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Title: Asporin in compressed periodontal ligament cells inhibits bone formation

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Abstract: Objective: During orthodontic tooth movement, bone resorption and inhibition of bone formation occur on the compressed side, thereby preventing ankylosis. Periodontal ligament (PDL) cells control bone metabolism and inhibition of bone formation on the compressed side by secreting bone-formation inhibitory factors such as asporin (ASPN) or sclerostin (encoded by SOST). The aim of this study was to identify the inhibitory factors of bone formation in PDL cells.
Design: In vitro, the changes in expression of ASPN and SOST and subsequent protein release in human PDL (hPDL) cells were assessed by semi-quantitative polymerase chain reaction (PCR), real-time PCR, and immunofluorescence in hPDL cells subjected to centrifugal force using a centrifuge (45, 90, 135, and 160 ×g). In vivo, we applied a compressive force using the Waldo method in rats, and examined the distribution of ASPN or sclerostin by immunohistochemistry.
Results: In vitro, hPDL cells subjected to 90 ×g for 24 h demonstrated upregulated ASPN and downregulated SOST expressions, which were confirmed by immunofluorescent staining. In addition, the formation of mineralized tissue by human osteoblasts was significantly inhibited by the addition of medium from hPDL cells cultured during compressive force as well as the addition of equivalent amounts of ASPN peptide. In vivo, asporin-positive immunoreactive PDL cells and osteoclasts were found on the compressed side, whereas few sclerostin-positive PDL cells were observed.
Conclusions: PDL cells subjected to an optimal compressive force induce the expression and release of ASPN, which inhibits bone formation during orthodontic tooth movement on the compressed side.

Suggested Reviewers:

Editors-in-Chief

Archives of Oral Biology

Dear Dr. Gordon B. Proctor and Dr. Graham R. Holland

Manuscript number: AOB-D-15-00419

Thank you very much for your e-mail dated September 10, 2015. Here we send a revised version of the paper entitled, "Asporin in compressed periodontal ligament cells inhibits bone formation" by Masae Ueda, Tetsuya Goto, Kayoko N. Kuroishi, Kaori K. Gunjigake, Erina Ikeda, Shinji Kataoka, Mitsushiro Nakatomi, Takashi Toyono, Yuji Seta, and Tatsuo Kawamoto. We have read the reviewers' comments carefully and have revised the manuscript accordingly. We marked the revised words or sentences in our manuscript by red color.

We appreciate the helpful suggestions offered by the reviewer, as the comments were very useful for revising this manuscript. I hope you now find that this manuscript is suitable for publication. All authors have checked and approved the changes.

Sincerely yours,

Tetsuya Goto October 7, 2015.

Tetsuya Goto

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*Conflict of Interest Form

Journal: Archives of Oral Biology	**Author name**: Masae Ueda

Declarations

The following additional information is required for submission. Please note that failure to respond to these questions/statements will mean your submission will be returned to you. If you have nothing to declare in any of these categories then this should be stated.

Please state any conflict of interests. A conflict of interest exists when an author or the author's institution has financial or personal relationships with other people or organisations that inappropriately influence (bias) his or her actions. Financial relationships are easily identifiable, but conflicts can also occur because of personal relationships, academic competition, or intellectual passion. A conflict can be actual or potential, and full disclosure to The Editor is the safest course.

There is no conflict of interest in this manuscript to be submitted.

Please state any sources of funding for your research

This study was supported by JSPS KAKENHI Grant Number 23792440.

Please state whether Ethical Approval was given, by whom and the relevant Judgement's reference number

All procedures were approved by the Research Ethics Committee of Kyushu Dental University (permission number: 14-8), and the Animal Research Committee of Kyushu Dental University (permission number: 15-004).

If you are submitting a Randomized Controlled Trial, please state the International Standard Randomised Controlled Trial Number **(ISRCTN)**

Not applicable.

Authors contribution (if count of Authors =>4)

Masae Ueda contributed to conception and design of the study, and acquisition, analysis and interpretation of data, drafted the article, revised it critically for important intellectual content, and submitted final approval of the version.

Tetsuya Goto and Kayoko N Kuroishi contributed to conception and design of the study, analysis and interpretation of data, and drafted the article, and submitted final approval of the version.

Kaori K Gunjigake and Erina Ikeda contributed to acquisition of data, revised it critically for important intellectual content, and submitted final approval of the version.

Shinji Kataoka, Mitsushiro Nakatomi, and Takashi Toyono contributed to analysis and interpretation of data, revised it critically for important intellectual content, and submitted final approval of the version.

Yuji Seta and Tatsuo Kawamoto contributed to conception and design of the study, revised it critically for important intellectual content, and submitted final approval of the version.

All authors have read and approved the final article.

RESPONSES TO REVIEWER #1:

We are grateful to reviewer for the critical comments and useful suggestions that have helped us to improve our paper. As indicated in the responses, we have taken all these comments and suggestions into account in the revised version of our paper. We marked the revised words or sentences in our manuscript by red color.

The points of improvement are as follows.

1- "Compressive periodontal ligament cells" doesn't sounds proper. Reconsideration of the title is requested.

As reviewer #1 suggested, we corrected the title as "Asporin in compressed periodontal ligament cells inhibits bone formation".

2- ***Why SOST didn't increase, even decrease, under CF, while it was considered as a candidate of anti-osteogenic molecules, when the authors began this study? There is almost no discussion about SOST. It may be recommended to delete SOST relating part of this manuscript to make the study simpler and clearer.***

Because sclerostin is well-known anti-osteogenic molecule, we had initially hypothesized that the *SOST* (sclerostin) as a candidate of the anti-osteogenic molecule in compressed PDL cells, however, it decreased under CF. Then we tried ASPN as another candidate. To show the involvement of ASPN as anti-osteogeneic molecule in compressed PDL cells, we needed the data of *SOST* as a negative control. Though we understand the recommendation of reviewer to delete *SOST* relating parts to make the study simply and clearer, we don't think we had better to

delete *SOST* relating part of this manuscript.

