ORIGINAL ARTICLE

Expression of *SOST*/sclerostin in compressed periodontal ligament cells

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Abstract *Background/purpose*: Bone resorption and inhibition of bone formation occur on the compressed side during orthodontic tooth movement. Bone formation inhibitory factors such as sclerostin (encoded by *SOST*) are secreted on the compressed side by periodontal ligament (PDL) cells. PDL cells control bone metabolism, and compressed PDL cells inhibit bone formation during orthodontic tooth movement. The aim of this study was to identify the inhibitory factors of bone formation in PDL cells.
Materials and methods: Changes in *SOST* expression and subsequent protein release from human PDL (hPDL) cells were assessed using the real-time polymerase chain reaction (PCR), semi-quantitative PCR, and immunofluorescence in hPDL cells subjected to centrifugal force (40*g* and 90*g*). To confirm the effects on bone formation, human alveolar bone-derived osteoblasts (hOBs) were grown with the addition of sclerostin peptide. *In vivo*, a compressive force was applied using the Waldo method in rats, and the distribution of sclerostin in PDL tissues was examined by immunohistochemistry.
Results: *SOST* expression was downregulated *in vitro* by centrifugation at 90*g* for 24 hours but upregulated by centrifugation at 40*g* based on real-time PCR, as was confirmed by immunofluorescence staining. The addition of sclerostin peptide significantly decreased the mineralized area in hOBs. However, slightly weakly sclerostin-positive PDL cells were observed on the compressed side *in vivo*.
Conclusion: These results indicate that PDL cells subjected to light compressive force exhibit increased expression of *SOST*/sclerostin, which inhibits bone formation on the compressed side during orthodontic tooth movement.
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Introduction

Applying orthodontic force to teeth results in their movement. During this process, bone resorption is dominant on the compression side, and bone formation is dominant on the tension side. Once mechanical forces are applied, the root moves close to the alveolar wall, resulting in a reduction of vasculature to the area.[1] Then, a cell-free zone or hyalinized area is temporarily formed. These areas must be removed to allow the teeth to move.[2] To maintain the optimal periodontal ligament (PDL) distance, PDL cells on the compressed side release osteoclast-inductive molecules, causing osteoclast resorption of alveolar bone. If excessive force is applied, the root of the tooth and alveolar bone become attached, resulting in ankylosis.[3,4] To avoid ankylosis, compressed PDL cells secrete factors that inhibit osteoblasts as well as activating factors for osteoclasts.

Regarding the effects of mechanical stress on osteoblastic bone formation, previous reports indicated that compressive force (CF) activates osteoblastic bone formation.[5−7] However, during orthodontic tooth movement, no activation of bone formation is observed on the compressed side. These contradictory findings suggest that bone formation on the compression side is likely to be inhibited by compressed PDL cells. The most likely candidate molecule for osteoblastic bone formation inhibition by PDL cells is sclerostin (encoded by *SOST*).[8,9] *SOST*/sclerostin inhibits bone formation by inhibiting Wnt signaling. Sclerostin is a Wnt antagonist that has been identified as an inhibitor of canonical Wnt signaling, and Wnt signaling and sclerostin are blocked by preventing the formation of Wnt-Fizzled (Fz)-lipoprotein receptor-related protein (LRP) 5/6 complexes.[10−15] That is, sclerostin inhibits the binding of the Wnt receptor by interacting with LRP5/6, and suppresses bone formation. However, the molecules involved in the inhibition of bone formation on the compressed side during orthodontic tooth movement remain unknown.

We investigated the changes in *SOST*/sclerostin expression and the inhibitory effects on bone formation of PDL cells subjected to mechanical CF.

