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Original Article

Fibrillin-1 regulates periostin expression during maintenance of periodontal homeostasis

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Abstract *Background/purpose:* Human periodontal ligament consists of elastic system fibers, mainly fibrillin-1 (FBN1). Periostin (POSTN) maintains periodontal homeostasis. A previous study showed that the expression of *Postn* in periodontal ligament cells was decreased in mice underexpressing *Fbn1*. However, the relationship between FBN1 and POSTN is not fully understood in the context of mechanical stress. FBN1 contributes to transforming growth factor β 1 (TGF- β 1) activation; TGF- β 1 upregulates the expression of *POSTN* in human periodontal ligament cells. This study examined whether FBN1 contributed to the maintenance of periodontal homeostasis in cultured human periodontal ligament cells.

Materials and methods: Human periodontal ligament fibroblasts (HPDLFs) were exposed to mechanical force via centrifugation. The expression of *POSTN* was examined by quantitative reverse transcription polymerase chain reaction. The phosphorylation of Smad2 in the TGF- β /Smad signaling pathway was monitored by western blotting.

Results: The expression levels of *FBN1* and *POSTN* were not significantly decreased by centrifugation. However, the expression of *POSTN* after centrifugation significantly decreased upon knockdown of *FBN1*. The phosphorylation of Smad2 after centrifugation was decreased, regardless of *FBN1* knockdown. Supplementation with 0.1 ng/ml recombinant human TGF- β 1 rescued *POSTN* expression after centrifugation in HPDLFs upon knockdown of *FBN1*.

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Conclusion: FBN1 regulates the expression of *POSTN* to maintain periodontal homeostasis via TGF- β /Smad signaling during centrifugation.

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Introduction

Periodontal tissue contains the periodontal ligament, which senses mechanical stress (e.g., occlusion). Under mechanical stress, human periodontal ligament cells promote collagen fiber production and remodeling according to the magnitude and direction of strain.^{1–3} They induce molecules such as prostaglandin-endoperoxide synthase 2 and respond to mechanical stress.⁴ The periodontal ligament thus contributes to the maintenance of periodontal homeostasis. However, the underlying mechanisms have been unknown.

The human periodontal ligament consists of two types of fibers: elastic system and collagen. Elastic system fibers are present in tissues that require elasticity, such as blood vessels; they work against external forces.^{5,6} Elastic system fibers in the periodontal ligament exist parallel to the long axis of the root.⁷ Elastic system fibers consist of microfibrils and elastin;⁸ they are classified into three types according to the relative proportions of microfibrils and elastin. Most elastic system fibers in the periodontal ligament are oxytalan fibers, which include only microfibrils.⁹ The major constituent protein of microfibrils is fibrillin-1 (FBN1),¹⁰ which is important for the storage of transforming growth factor β 1 (TGF- β 1) via latent TGF- β -binding protein 1 (LTBP1);^{11–13} it also controls TGF- β 1 activity in the matrix.¹⁴ FBN1 is presumed to regulate human periodontal ligament cell alignment.¹⁵

The periodontal ligament also contains periostin (POSTN).¹⁶ POSTN is an important mediator of the biomechanical properties of fibrous connective tissue.¹⁷ In a previous study, *Postn*-null mice showed irregular periodontal ligament, alveolar bone resorption, and external root resorption because of occlusal loading.¹⁸ Those findings suggested that POSTN maintains periodontal tissue structure and contributes to periodontal ligament homeostasis. Mechanical force causes the release of signaling molecules (e.g., cytokines and colony-stimulating factors) via blood vessels.¹⁹ Although some studies have described homeostasis of the extracellular matrix, the underlying mechanisms have been unknown. Here, we focused on FBN1 and POSTN. A previous study reported that the expression of *Postn* in periodontal ligament cells was decreased in mice underexpressing *Fbn1*;²⁰ however, it has been unclear whether FBN1 regulates the expression of *POSTN* under mechanical force. Thus, we investigated whether FBN1 maintained the expression of *POSTN* in human periodontal ligament fibroblasts (HPDLFs) under mechanical force.