3- Authors described that the optimal CF for ASPN expression 12 in hPDL cells w-as 90 ×g. What does "optimal" mean? Optimal for what?

The reason why we defined 90 x g as "optimal" force was that we found the greatest expression of *ASPN* mRNA when we added 90 x g. This is our definition in this manuscript.

Also, from the clinical point of view, we added a reference which mentioned that 33.5 g/cm^2 was concluded to be as a clinical orthodontic force (Reference No.27). The 90 x g CF is equivalent to 36.0 g/cm^2, which is closed to 33.5 g/cm^2. Therefore, this is the other reason why we thought 90 x g CF as the optimal force.

4- The title of material and methods 2.6 should include orthodontic tooth movement model.

As reviewer #1 suggested, we have corrected the sub title of material and methods 2.6 as, "Orthodontic tooth movement model and immunocytochemistry".

5- Figure 3 'control cells' should be 'control tissues'. There is no explanation of condition for figure E and J in figure legends. In Figure 3, ASPN positive cells are observed even at tension side as indicated by white arrows. How do the author explain the presence of ASPN cells at the tension side? Figure 3D shows root resorption and cells in a resorption cavity of the root are ASPN positive. Does this finding have biological or pathological meaning?

According the comment of reviewer #1, we corrected figure legends, results, and discussion.

We changed "control cells" to "control tissues" in figure legends. And, we added explanation that figure 3E and J were the low power images that contain both the tension side and the compressed side in figure legends and results.

Though we did not performed quantitative analysis in terms of the expression of ASPN in PDL tissues, by the images of Fig. 3B, 3D, and 3E we thought stronger immunopositive staining for ASPN represents greater expression of ASPN at compressed side of PDL.

Regarding with the root resorption, we think root resorption has occurred in the process of tooth movement by Waldo method using elastics. Therefore, we think the root resorption by Waldo method in rat is a kind of biological meaning.

6- How do PDL cells produce ASPN responding to CF? Directly respond or indirectly respond through cytokines? Discussion about molecular mechanism should be added.

In this study, we found the involvement of ASPN responding to CF. Our previous study (Reference No.3) indicated compressive force to PDL cells induce IL1-□□release, then bind to the receptor on PDL cells and induced the expression of RANKL. This may be an indirect case as reviewer #1 suggested. However, in terms of ASPN expression by CF the mechanism of the increase of ASPN by mechanical stress such as CF is not understood including whether direct effect of indirect effect, we have just started the experiment to clarify the mechanism between mechanical stress and ASPN expression in PDL cells. Therefore, we added brief comments, "However, the relation between the expression of ASPN in PDL cells and mechanical stress such as CF has not been understood. Additional study is needed to elucidate the molecular

mechanism.", in Discussion.

7- In 4th paragraph of Discussion, 'However, the main activity of osteoblasts is bone resorption, not activation of bone formation.'

What does this mean? Main activity of osteoblasts is bone formation.

As reviewer #1 suggested, the sentence is difficult to understand. We changed the sentence as, "However, *in vivo* bone resorption occurred, but the activation of bone formation does not occurred at the compressed side."

8- How about the degree of compressive force in the animal experiment where ASPN was strongly expressed? Was it excessive CF or optimal CF?

As reviewer #1 pointed, the root resorption by compressed force seems to be induced by excessive force, but we often found such root resorption by Waldo method in rat. Also, if the force was excessive, hyalinization or necrosis of PDL would be occurred at the compressed side. Therefore, we think the compressive force by Waldo methods in rat is not excessive but optimal force.

Abstract

Objective: During orthodontic tooth movement, bone resorption and inhibition of bone formation occur on the compressed side, thereby preventing ankylosis. Periodontal ligament (PDL) cells control bone metabolism and inhibition of bone formation on the compressed side by secreting bone-formation inhibitory factors such as asporin (ASPN) or sclerostin (encoded by *SOST*). The aim of this study was to identify the inhibitory factors of bone formation in PDL cells.

Design: *In vitro*, the changes in expression of ASPN and *SOST* and subsequent protein release in human PDL (hPDL) cells were assessed by semi-quantitative polymerase chain reaction (PCR), real-time PCR, and immunofluorescence in hPDL cells subjected to centrifugal force using a centrifuge (45, 90, 135, and 160 ×g). *In vivo*, we applied a compressive force using the Waldo method in rats, and examined the distribution of ASPN or sclerostin by immunohistochemistry.

Results: *In vitro*, hPDL cells subjected to 90 ×g for 24 h demonstrated upregulated *ASPN* and downregulated *SOST* expressions, which were confirmed by immunofluorescent staining. In addition, the formation of mineralized tissue by human osteoblasts was significantly inhibited by the addition of medium from hPDL cells cultured during compressive force as well as the addition of equivalent amounts of ASPN peptide. *In vivo*, asporin-positive immunoreactive

PDL cells and osteoclasts were found on the compressed side, whereas few sclerostin-positive PDL cells were observed.

Conclusions: PDL cells subjected to an optimal compressive force induce the expression and release of ASPN, which inhibits bone formation during orthodontic tooth movement on the compressed side.

[Highlights]

1. Periodontal ligament (PDL) cells and bone metabolism are greatly related.
2. PDL cells inhibit of bone formation by secreting anti-osteogenic factors.
3. PDL cells subjected to an optimal compressive force induce asporin (ASPN).
4. PDL cells subjected to an optimal compressive force downregulate *SOST*/sclerostin.
5. ASPN inhibits bone formation during orthodontic treatment on the compressed side.