Materials and methods

Human periodontal ligament (hPDL) cells were isolated from healthy PDLs of premolar teeth that were extracted for orthodontic reasons. All patients gave informed consent prior to providing samples. All cells were cultured in Alpha Minimum Essential Medium (αMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Cellgro; Mediatech Inc., Herndon, VA, USA), 50 U/mL penicillin G (Gibco-BRL), 50 μg/mL fungizone (Gibco-BRL), and 50 μg/mL gentamicin (Gibco-BRL), and incubated at 37°C in a 5% CO_2 incubator. Cells underwent three to eight passages prior to use in experiments. All procedures were approved by the Research Ethics Committee of Kyushu Dental University (Kitakyushu, Japan; permission number, 14-8).

We performed reproduction of the compressed side during clinical orthodontic tooth movement by centrifugation because centrifugation enables sufficient external mechanical stimulation, as described in previous studies.[16−18] We applied centrifugation according to the procedure described by Redlich et al,[19] with some modifications. When cells reached subconfluence in culture flasks (Becton Dickinson, San Jose, CA, USA), the medium was changed to HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate (Invitrogen Co., Carlsbad, CA, USA), and 90 μL HCl (Nacalai Tesque Inc., Kyoto, Japan) was added to stabilize the pH. Cells were then incubated at 37°C (Yamato Scientific Co., Ltd., Tokyo, Japan) for 24 hours. Next, cell-culture flasks were centrifuged at 40 and 90 *g* (Kubota Co., Tokyo, Japan) at 37°C for 24 hours.

Force was calculated using the following equation:

$$P = \left(m \times r \times rpm^2 \times \pi^2\right)/\left(A \times 9.8 \times 900\right), \tag{1}$$

where *P* is kg compression/cm^2 of cells; *m* is the mass of the medium (0.005 kg); *r* is the radius (0.1 m); rpm denotes revolution/min (40 g and 90 g); and *A* is the area of contact between the medium and cells (12.5 cm^2). The CFs of 40*g* and 90*g* correspond to 16.1 g/cm^2 and 36.2 g/cm^2, respectively.

RNA was isolated from CF-treated hPDL cells and purified using RNAqueous Total RNA Isolation Kit (Ambion) according to the manufacturer's instructions. Total RNA from CF-treated hPDL cells was treated with DNase and reverse-transcribed with random primers using a Superscript First-Strand Kit (Invitrogen Co.). Real-time polymerase chain reaction (RT-PCR) was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and Eco Real Time PCR System (Illumina, San Diego, CA, USA). mRNA detection was performed using predeveloped proprietary TaqMan primers [β-*actin* (ACTB: Hs99999903_m1) and *SOST* (SOST: Hs00228830_m1); Applied Biosystems]. These analyses were conducted to determine the levels of β-*actin* for data normalization. The cycling conditions were 95°C for 15 seconds and 60°C for 60 seconds, for 40 cycles. The expression levels of target genes were normalized to β-*actin* expression and are presented relative to the control.

RNA was isolated from CF-treated hPDL cells as described above, followed by DNase digestion. cDNA was synthesized from 2 μL total RNA in 30 μL reaction buffer containing 500mM dNTPs, 20 U ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U Superscript II Reverse Transcriptase (Invitrogen Co., Life Technologies). The following primers were used for amplification: *GAPDH*, 5′-TGA AGG TCG GTG TCA ACG GA-3′ and 5′-TAC TGG TGT CAG GTA CGG TAG-3′; *SOST*, 5′-GGA CTC CAG TGC CTT TTG AA-3′ and 5′ =-GTT CCA GTG AAG GTC TTA AGT C-3′. The PCR program consisted of an initial denaturation step (*GAPDH* and *SOST*, 94°C for 2 minutes) followed by 40 cycles of denaturation (*GAPDH*, 94°C for 30 seconds; *SOST*, 94°C for 60 seconds), annealing (*GAPDH*, 55°C for 1 minute; *SOST*, 62°C for 1 minute), and extension at 72°C for 1 minute. The PCR products were subjected to electrophoresis in 5% agarose gels and visualized with ethidium bromide. *GAPDH* expression was used as an internal control.