Materials and methods

Cell isolation and culture

HPDLFs were purchased from Lonza Japan, Inc. (Tokyo, Japan). The research protocol was approved by the Research Ethics Committee of Kyushu Dental University (permission number: 17–37). HPDLFs were cultured in alpha-minimum essential medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Biosera, Nuaillé, France), 100 U/mL penicillin G (Gibco BRL), 100 μ g/ml streptomycin (Gibco BRL) and 100 μ g/ml amphotericin B (Gibco BRL); they were incubated at 37 °C with 5% CO₂. Cells from passages four through six were used for all experiments.

Mechanical force application

To simulate the compression side during orthodontic tooth movement, we applied mechanical force to HPDLFs by centrifugation using a previously published protocol.²¹ HPDLFs were cultured at 33.6×10^3 cells/well in six-well plates until they reached sub-confluence; the medium was subsequently exchanged for Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 90 μ L/well HCl (Nacalai Tesque Inc., Kyoto, Japan) to stabilize pH and cells were incubated at 37 °C for 24 hours (Yamato Scientific Co., Ltd., Tokyo, Japan). Finally, a centrifuge (Kubota Co., Tokyo, Japan) in the incubator was used to apply mechanical forces of 40, 90, and 135 g for 12 and 24 hours.

Cell damage assay

Lactate dehydrogenase release from cells was quantified to evaluate cell damage caused by mechanical force. After centrifugation treatment, we measured the absorbance (optical density [OD] value) of formazan dye at 490 nm with a spectrophotometer (iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Cytotoxicity Lactate Dehydrogenase Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan). The supernatant from cells not subjected to mechanical force was used as a low control; the supernatant with lysis buffer added 30 minutes before collection was used as a high control. After the background control OD value was subtracted from the OD value of each well, cytotoxicity was calculated using the following formula: cytotoxicity (%) = (OD value of

experimental group – OD value of low control)/(OD value of high control – OD value of low control) × 100%.

HPDLF treatments

For gene silencing experiments, small interfering RNA targeting human *FBN1* (5'-ACCGGTTTACCCGTTGATATT-3') was purchased from Qiagen (Qiagen, Venlo, Limburg, the Netherlands); the expression of *FBN1* was suppressed using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen Co., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The All Stars Negative control (Qiagen) was used as a scrambled control for gene silencing experiments. For stimulatory experiments, 0.001, 0.01, 0.1, 1.0, and 10 ng/ml recombinant human TGF-β1 (PeproTech, Cranbury, NJ, USA) were used.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from HPDLFs and purified using the RNAqueous Total RNA Isolation Kit (Ambion, Inc., Austin, TX, USA), in accordance with the manufacturer's instructions. Total RNA was treated with DNase (Thermo Fisher Scientific, Waltham, MA, USA); 0.5 μg RNA was reverse transcribed into cDNA using SuperScript VILO Master Mix (Invitrogen Co.). The expression levels were evaluated by quantitative reverse transcription polymerase chain reaction using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and QuantStudio 3 Real-Time PCR System (Applied Biosystems). Primers for the following gene transcripts were used: *FBN1*, *POSTN*, *bone morphogenetic protein 2 (BMP2)*, *connective tissue growth factor (CTGF)*, and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* (Table 1). Polymerase chain reaction amplification consisted of 40 cycles of denaturation at 95 °C for 15 seconds, followed by annealing and extension at 60 °C for 1 minute. The specificity of the polymerase chain reaction product was confirmed by melt curve analysis. *GAPDH* was used as a reference gene. Relative expression was quantified using the ΔΔCt method.

Table 1 Primers used for quantitative reverse transcription-polymerase chain reaction. *BMP2*: bone morphogenetic protein 2, *CTGF*: connective tissue growth factor, *FBN1*: fibrillin-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, *POSTN*: periostin.