[Title]

Asporin in compressed periodontal ligament cells inhibits bone formation

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Compressive force induces asporin

[Keywords]

Periodontal ligament; Compressive force; Orthodontic tooth movement; Asporin; PLAP-1;

Sclerostin

1. Introduction

During the application of orthodontic force to teeth, bone resorption is dominant on the compressed side, whereas bone formation is dominant on the tension side. Upon application of mechanical forces, the root shifts toward the alveolar wall, resulting in reduced vasculature to the area.[1] In this manner, a cell-free or hyalinized zone is formed temporally, which ultimately results in tooth movement.[2] To maintain the optimal periodontal ligament (PDL) distance, PDL cells on the compressed side release osteoclast inductive molecules such as interleukin-1 (IL-1) or receptor activator of nuclear factor kappa-B ligand (RANKL),[3-6] resulting in osteoclast resorption of alveolar bone. If excessive force is applied, the root of the tooth and alveolar bone become attached and form ankylosis.[7, 8] To avoid ankylosis, compressed PDL cells secrete inhibitory factors for osteoblasts and activating factor for osteoclasts.

Mechanical stress produces a compressive force (CF) that affects osteoblastic bone formation.[9-11] However, during orthodontic tooth movement, no activation of bone formation is observed on the compressed side. These contradictory findings may be explained by active inhibition of bone formation on the compressed side, which is likely inhibited by factors from the compressed PDL cells. The candidate inhibitory molecules for osteoblastic bone formation by PDL cells are sclerostin (encoded by *SOST*)[12, 13] and periodontal ligament-associated protein

1/asporin (PLAP-1/ASPN).[14-18] *SOST* inhibits bone formation by inhibiting Wnt signaling.[19-21] ASPN binds directly with bone morphogenetic protein (BMP)-2, and inhibits transforming growth factor (TGF)-β/Smad signaling, resulting in the inhibition of bone formation.[14-16] Recently, upregulated expression of ASPN in PDL cells was demonstrated *in vitro* during CF;[22] however, the types of molecule involved in the inhibition of bone formation on the compressed side during orthodontic tooth movement are unknown.

We investigated the inhibitory effects of bone formation by PDL cells subjected to mechanical CF, by measuring expression changes in *ASPN* and *SOST* in PDL cells subjected to CF.

2. Materials and Methods

2.1. Cell culture

Human periodontal ligament (hPDL) cells were isolated from healthy PDLs of premolar teeth that were extracted for orthodontic reasons. All the patients gave informed consent before providing the samples. All cells were cultured in alpha-minimum essential medium (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 3 to 8 passages prior to use in the experiments. All procedures were approved by the Research Ethics Committee of Kyushu Dental University (permission number: 14-8).

2.2. Application of CF *in vitro*

We applied mechanical force via centrifugation according to a previous report with some modifications.[23] When the cells reached subconfluence in culture flasks (Becton Dickinson, San Jose, CA, USA), the medium was changed to HEPES-buffered Dulbecco's modified eagle medium without bicarbonate (Gibco, Invitrogen Co., Carlsbad, CA, USA), and 90 μL HCl

(Nacalai Tesque, Inc., Kyoto, Japan) was added to stabilize the pH; then, cells were incubated in the incubator (Yamato Scientific Co., Ltd., Tokyo, Japan) at 37°C for 24 h. Next, cell culture flasks were centrifuged at 40, 90, 135, and 160 ×*g* (Kubota Co., Tokyo, Japan) in the incubator at 37°C for 24 h.

Force was calculated using the following equation: $P = (m \times r \times rpm^2 \times \pi^2) / (A \times 9.8 \times 900)$, where P = kg compression / cm^2 of cells; m = mass of medium (0.005 kg); r = radius (0.1 m); rpm = revolution / min (600, 900, 1100, and 1200); and A = area of contact between medium and cells (12.5 cm^2). The CFs of 40, 90, 135, and 160 ×*g* corresponded to 16.0, 36.0, 53.9, and 63.9 *g*/cm^2, respectively.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was isolated from CF-treated hPDL cells and purified using RNAqueous (Ambion; Life Technologies, Austin, Texas, USA) according to the manufacturer's instructions followed by DNase digestion. cDNA was synthesized from 2 µL total RNA in 30 µL reaction buffer containing 500 mM dNTPs, 20 U ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U Superscript II reverse transcriptase (Invitrogen; Life Technologies, Carlsbad, CA, USA). 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′; *ASPN*, 5′ TCC TAG ACT GGT CTT CTA CAC T 3′ and 5′ GTG CTC AAC ATG TAA CGA GTC T 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 min; *ASPN*, 95°C for 9 min) followed by 40 cycles of denaturation (*GAPDH*, 94°C for 30 s; *ASPN*, 95°C for 60 s; *SOST*, 94°C for 60 s), annealing (*GAPDH* and *ASPN*, 55°C for 1 min; *SOST*, 62°C for 1 min), and extension at 72°C for 1 min. The PCR products were subjected to electrophoresis in 2% (*ASPN*) and 5% (*SOST*) agarose gels, and visualized with ethidium bromide. *GAPDH* expression was used as an internal control.