Next, the release of sclerostin was determined by enzyme-linked immunosorbent assay (ELISA) using culture medium from hPDL cells treated with/without CF. hPDL

cells were cultured as mentioned above; 100 μL medium from each sample was pipetted into 96-well plastic plates and incubated at 4°C overnight. After incubation, the plates were washed with phosphate buffered saline (PBS)—Tween (0.5%, Tween 20) and blocked with Blocking One (Nacalai Tesque Inc.) for 30 minutes at room temperature. The plates were then incubated with a sclerostin antibody [rabbit polyclonal immunoglobulin IgG, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-130258] for 1 hour at room temperature. After washing, donkey antigoat IgG-horseradish peroxidase (1:5000 dilution; Santa Cruz Biotechnology) was added and incubated for 1 hour at room temperature. After further washes, alkaline phosphatase activity was detected by ELISA in the buffer. The ELISA buffer was obtained by adding OPD tablets (Wako, Osaka, Japan) and 4 μL H_2O_2 in 12 mL 0.1M citrate phosphate buffer. The optical density was measured at 490 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

hPDL cells treated with/without CF were fixed with 4% paraformaldehyde (PFA). The distribution of sclerostin was also examined immunocytochemically using rabbit polyclonal antisclerostin (1:100 dilution; Santa Cruz Biotechnology) as the primary antibody and goat antirabbit IgG (1:400 dilution; Invitrogen Co., Life Technologies) as the secondary antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA).

Male Sprague—Dawley (SD) rats weighing 200—250 g were used for all in vivo experiments. All procedures were approved by the Animal Research Committee of Kyushu Dental University (permission number, 15-004). An elastic band was inserted into male SD rats between their first and second upper molars. Untreated animals were used as a control group. Rats were then perfused through the left ventricle with 4% PFA in 0.2M phosphate buffer (pH 7.4) containing 0.2% picric acid.[20] Maxillary bones, including upper molars, were then removed, postfixed with PFA overnight, decalcified with 10% EDTA solution, and embedded in paraffin; then 5-μm horizontal sections of the molars in the maxilla were prepared. The distribution of sclerostin was also examined immunohistochemically using the same procedure. Positive reactions were visualized with 0.02% 3,3′-diaminobenzidine (DAB; Dojindo, Kumamoto, Japan) and 0.02% hydrogen peroxidase solution (Wako).

Finally, human alveolar bone-derived osteoblasts (hOBs; Cell Applications Inc., San Diego, CA, USA) were grown in 24-well plates at a density of 2.0×10^4 cells/well. After reaching near-confluence, half the medium was replaced with the supernatant of the culture medium taken from hPDL cells treated with/without CF. Alternatively, sclerostin peptide (Acris Antibodies GmbH, Herford, Germany) was added to confirm the effects on bone formation based on the sclerostin protein concentration as determined by ELISA. We used this sclerostin peptide referring to the characterization of sclerostin described by Veverka et al.[21] The medium was changed every 3 days, and cells were cultured for 4 weeks. After incubation, hOBs were washed with PBS and fixed with 4% PFA for 10 minutes at room temperature. After washing, hOBs were treated with 5% silver nitrate solution for 30 minutes

at room temperature. After further washes, hOBs were fixed with 3% sodium thiosulfate solution for 5 minutes at room temperature. The plates were dried for 12 hours after washing. Quantitative data were obtained by Image J (NIH, Bethesda, MD, USA) analysis of the images taken with a microscope.

Statistical analysis

One-way analysis of variance followed by individual post hoc comparisons (Scheffé) was used to analyze significant differences.