Gene	Primers
FBN1	5'-GGAACGTGAAGGAAACCAGA-3' 5'-GGCAAATGGGGACAATACAC-3'
POSTN	5'-ATTGATGGAGTGCCTGTG-3' 5'-CCTTGGTGACCTCTTCTTG-3'
BMP2	5'-ATGGATTCTGTTGGGAAGTG-3' 5'-GTGGAGTTCAGATGATCAGC-3'
CTGF	5'-CAGCATGGACGTTCTGTG-3' 5'-AACACCGGTTTGGTCTTGG-3'
GAPDH	5'-TGAAGTTCGGAGTCAACGGAT-3' 5'-TCACCCATGACGAACATGG-3'

Western blotting

Cells were lysed in sample buffer supplemented with protease and phosphatase inhibitor cocktails (both from Nacalai Tesque). Protein concentrations were determined using protein assay reagent (Cytoskeleton, Denver, CO, USA); for each sample, 20 μg protein were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated for 12 hours at room temperature with primary antibodies against Smad2/3 (1:2000, Cat. No.: 8685; Cell Signaling Technology, Beverly, MA, USA) and phospho-Smad2 (1:1000, Cat. No.: 3108; Cell Signaling Technology); they were incubated for 1 hour at room temperature with a primary antibody against β-actin (1:5000, Cat. No.: sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then washed and incubated at room temperature for 1 hour with horseradish peroxidase-conjugated secondary antibody (1:25,000, Cat. No.: 715,035,150; Jackson ImmunoResearch, West Grove, PA, USA). Protein bands were visualized using Western Chemiluminescent HRP Substrate (Millipore, Boston, MA, USA). Images were quantified by ImageJ software (version 1.53e, National Institutes of Health, Bethesda, MD, USA). The densities of protein bands were normalized to β-actin.

Statistical analysis

Data are shown as means ± standard errors. Two-way analysis of variance was used to analyze cytotoxicity after exposure to mechanical force. One-way analysis of variance was used to analyze changes in the expression levels of genes after exposure to mechanical force and *FBN1* knock-down; it was also used to analyze changes in phospho-Smad2 levels. To identify statistically significant differences between groups, Tukey's test was used for post hoc multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Evaluation of cytotoxicity after exposure to mechanical force

To evaluate the effect of mechanical force on HPDLFs, cytotoxicity was investigated. After application of 135 g for 12 or 24 hours, cytotoxicity was significantly increased compared with application of 40 g for 12 or 24 hours ($p < 0.05$). However, there were no significant differences in cytotoxicity between 40 and 90 g for 12 or 24 hours (Fig. 1). The mechanical forces created by centrifugation at 40g and 90 g did not cause different degrees of cytotoxicity, consistent with previous findings.²² Thus, subsequent experiments were conducted using 90 g for 24 hours.

Changes in expression levels of *FBN1* and *POSTN* in HPDLFs after exposure to mechanical force.

To evaluate the effects of mechanical force on HPDLFs, expression levels of *FBN1* and *POSTN* were examined. There were no significant differences in the expression of *FBN1* (Fig. 2A) or *POSTN* (Fig. 2B).

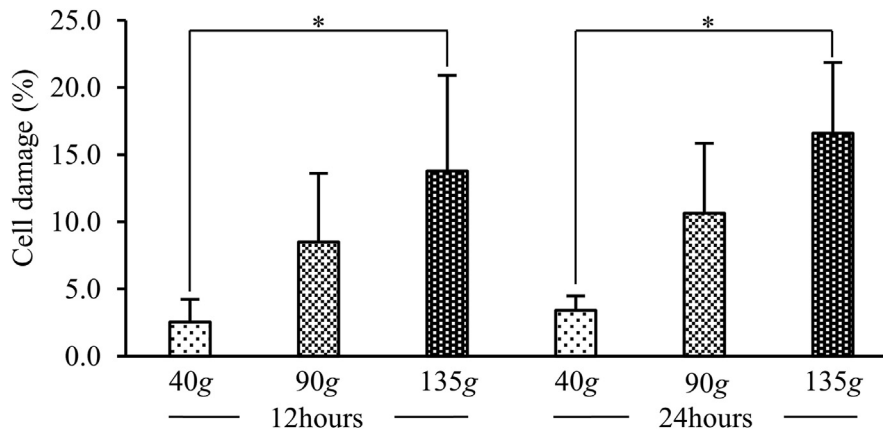


Figure 1 Cytotoxicity under mechanical force. Cytotoxicity was significantly increased by the application of 135 g for 12 and 24 hours compared with 40 g. However, it was not significantly increased by the application of 90 g for 12 and 24 hours compared with 40 g ($n = 3$). For subsequent experiments, 90 g mechanical force was applied for 24 hours. * $p < 0.05$, 40 g versus 90 g versus 135 g.