2.4. Real-time PCR analysis

Total RNA samples from CF-treated hPDL cells were treated with DNase and reverse transcribed with random primers using a Superscript First-Strand Kit (Invitrogen; Life Technologies). Real-time 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 pre-developed proprietary TaqMan primers (*β-actin* [ACTB: Hs99999903_m1] and *ASPN* [ASPN: Hs01550903_m1]; Applied

Biosystems). These analyses were conducted to determine the levels of *β-actin* for data normalization. Cycling conditions were 95°C for 15 s and 60°C for 60 s, for 40 cycles. The expression levels of target genes were normalized to *β-actin* expression and presented relative to the control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The protein release of ASPN was detected by ELISA using the culture medium of hPDL cells treated with or 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 PBS-Tween (0.5%, Tween 20) and blocked with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 30 min at room temperature. Then the plates were incubated with anti-human asporin (1:500 dilution; Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. After washing, donkey anti-goat IgG-HRP (1:5000 dilution; Santa Cruz Biotechnology) was added and incubated for 1 h at room temperature. After further washes, the 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 0.1 M citrate phosphate buffer. The optical density was measured at 490 nm using a microplate

reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Orthodontic tooth movement model and immunocytochemistry

All procedures were approved by the Animal Research Committee of Kyushu Dental University (permission number: 15-004). Using male Sprague-Dawley (SD) rats weighing 200–250 g, we inserted an elastic band between their first and second upper molars. Untreated animals were used as a control group. Then the rats were perfused through the left ventricle with 4% paraformaldehyde (PFA). The maxillas were dissected, decalcified in 10% EDTA solution, and embedded in paraffin; then 5 μm horizontal sections of the molars in the maxilla were prepared. Immunohistochemical analysis was performed using rabbit polyclonal anti-asporin (1:100 dilution; Funakoshi Co., Tokyo, Japan) and anti-sclerostin (1:100 dilution; Santa Cruz Biotechnology) as the primary antibodies and goat anti-rabbit IgG (1:400 dilution; Invitrogen) as the secondary antibody. Positive reactions were visualized with 0.02% 3,3′-diaminobenzidine (DAB; Dojindo, Kumamoto, Japan) and 0.02% hydrogen peroxidase solution (Wako). hPDL cells were fixed with 4% PFA, and the expressions of ASPN and sclerostin were also examined immunocytochemically using the same procedure. Cell nuclei were stained with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA).

2.7. Bone formation assay

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 per well. After reaching near-confluence, half of the medium was replaced with the supernatant of the culture medium of hPDL cells treated with/without CF. Alternatively, ASPN (E-15) peptide (Santa Cruz Biotechnology) was added to confirm the effects of bone formation. 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 min at room temperature. After washing, hOBs were treated with 5% silver nitrate solution for 30 min at room temperature. After further washes, hOBs were fixed with 3% sodium thiosulfate solution for 5 min at room temperature. After washing, the plates were dried for 12 h. Quantitative data were obtained by Image J (NIH, Bethesda, MD, USA) analysis of the images taken with a microscope.

2.8. Statistical Analysis

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

3. Results

To evaluate the differential mRNA expression of bone-formation inhibitory factors, the expressions of *ASPN* and *SOST* mRNA were measured by RT-PCR in hPDL cells treated with or without CF (90 ×*g*) (Fig. 1A). Compared to controls, CF-treated hPDL cells showed higher expression of *ASPN* mRNA but lower expression of *SOST* mRNA, as detected by semi-quantitative RT-PCR. To investigate the effect of the magnitude of CF, we used real-time RT-PCR to examine the expression of *ASPN* mRNA in hPDL cells that were treated at various magnitudes of CF (Fig. 1B). Increased *ASPN* mRNA expression was the greatest in cells treated with CF of 90 ×*g*; *ASPN* mRNA expression was significantly decreased when cells were treated with CF over 90 ×*g* (i.e., 135 and 160 ×*g*). Therefore, the optimal CF for *ASPN* expression in hPDL cells was 90 ×*g*.

Next, we used ELISA to determine the level of ASPN released from CF-treated hPDL cells. We found that ASPN protein released from hPDL cells was significantly increased (mean, 3.9 ng/mL) compared to control (mean, 2.8 ng/mL) (Fig. 1C).

To confirm the distribution of ASPN and sclerostin in cultured hPDL cells, we used immunofluorescence labeling. Similar to the observed increase in *ASPN* mRNA expression, the ASPN in hPDL cells subjected to CF (Fig. 2B) showed greater immunopositivity than the

control (Fig. 2A). Conversely, sclerostin showed lower immunopositivity in CF-treated hPDL cells (Fig. 2D) compared to untreated cells (Fig. 2C), which was consistent with the decreased *SOST* mRNA expression.

Because of the difficulty of using human tissue *in vivo*, we assessed the molars of SD rats using elastic bands to apply orthodontic force (Fig. 3A). Intensive immunopositive staining for ASPN was distributed on the compressed side, especially in PDL cells and osteoclasts (Fig. 3D), compared the tension side or control (Fig. 3B, C). Strong immunopositive staining for ASPN at compressed side was confirmed also in the low power image that contains both tension and compressed sides (Fig. 3E), and the low power image of the control (Fig. 3F). Immunopositive staining for sclerostin was scarcely distributed in PDL cells on the tension side (Fig. 3G), and weak immunopositive cells were found in the control and on the compressed side (Fig. 3H, I). Weak immunopositive staining for sclerostin was confirmed also in the low power image that contains both tension and compressed sides (Fig. 3J), as well as compared the control (Fig. 3K).

To examine the inhibitory effects of bone formation by hPDL cells, hOBs were cultured with the supernatant of hPDL cells treated with 90 ×*g* CF for 4 weeks. The mineralized area was significantly decreased in hOBs cultured with the supernatant from CF-treated cells,

compared to that in cells cultured with the supernatant of untreated cells (Fig. 4A-C). Finally, to confirm that the inhibitory effects were attributable to ASPN, hOBs were cultured with ASPN peptide (2.8 or 3.9 ng/mL). We determined that the concentrations of ASPN peptide at 2.8 and 3.9 ng/mL were equivalent with the control and centrifuged levels of ASPN, respectively (Fig. 1C). The addition of 3.9 ng/mL ASPN significantly decreased the mineralized area compared to control cells cultured with 2.8 ng/mL ASPN (Fig. 4D-F).