Results

To evaluate the differential mRNA expression patterns of a bone formation inhibitory factor, SOST mRNA expression in hPDL cells treated with/without CF (40g and 90g) was measured using real-time RT-PCR (Figure 1A). Although 90g CF-treated hPDL cells exhibited lower SOST mRNA
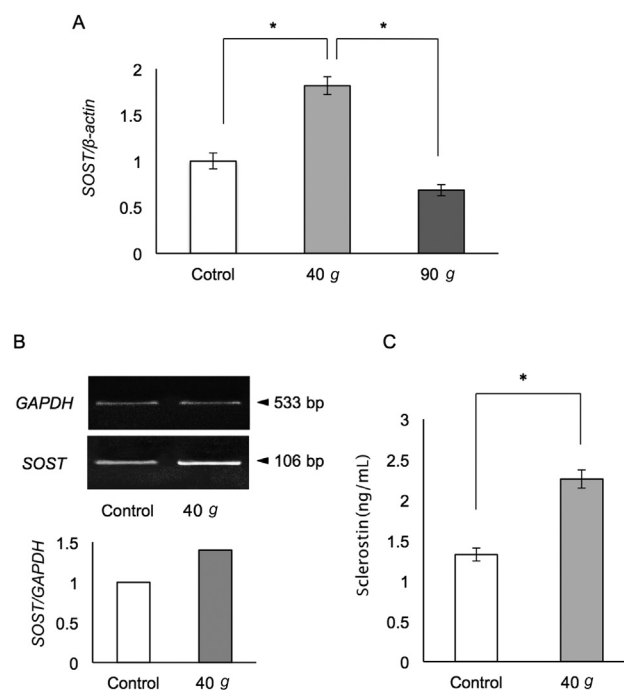

Figure 1 Effects of CF on SOST expression in hPDL cells. (A) Quantitative expression of SOST mRNA in hPDL cells subjected to a CF of 40g for 24 hours was analyzed by real-time PCR. Data indicate expression relative to the control (β-actin) ($n = 5$). Values are presented as the means ± SE. * P < 0.01. (B) RT-PCR analysis of SOST mRNA expression in CF-treated hPDL cells. CF was applied at 40g for 24 hours. SOST mRNA expression was analyzed relative to GAPDH expression. (C) Enzyme-linked immunosorbent assay (ELISA) analysis was used to determine the amount of sclerostin in the supernatant of CF-treated cells (40g) for 24 hours ($n = 10$). Values are presented as the means ± SEM. * P < 0.01. CF = compressive force; hPDL = human PDL; PDL = periodontal ligament; RT-PCR = real-time polymerase chain reaction; SEM = standard error of the mean.

expression, 40*g* CF-treated hPDL cells showed higher expression of *SOST* mRNA compared to the control. In addition, to investigate the effect of CF (40*g*), we used RT-PCR to determine the expression of *SOST* mRNA in hPDL cells treated with CF (40*g*; Figure 1B). *SOST* mRNA expression increased in 40*g* CF-treated hPDL cells based on both real-time PCR and RT-PCR; *SOST* mRNA expression was significantly decreased when cells were treated with 90*g*.

Next, we used ELISA to determine the levels of sclerostin released from CF-treated hPDL cells. We found that hPDL cells secreted significantly higher levels of sclerostin (mean, 2.3 ng/mL) compared with controls (mean, 1.3 ng/mL; Figure 1C).

To confirm the distribution of sclerostin in cultured hPDL cells, immunofluorescence labeling was used. Similar to the increase in *SOST* mRNA expression in hPDL cells treated with 40*g* CF (Figure 2B), hPDL cells subjected to CF showed greater sclerostin immunopositivity than the controls. Conversely, cells treated with a CF of 90*g* exhibited lower sclerostin immunopositivity (Figure 2C) compared to untreated cells (Figure 2A), consistent with the decreased *SOST* mRNA expression based on real-time PCR.

We assessed sclerostin localization in PDL tissues of SD rats by using an elastic band between their first and second upper molars to apply orthodontic force. Immunopositive staining for sclerostin was a little scarce in PDL tissues (Figure 3D) compared to the control (Figure 3A). Slightly weak immunopositive staining for sclerostin was also confirmed in the compressed (Figure 3E) and tension PDL tissues (Figure 3F), as well as on the mesial (Figure 3B) and distal sides (Figure 3C) in coronal tissues of the first upper molar distal root.