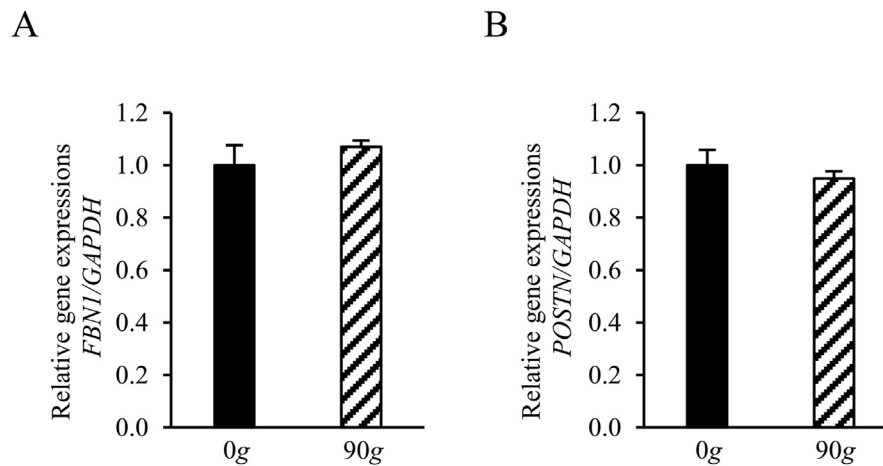


Figure 2 Changes in the expression levels of *FBN1* and *POSTN* under mechanical force in HPDLFs, evaluated by quantitative reverse transcription-polymerase chain reaction ($n = 3$). The relative expression levels are shown with the 0 g group regarded as 1. There were no significant differences in the expression of *FBN1* (A) and *POSTN* (B) upon exposure to 90 g for 24 hours. *FBN1*: fibrillin-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, HPDLFs: human periodontal ligament fibroblasts, *POSTN*: periostin.

Mechanical force-induced changes in expression levels of *POSTN* in HPDLFs upon knockdown of *FBN1*.

To clarify the involvement of *FBN1* in *POSTN* expression under mechanical force, *FBN1* was knocked down and changes in *POSTN* gene expression were examined. First, *FBN1* knockdown was confirmed (Fig. 3A). *POSTN* expression was significantly decreased upon suppression of *FBN1* expression. This reduction of *POSTN* expression was greater after exposure to mechanical force (Fig. 3B).

Mechanical force-induced changes in expression levels of *BMP2* and *CTGF* in HPDLFs upon knockdown of *FBN1*.

BMP2 and *CTGF* function to upregulate the expression of *POSTN*. Therefore, to determine the involvement of *POSTN* in expression of other genes, expression levels of *BMP2* and *CTGF* were examined. Centrifugation caused a significant increase in *BMP2* expression; however, there were no significant differences in the expression levels of *BMP2* upon knockdown of *FBN1* (Fig. 4A). Additionally, there was no significant difference in the expression of *CTGF* under mechanical force, regardless of *FBN1* knockdown (Fig. 4B).

Mechanical force-induced changes in Smad2 phosphorylation in HPDLFs upon knockdown of *FBN1*.

TGF- β 1 binds to *FBN1* via LTBP1; it is released from LTBP1 to activate and upregulate *POSTN*. Therefore, to elucidate the involvement of *FBN1* in *POSTN* expression, we examined the phosphorylation of Smad2, which functions downstream of the TGF- β /Smad signaling pathway. Knockdown of *FBN1* caused a significant decrease in the phosphorylation of Smad2. Surprisingly, centrifugation enhanced the decrease in Smad2 phosphorylation (Fig. 5A and B).

Effects of TGF- β 1 supplementation in HPDLFs upon knockdown of *FBN1* during exposure to mechanical force.

To explore the possibility that TGF- β 1 affects the expression of *POSTN*, the medium was treated with 0.001, 0.01, 0.1, 1.0, or 10 ng/ml TGF- β 1. The expression of *POSTN* was significantly increased upon supplementation with ≥ 0.1 ng/ml TGF- β 1 ($p < 0.01$) (Fig. 6A). Importantly, the mechanical force-induced reduction of *POSTN* expression upon knockdown of *FBN1* in HPDLFs was rescued by supplementation with 0.1 ng/ml TGF- β 1 ($p < 0.01$) (Fig. 6B).