4. Discussion

Through experiments that used tooth compression, we found that ASPN produced by PDL cells on the compressed side inhibited bone formation during orthodontic tooth movement. Therefore, ASPN is an important factor associated with the prevention of ankylosis on the compressed side of compressed teeth.

ASPN and sclerostin are two candidate factors involved in the inhibition of bone formation. In the present study, we showed that ASPN is likely involved in the inhibition of bone formation on the compressed side. ASPN binds directly with BMP-2 and inhibits TGF-β/Smad signaling, thereby inhibiting bone formation.[14-16] The leucine-rich repeat or D repeat polymorphism of ASPN has a high affinity for BMP-2.[24, 25] Also, Interestingly, it has been reported that TGF-β was no correlation of the level between bone remodeling markers, but the high level of TGF-β expression found in both compressed and tension sides of hPDL cells, which represents an overall anabolic response inducing extracellular matrix (ECM) synthesis and bone formation that can differentially be modulated by tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10).[26] Thus, further studies are needed to understand the relationship between cytokines potentially involved during orthodontic tooth movement.

In the present study, we found that the expression of *ASPN* in CF-treated hPDL cells was significantly increased under 90 ×*g*. Therefore, we concluded that the optimal CF for *ASPN* mRNA expression was 90 × g (36.0 *g*/cm^2), which is almost the same with the finding that 33.5 *g*/cm^2 is clinical orthodontic force.[27] On the other hand, the expression of *SOST* was not increased by CF. These results were confirmed by immunocytochemical staining for each molecule. Furthermore, our *in vivo* study using rat molars demonstrated increased ASPN immunoreactivity on the compressed side of the periodontium. These findings suggested that at the compressed side of root movement not only bone resorption by osteoclast but also inhibition of bone formation by ASPN would be occurred. In addition, strong immunoreactivity in osteoclasts demonstrated that osteoclasts as well as PDL cells might be involved in the inhibition of bone formation at the compressed side. The expression and release of ASPN was increased during CF, which would result in the inhibition of bone formation by osteoblasts via inhibition of TGF-β/Smad signaling. Generally, optimal mechanical stress induces bone formation by osteoblasts; however, ASPN released by PDL cells may inhibit alveolar bone formation at the periodontal space of the compressed side during orthodontic tooth movement. Previous study indicated mineralization-related cytokines such as fibroblast growth factor-2 (FGF-2) or bone morphogenetic protein -2 (BMP-2) would regulate the expression of ASPN in

PDL cells,[28] however, the relation between the expression of ASPN in PDL cells and mechanical stress such as CF has not been understood. Additional study is needed to elucidate the molecular mechanism. Interestingly, our *in vivo* data indicate strong immunoreactivity of ASPN in the osteoclast-like cells on the compressed side, suggesting that PDL cells and osteoclasts cooperatively inhibit bone formation to prevent ankylosis on the compressed side.

We found that a CF of 90 ×*g* (36.0 g/cm^2) induced the highest expression of *ASPN* mRNA and ASPN protein production. On the other hand, a previous study showed upregulation of *RUNX2* expression in mature osteoblasts that were subjected to 200 ×*g* (40.3 g/cm^2) of CF,[29] suggesting that mechanical force stimulates bone formation on the compressed side during orthodontic tooth movement. However, *in vivo* bone resorption occurred, but the activation of bone formation does not occurred at the compressed side. To address this contradiction, the inhibitory effects of bone formation by ASPN released by CF-treated PDL cells were examined. It has been reported that a CF of 30 ×*g* for 30 min could upregulate the expression of ASPN and suppress the expression of BMP-2 back to baseline levels.[22] In the present study, we found that the maximum expression of *ASPN* in PDL cells and maximum ASPN release in the medium were achieved at 90 ×*g* (36.0 g/cm^2) CF. Previously, we found that a CF (2.0 or 5.0 g/cm^2) for 2–4 days increased RANKL and IL-1β expression in hPDL cells,[3] but the increase in ASPN

needed greater mechanical force. These results suggest that even a weak CF could induce bone resorption, as the increase in optimal CF in PDL cells inhibited bone formation. In addition, excessive CF inhibited bone formation via the release of ASPN by PDL cells.

Inhibition of bone formation was confirmed using the culture supernatant from CF-treated cells. We found that the supernatant of hPDL cells cultured with the 90 $\times g$ CF for 24 h significantly inhibited bone formation by human osteoblastic cells. Furthermore, ASPN peptide significantly inhibited bone formation by human osteoblastic cells when added at the equivalent density of ASPN released in the supernatant of hPDL cells cultured with 90 $\times g$ CF for 24 h. These findings suggest that CF induces the production of bone formation inhibitory factors such as ASPN, explaining in part why bone formation is not activated on the compressed side during orthodontic tooth treatment.

5. Conclusions

Thus, we elucidated key components in the mechanism of inhibition of bone formation on the compressed side of teeth subject to CF during orthodontic tooth movement. PDL cells subjected to optimal mechanical forces induced the bone formation inhibitory factor ASPN, which prevents ankylosis. The expression of ASPN could be an important indicator for finding the optimal CF in tooth movement.

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Fig. 1

Effects of compressive force (CF) on expression of *ASPN* and *SOST* in hPDL cells. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *ASPN* and *SOST* mRNA expression in CF-treated hPDL cells. CF was applied at 90 ×g for 24 h. *ASPN* and *SOST* mRNA expression relative to *GAPDH* expression was analyzed. (B) Quantitative expression of *ASPN* mRNA in hPDL cells under CF at 45, 90, 135, and 160 ×*g* for 24 h was analyzed by real-time RT-PCR. Data indicate expression relative to control (*β-actin*) (n = 5). The values are presented as means ± S.E. **P < 0.01. (C) Enzyme-linked immunosorbent assay (ELISA) analysis was used to determine the amount of ASPN in the supernatant of CF-treated cells (90 ×g) for 24 h. (n = 5). The values are presented as means ± S.E. **P < 0.01.

