.36
LB of mean CI	1.26	2.37
UB of mean CI	1.91	4.35
P-value (t-test)	0.000774	
P-value (KS-test)	0.000182	

B Distal (Tensile) site

Relative intensity of Sclerostin to Nucleus

	Control	OTM
Mean	3.05	1.62
LB of mean CI	2.44	1.15
UB of mean CI	3.67	2.08
P-value (t-test)	0.000891	
P-value (KS-test)	0.000729	

OTM: Orthodontic Tooth Movement

LB: Lower bound, UB: Upper bound, CI: Confidence interval

A **B**

3D Projection (Sclerostin / Nuclei)

Control side

Orthodontic Movement

Mesial ← → Distal
Orthodontic Force ← →

Nishiyama, Matsumoto et al., Figure3

Nishiyama, Matsumoto et al., Figure4