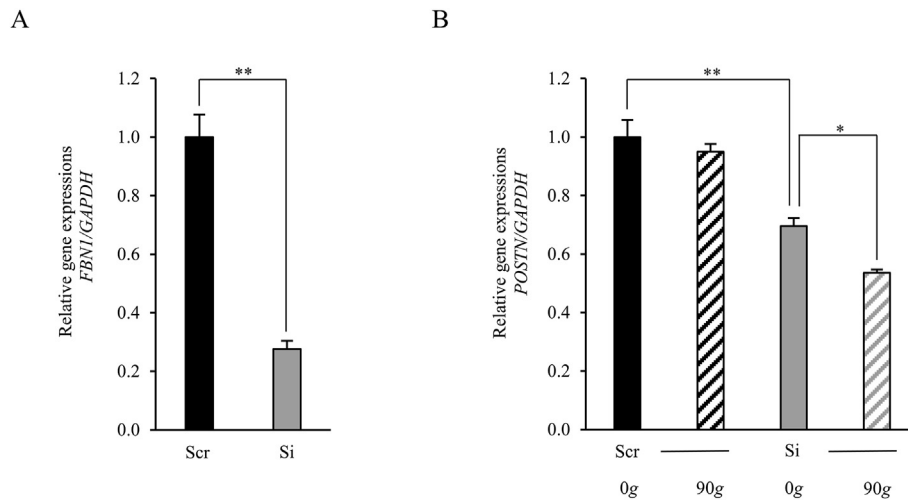


Figure 3 Changes in the expression levels of *FBN1* and *POSTN* under mechanical force in HPDLFs upon knockdown of *FBN1*, evaluated by quantitative reverse transcription-polymerase chain reaction. (A) Expression of *FBN1* in HPDLFs. The relative expression levels are shown with Scr regarded as 1. Expression of *FBN1* was decreased compared with the Scr group ($p < 0.01$) ($n = 3$). This result confirmed reduced expression of *FBN1*. (B) Expression of *POSTN* under mechanical force of 90 g in HPDLFs upon knockdown of *FBN1*. The relative expression levels are shown with Scr-0 g regarded as 1. Expression of *POSTN* in Si-0 g was decreased compared with Scr-0 g ($p < 0.01$) ($n = 3$). Similarly, the expression of *POSTN* in Si-90 g was significantly decreased compared with Si-0 g ($p < 0.05$) ($n = 3$). (A) * $p < 0.05$, Scr versus Si. (B) * $p < 0.05$ or ** $p < 0.01$, Scr-0 g versus Scr-90 g versus Si-0 g versus Si-90 g *FBN1*: fibrillin-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, HPDLFs: human periodontal ligament fibroblasts, *POSTN*: periostin, Scr: scrambled siRNA, Si: siRNA against *FBN1*.

Discussion

To our knowledge, this is the first study to show that *FBN1* maintains the expression of *POSTN* under mechanical force. Our study examined the interrelationship between *FBN1* and *POSTN* in HPDLFs after exposure to mechanical force. The expression levels of *POSTN* in HPDLFs did not significantly decrease under mechanical force. However, the expression of *POSTN* significantly decreased upon knockdown of *FBN1*. These findings suggest that *FBN1* is important for maintaining periodontal tissue homeostasis under mechanical force.

Regarding *FBN1* expression under mechanical force, a previous study showed that *FBN1* expression did not change when cyclic stretching was applied to human periodontal ligament cells.²³ Similarly, the present study showed that *FBN1* expression did not change after centrifugation (Fig. 2A). These results suggest that *FBN1* expression does not change under mechanical force. To our knowledge, our report is one of few concerning *FBN1* expression under mechanical force.

Regarding *POSTN* expression under mechanical force, Panchamanon et al. subjected human periodontal ligament cells to compression force using weighted coins; they found that *POSTN* expression increased with compression force of 1.0 g/cm² and decreased with compression force of 2.0 g/cm².²⁴ The magnitude of the force used in the previous study was equivalent to a centrifugal force of 1.9 g to 3.8 g, which considerably differed from the centrifugal force of 90 g used in our study. Because we observed no changes in the expression levels of *FBN1* and *POSTN* under mechanical force, we presume that *FBN1* contributes to the maintenance of *POSTN* expression.