Fig. 2

Immunohistochemical localization of ASPN and sclerostin in CF-treated hPDL cells. Immunohistochemical staining of ASPN (red), sclerostin (green), and the nuclei of hPDL cells (blue). Control cells (A, C) and CF-treated cells (90 ×g) for 24 h (B, D). Bars = 50 μm.

Fig. 3

Localization of ASPN in rat PDL tissues *in vivo*. Elastic bands were placed between the first (M1) and second molars (M2) of SD rats according to the Waldo method (A). DAB staining of ASPN (B-F) and sclerostin (G-K). Control tissues without CF (C, F, H, K), tension side (B, G), and compressed side (D, I). The low power image that contains both the tension side and the compressed side (E, J), compared the control (F, K). White arrows indicate the expression of ASPN (B, E) in PDL cells. Black arrows indicate the expression of ASPN (D, E) in osteoclasts. (PD = periodontal ligament, R = tooth root, AB = alveolar bone, P = dental pulp). Bars = 50 μm.

Fig. 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 supernatant from hPDL cells without CF (A) and with CF (B). Von Kossa staining was performed after culturing human osteoblast-like cells in medium from hPDL cells plus the expression amount of ASPN protein without CF (D) and with CF (E). Comparison of changes in the area of von Kossa-positive staining (C, F; n = 10). Bars = 1 mm. The values are presented as means ± S.E. $^{**}P < 0.01$.

[All figure captions]

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Effects of compressive force (CF) on expression of *ASPN* and *SOST* in hPDL cells.

Fig. 2

Immunohistochemical localization of ASPN and sclerostin in CF-treated hPDL cells.

Fig. 3

Localization of ASPN and sclerostin in rat PDL tissues *in vivo*.

Fig. 4

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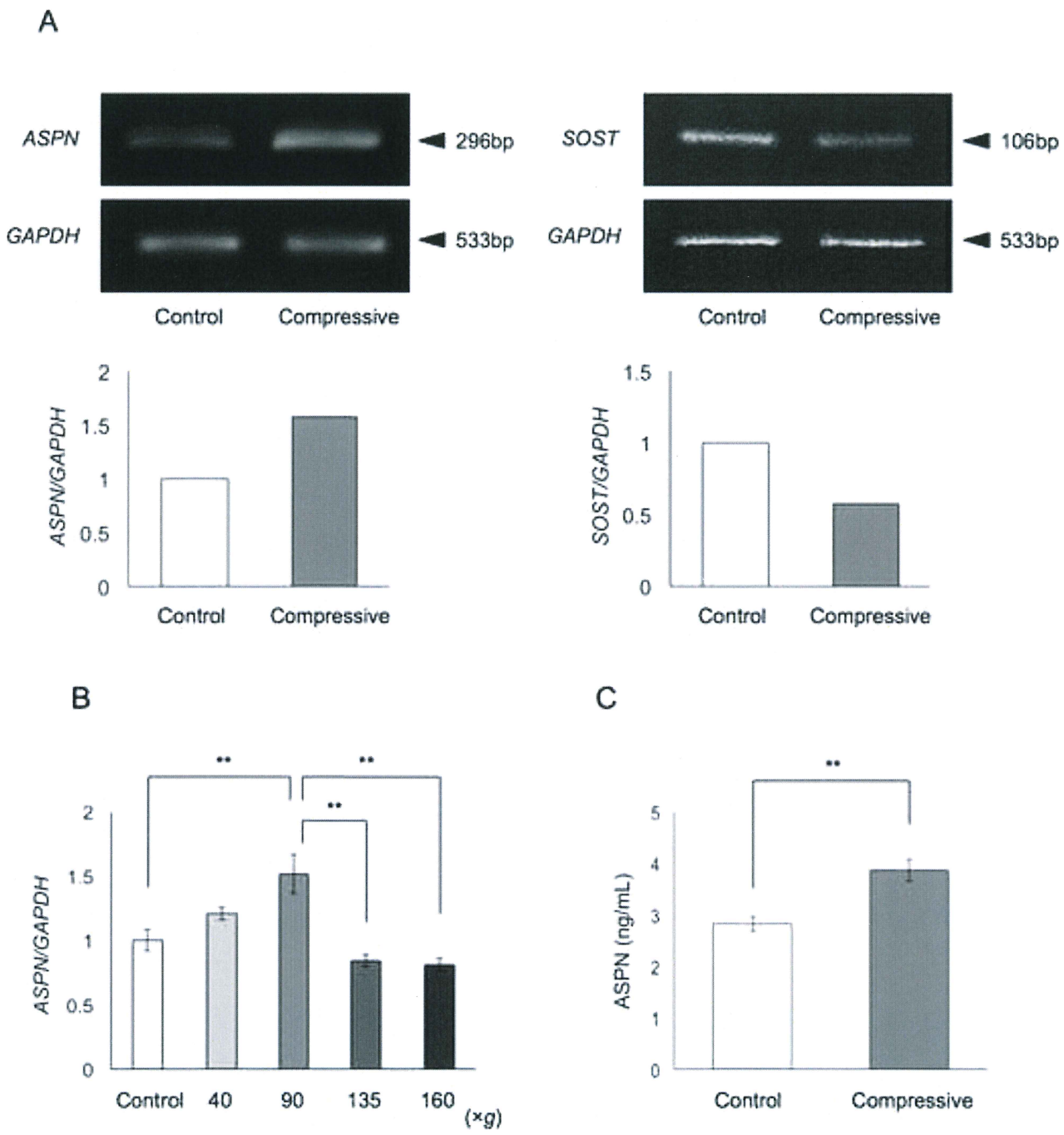