To examine the inhibitory effects of bone formation by hPDL cells, hOBs were cultured with the supernatant from hPDL cells treated with 40*g* CF for 4 weeks. The mineralized area was significantly decreased in hOBs cultured with the supernatant from CF-treated cells compared to cells cultured with the supernatant from untreated cells (Figures 4A–4C). Finally, to confirm that the inhibitory effects were attributable to sclerostin, hOBs were cultured with sclerostin peptide (1.3 ng/mL or 2.3 ng/mL). We determined that sclerostin peptide concentrations at 1.3 ng/mL and 2.3 ng/mL were equivalent to the control and centrifuged levels of sclerostin, respectively (Figure 1C). The addition of 2.3 ng/mL sclerostin significantly decreased the mineralized area compared to cells cultured with 1.3 ng/mL sclerostin (Figures 4D–4F).

Discussion

We determined the bone-formation inhibitory effects of *SOST*/sclerostin produced by PDL cells subjected to light CF during orthodontic tooth movement. *SOST* was found to be an important factor in the prevention of ankylosis on the compressed side.[22,23] *SOST*/sclerostin is a candidate factor in the inhibition of bone formation.[8,9] *SOST*/sclerostin inhibits the binding of Wnt receptor by interacting with LRP5/6 and suppresses bone formation.[10–15] In the present study, we showed that *SOST*/sclerostin in PDL cells is likely to be involved in the inhibition of bone formation on the compressed side during orthodontic tooth movement.

In vitro, we confirmed the expression of *SOST*/sclerostin in hPDL cells without CF. Expression of *SOST*/sclerostin in hPDL cells increased significantly under a CF of 40*g*, but decreased under a CF of 90*g* based on real-time PCR. These results were confirmed by immunocytochemical staining of hPDL cells subjected to CF. Additionally, the release of sclerostin in hPDL cells significantly increased under a CF of 40*g* based on ELISA. Therefore, we concluded that the CF needed for high *SOST*/sclerostin expression and release is 40*g* (16.1 g/cm^2), which differs slightly from the optimal clinical orthodontic force (33.5 g/cm^2).[24]

In vivo, slightly weak immunoreactivity for sclerostin was found on the compressed side of the periodontium of rat molars. This may have been due to the excessive CF induced by using an elastic band[25]; further studies are required with a rat model that allows light CF to be applied.

These findings suggest that *SOST* expression and sclerostin release by PDL cells are increased during light CF, which might have an effect on osteoblasts and bone formation.

To investigate the inhibitory effects on bone formation of *SOST*/sclerostin released by CF-treated PDL cells, we examined the effects on bone formation using hOBs with/without the supernatant from 40*g* CF-treated hPDL cells or sclerostin peptide. We found that the mineralized area was significantly decreased in hOBs cultured with the supernatant from 40*g* CF-treated hPDL cells compared to hOBs cultured with the supernatant from untreated hPDL cells. Furthermore, compared to the control, the mineralized area was significantly decreased in hOBs by addition of sclerostin peptide.

We found that the maximum *SOST* expression in PDL cells occurred at 40*g* (16.1 g/cm^2), and the expression of *SOST*/sclerostin in hPDL cells decreased after exposure to a CF of
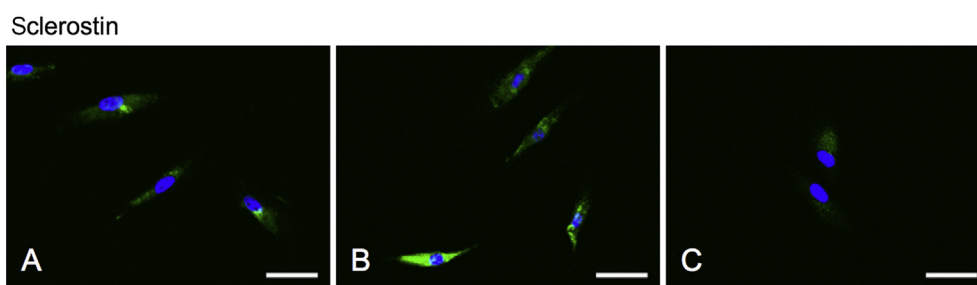
Sclerostin