Factors involved in the expression of *POSTN* include *BMP2*,²⁵ *CTGF*,²⁶ and *TGF- β 1*.²⁷ To evaluate these factors, we examined changes in their expression levels upon knockdown of *FBN1*. The expression of *BMP2* was significantly increased after exposure to mechanical force, regardless of *FBN1* knockdown (Fig. 4A). However, there were no significant differences in the expression levels of *CTGF* (Fig. 4B). *BMP2* induces new bone formation;²⁸ it also activates osteoclasts.²⁹ The increased expression of *BMP2*—in response to tension force in the previous study³⁰ and compressive force in the present study—suggests increased bone metabolism. In the present study, upon knockdown of *FBN1*, the expression of *BMP2* was increased, whereas the expression of *POSTN* was decreased. These findings implied that *BMP2* was not involved in the interaction between *FBN1* and *POSTN*. Previous studies have shown that *Ctgf* is highly expressed in rat osteoblasts during bone formation.³¹ *CTGF* may be important during bone formation. However, upon knockdown of *FBN1*, we found that the expression of *CTGF* did not change, whereas the expression of *POSTN* was decreased (Fig. 4B). Our findings indicate that *CTGF* is not involved in the interaction between *FBN1* and *POSTN*.

The phosphorylation of Smad2, which functions downstream of the *TGF- β 1* signaling pathway, was decreased after exposure to mechanical force, regardless of *FBN1* knockdown (Fig. 5). A previous study showed that *TGF- β 1* was induced by intermittent compressive force on human periodontal ligament cells and the expression of *POSTN* was induced by *TGF- β 1*,²⁷ suggesting enhancement of Smad2 phosphorylation. Similar to our results regarding the expression of *POSTN* upon knockdown of *FBN1*, the expression of *POSTN* may depend on the *TGF- β 1* signaling