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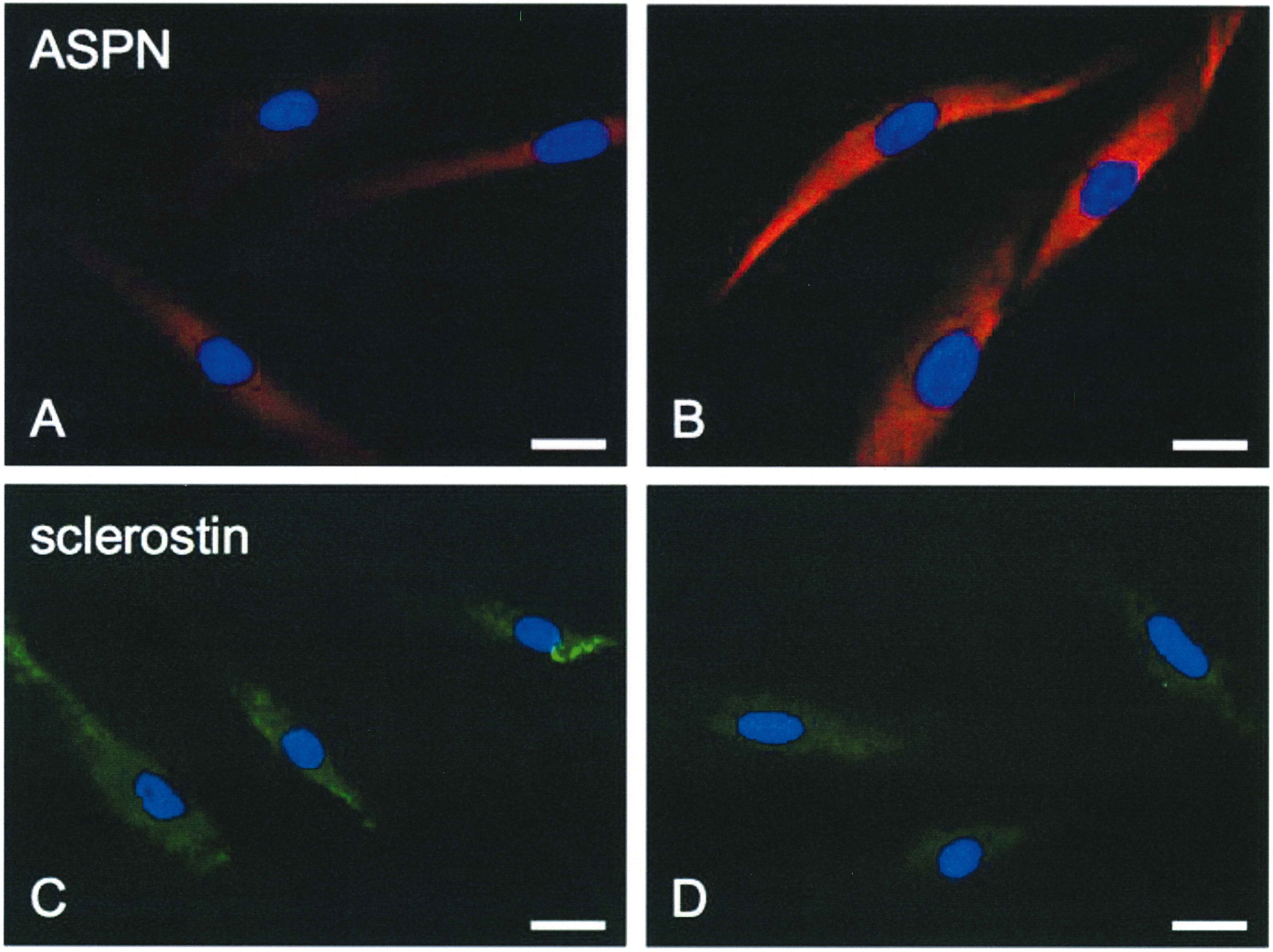

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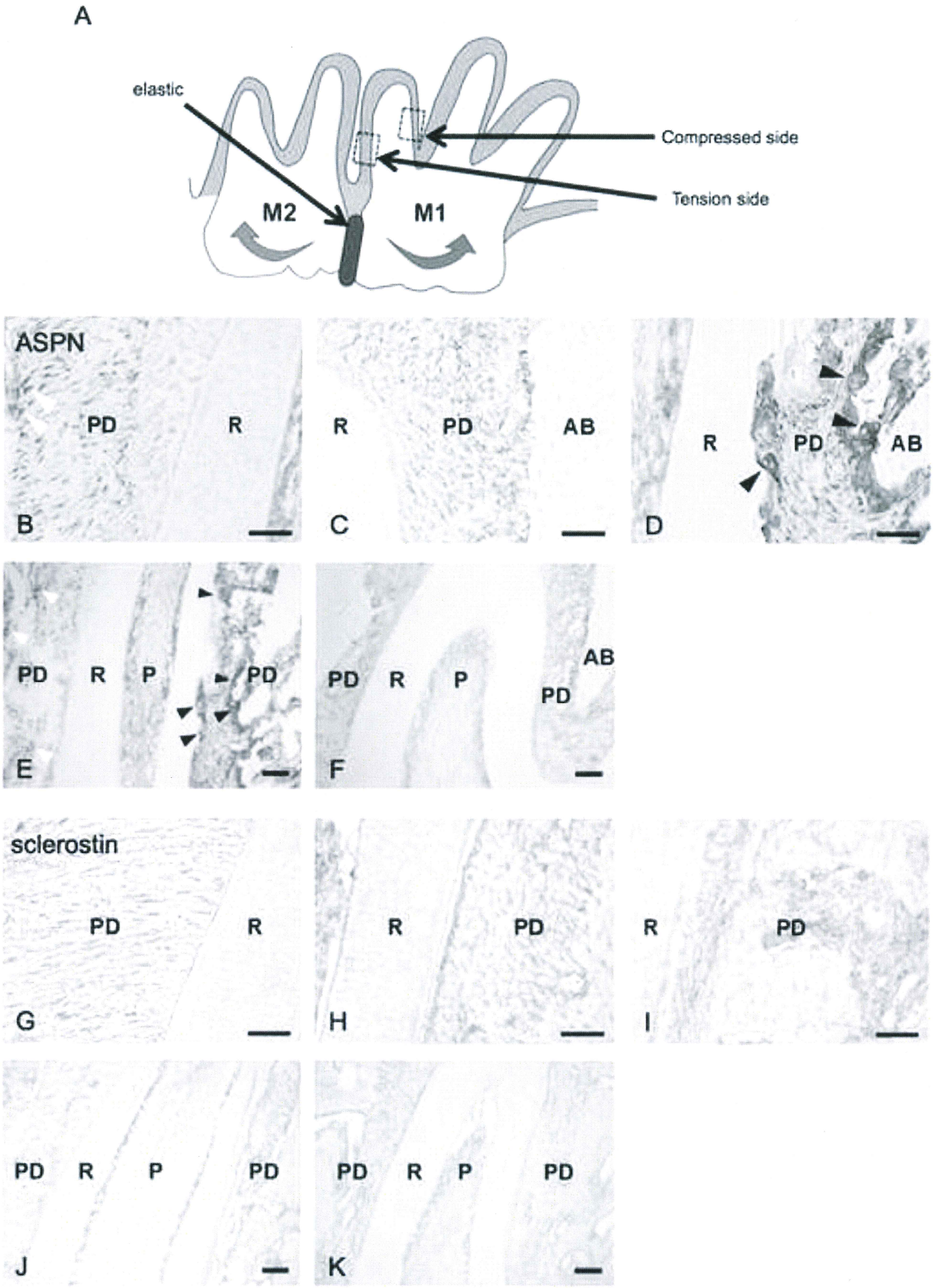