Figure 2 Immunocytochemical localization of sclerostin in CF-treated hPDL cells. Immunocytochemical staining of sclerostin (green) and hPDL cell nuclei (blue). Control cells (A) and CF-treated cells [(B) 40*g* for 24 hours; (C) 90*g* for 24 hours]. Bars = 50 μm. CF = compressive force; hPDL = human periodontal ligament.
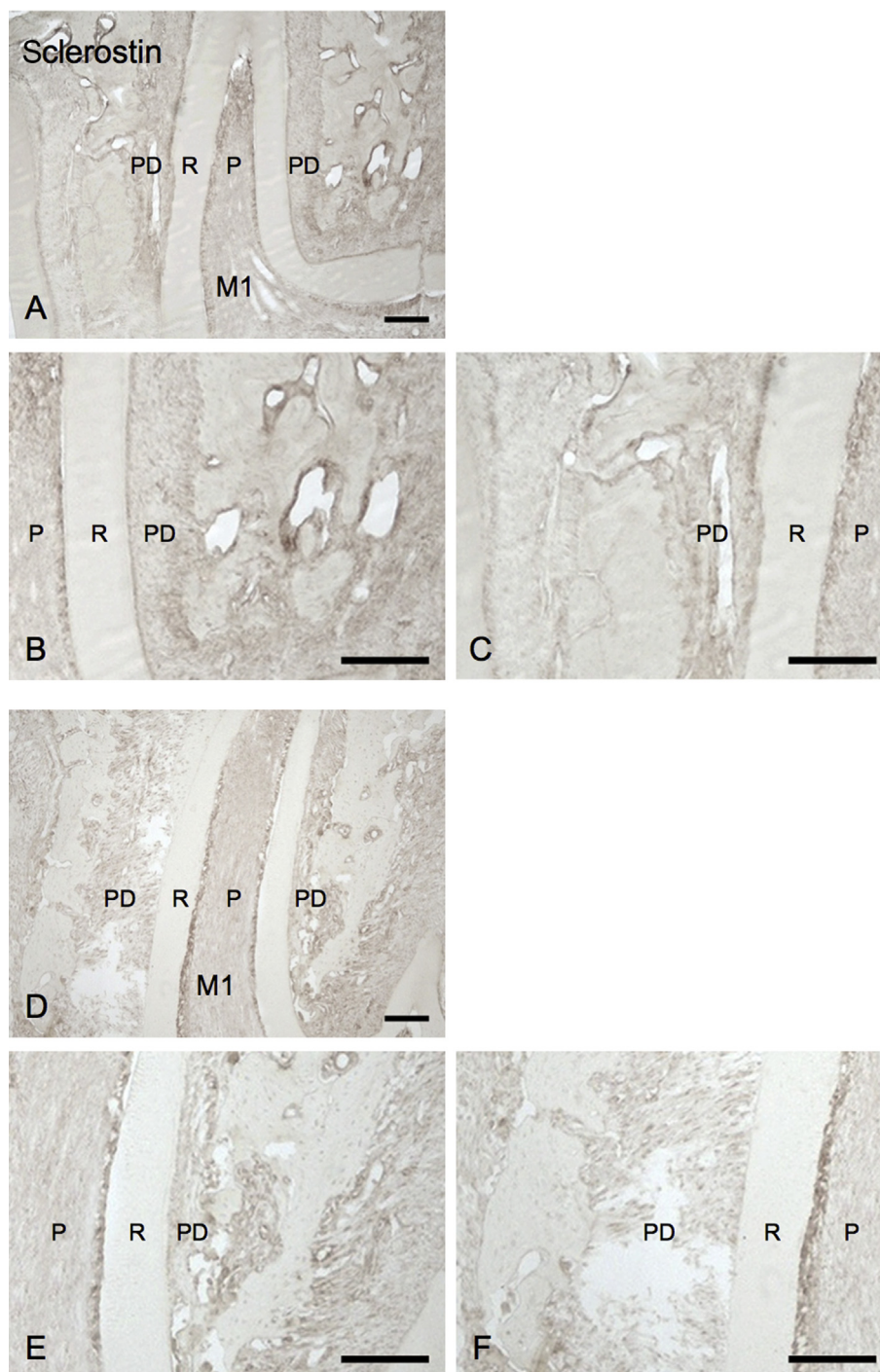
Figure 3 Localization of sclerostin in rat PDL tissues. Elastic bands were placed between the first (M1) and second molars (M2) of SD rats according to the Waldo method. DAB staining of sclerostin. (A—C) Control tissues without CF, including mesial (B) and distal (C) sides in coronal tissues of the M1 distal root. (D—F) Tissues with CF, including compressed (E) and tension tissues (F) by the Waldo method. Slightly weak immunopositive staining for sclerostin was confirmed on the mesial (B), distal (C), compressed (E), and tension (F) sides in PDL tissues of the M1 distal root. CF = compressive force; DAB = 3,3′-diaminobenzidine; hPDL = human PDL; M1 = first molar; P = dental pulp; PDL = periodontal ligament; R = tooth root; SD = Sprague—Dawley. Bars = 100 μm.