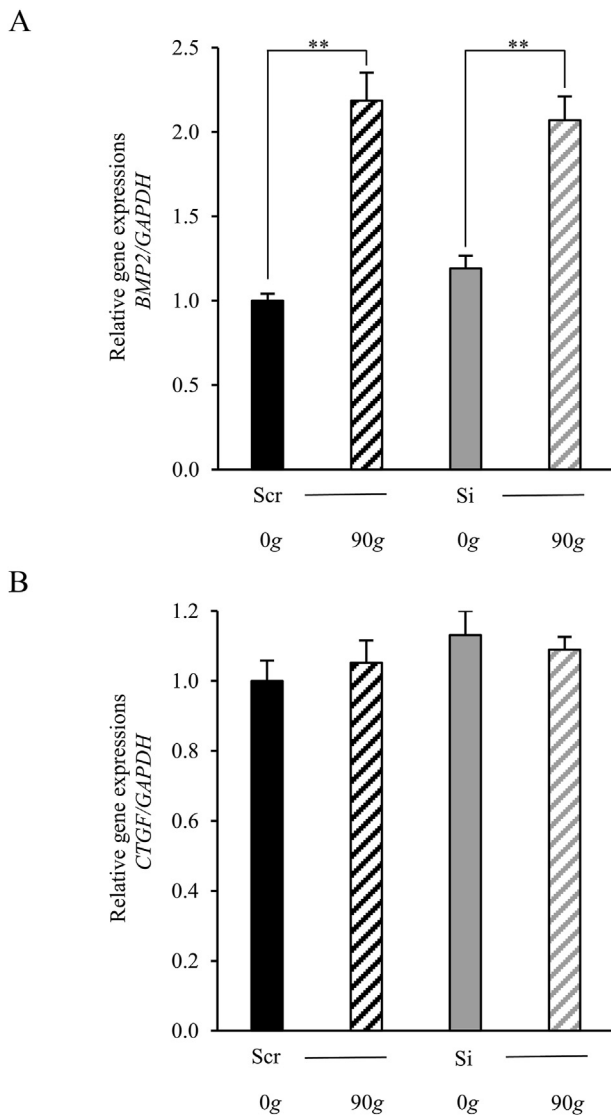


Figure 4 Changes in the expression levels of *BMP2* and *CTGF* under mechanical force in HPDLFs upon knockdown of *FBN1*, evaluated by quantitative reverse transcription-polymerase chain reaction. The relative expression levels are shown with Scr-0 g regarded as 1. (A) Expression of *BMP2* was significantly increased under mechanical force of 90 g, regardless of *FBN1* knockdown ($p < 0.01$) ($n = 3$). (B) There was no change in the expression of *CTGF* under mechanical force of 90 g, regardless of *FBN1* knockdown ($n = 3$). ** $p < 0.01$ versus Scr-0 g. *BMP2*: bone morphogenetic protein 2, *CTGF*: connective tissue growth factor, *FBN1*: fibrillin-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, HPDLFs: human periodontal ligament fibroblasts, *POSTN*: periostin, Scr: scrambled siRNA, Si: siRNA against *FBN1*.

pathway. Furthermore, in a study by Panchamanon et al., *TGF- β 1* and *POSTN* expression levels increased when human periodontal ligament cells were subjected to a compression force of 1.0 g/cm.²⁴ These results indicate an important role for *FBN1* in the expression of *POSTN* under mechanical force via Smad2 phosphorylation and TGF- β 1 signaling.

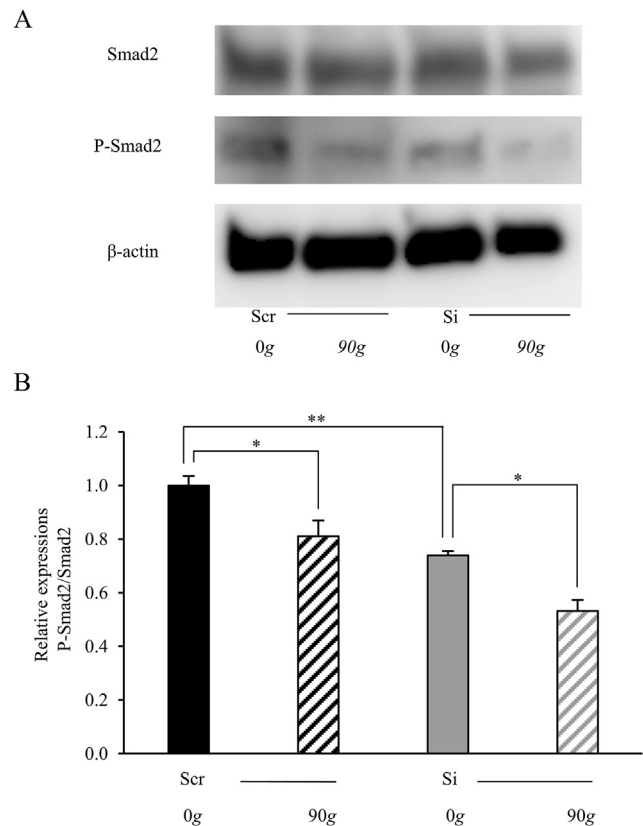


Figure 5 Changes in the phosphorylation of Smad2 under mechanical force in HPDLFs upon knockdown of *FBN1*, evaluated by western blotting. β -actin was used as the internal loading control. (A) *FBN1* knockdown caused a decrease in the phosphorylation of Smad2. Furthermore, centrifugation caused a decrease in the phosphorylation of Smad2. (B) The phosphorylation rate of Smad2 shown in (A) was quantified by ImageJ software. The relative expression levels are shown with Scr-0 g regarded as 1. The phosphorylation of Smad2 was significantly decreased under mechanical force of 90 g, regardless of *FBN1* knockdown ($p < 0.05$) ($n = 3$). * $p < 0.05$ ** $p < 0.01$, Scr-0 g versus Scr-90 g versus Si-0 g versus Si-90 g *FBN1*: fibrillin-1, HPDLFs: human periodontal ligament fibroblasts, Scr: scrambled siRNA, Si: siRNA against *FBN1*.