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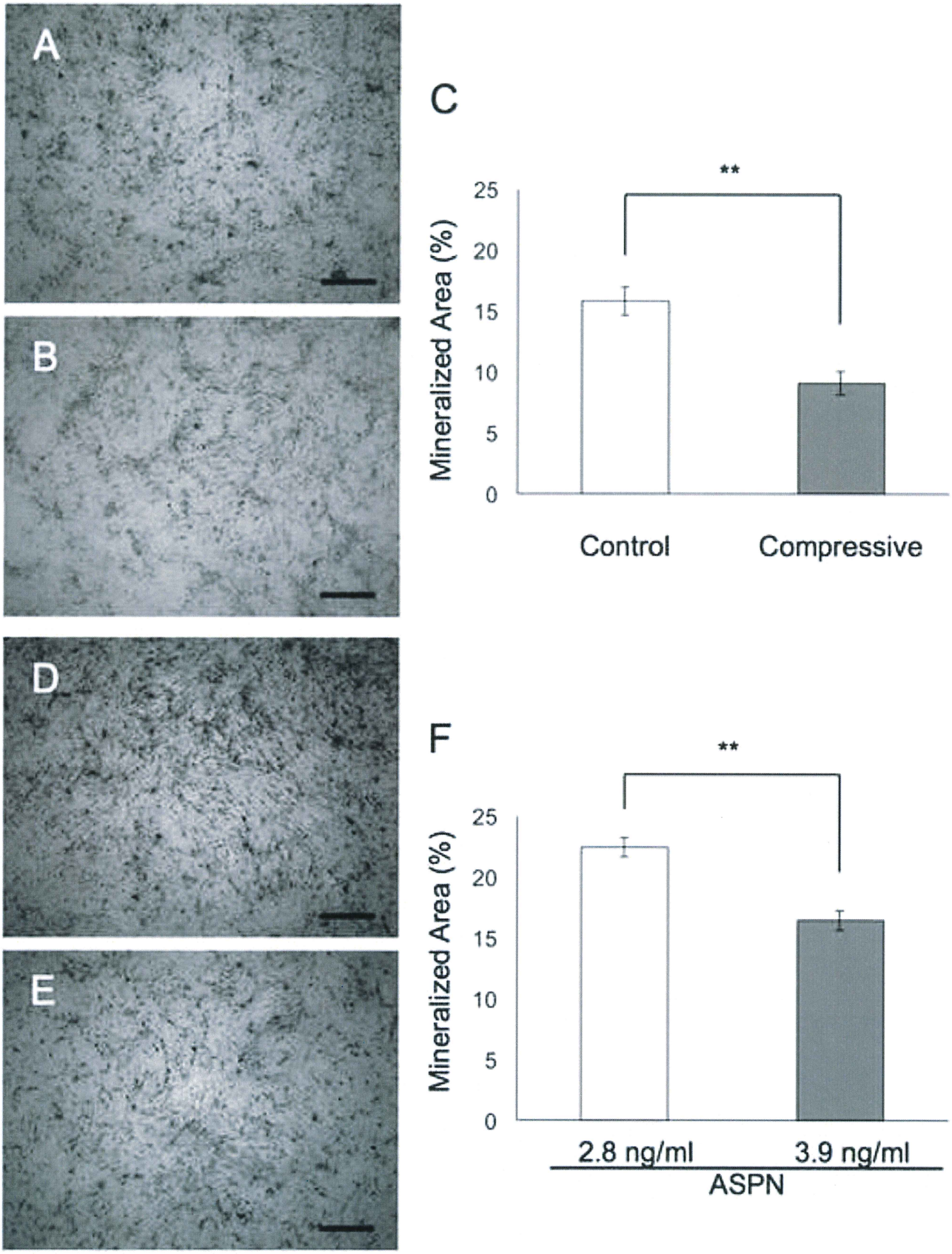

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