90g (36.2 g/cm^2), which is close to the optimal clinical orthodontic force (33.5 g/cm^2).[24] Generally, continuous force is the main mechanism of orthodontic tooth movement, but the degree of force decreases slowly over time. Therefore we suggest that the production of bone-formation inhibitory factors such as *SOST*/sclerostin by PDL cells increases when the level of continuous force decreases, i.e., when subjected to light CF, which may inhibit alveolar bone formation at the periodontal space on the side to which light CF is applied during orthodontic tooth movement. We
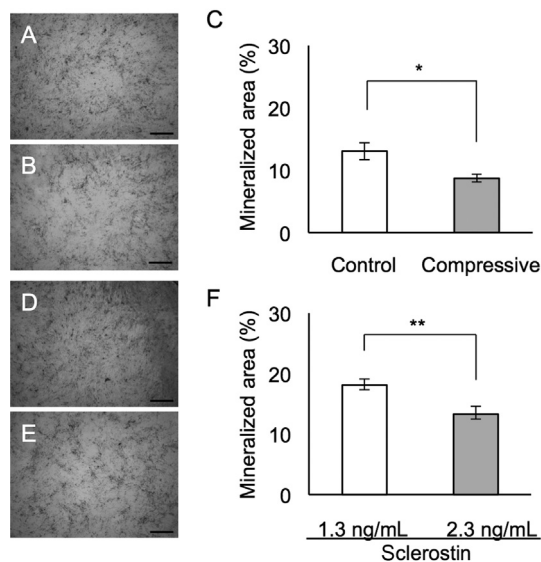
Figure 4 Changes in the mineralization of human osteoblast cells by CF. von Kossa staining was performed after culturing human osteoblast-like cells in medium plus the supernatant from hPDL cells without (A) and with CF (B). von Kossa staining was performed after culturing human osteoblast-like cells in medium from hPDL cells plus the amount of sclerostin protein produced without (D) and with CF (E). Comparison of changes in the area of von Kossa-positive staining (C, F; $n = 10$). Bars = 1 mm. Values are presented as the means ± SE. * P < 0.05, ** P < 0.01. CF = compressive force; hPDL = human periodontal ligament; SE = standard error.

suggest that other antiosteogenic factors might play roles under optimal clinical orthodontic force.[26,27]

In conclusion, *SOST* expression and sclerostin release by PDL cells increase when they are subjected to light CF, which inhibits bone formation by osteoblasts. That is to say, *SOST*/sclerostin may contribute to inhibition of bone formation on the compressive side during orthodontic treatment, and support bone metabolism.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jds.2016.02.006.

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