To further clarify the involvement of TGF- β 1 in *POSTN* expression, we evaluated changes in *POSTN* expression upon TGF- β 1 supplementation to HPDLFs. We found that the expression of *POSTN* was rescued (Fig. 6B), implying that the expression of *POSTN* under mechanical force depends on TGF- β /Smad signaling. A previous study reported that *FBN1* binds to TGF- β 1 via latency-associated propeptide and LTBP1; TGF- β 1 is activated upon release from LTBP1.^{12,14,32} *FBN1* is important for the storage of TGF- β 1. The present study demonstrated that the expression of *POSTN* was decreased upon knockdown of *FBN1*. The underlying mechanism is that the expression of *POSTN* depends on active TGF- β 1 release from latency-associated propeptide. *FBN1* presumably binds to integrins present on the cell membrane and participates in cell signaling;³³ the integrin expression levels are increased with 24 hours of

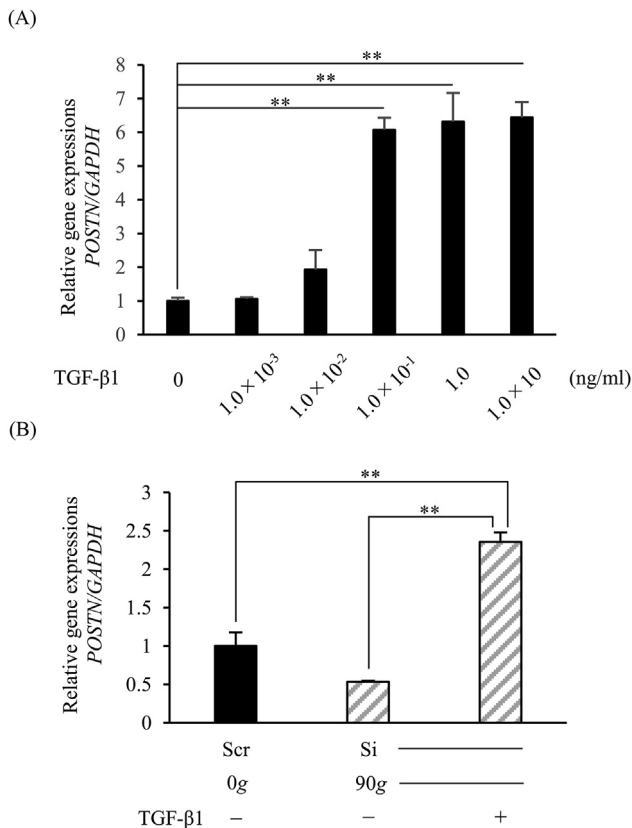


Figure 6 Changes in the expression of *POSTN* upon treatment with TGF-β1, evaluated by quantitative reverse transcription-polymerase chain reaction. (A) Expression of *POSTN* was increased by supplementation with 0.1, 1.0, or 10 ng/ml TGF-β1 ($p < 0.01$) ($n = 3$). (B) Expression of *POSTN* in Si-90 g was rescued by supplementation with 0.1 ng/ml TGF-β1 ($p < 0.01$) ($n = 3$). (A) $**p < 0.01$, versus control. (B) $**p < 0.01$, versus Scr-0 g and Si-0 g (without TGF-β1 supplementation). *FBN1*: fibrillin-1, *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase, HPDLFs: human periodontal ligament fibroblasts, *POSTN*: periostin, Scr: scrambled siRNA, Si: siRNA against *FBN1*.

cyclic extension.³⁴ These findings suggest that integrin expression decreases during compression; TGF-β1 cell signaling was suppressed by reducing the expression of *FBN1* for integrin binding. A previous study showed that shear stress significantly reduced the expression levels of the two major *TGF-β* receptors (*types I* and *II*) in mouse embryo mesenchymal progenitor cells.³⁵ The suppression of Smad2 phosphorylation in this study may be related to decreased *TGF-β* receptor expression. Additionally, a previous study showed that angiotensin II was involved in the TGF-β1-mediated alteration of *Postn* expression.³⁶ Another study reported that mechanical force to HPDLFs led to enhanced secretion of angiotensin II.³⁷ Further studies are needed to elucidate the detailed mechanism underlying *POSTN* expression in response to mechanical force.

Regarding the clinical significance of our findings, a previous study revealed fewer oxytalan fibers in the central region of mouse periodontal ligament;⁷ periodontal tissue homeostasis might be maintained by controlling the

application of orthodontic force. In conclusion, we demonstrated that *FBN1* contributes to the maintenance of *POSTN* expression in HPDLFs after centrifugation treatment. Our results may help elucidate the mechanisms involved in the maintenance of periodontal tissue homeostasis.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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