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 β1 (TGF-β1) activation; TGF-β1 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-β1 rescued POSTN expression after centrifugation in HPDLFs upon knockdown of FBN1.
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. They induce molecules such as prostaglandin-endoperoxide synthase 2 and respond to mechanical stress. 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. Elastic system fibers in the periodontal ligament exist parallel to the long axis of the root. Elastic system fibers consist of microfibrils and elastin; 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. The major constituent protein of microfibrils is fibrillin-1 (FBN1), which is important for the storage of transforming growth factor β1 (TGF-β1) via latent TGF-β1-binding protein 1 (LTBP1); it also controls TGF-β1 activity in the matrix. FBN1 is presumed to regulate human periodontal ligament cell alignment.

The periodontal ligament also contains periostrin (POSTN). POSTN is an important mediator of the biomechanical properties of fibrous connective tissue. In a previous study, Postn-null mice showed irregular periodontal ligament, alveolar bone resorption, and external root resorption because of occlusal loading. 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. 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; 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.

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 (Biostera, Nuaille, France), 100 U/mL penicillin G (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL) and 100 μg/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. 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 μL/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′-ACCGGTTTTACCTGTATT-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 (5′ → 3′)                        |
|--------|------------------------------------------|
| FBN1   | 5′-GGAACTGTAAAGAAACACCAGA-3′             |
|        | 5′-GCGGAAATGGGACACATACAC-3′              |
| POSTN  | 5′-ATTGATGGAGTGCCCTGTG-3′                |
|        | 5′-CCTTGGTGACCTCTTCTTG-3′               |
| BMP2   | 5′-ATGATGCTGTTGAGAAGTG-3′                |
|        | 5′-GGCTAGTCACTGATGACTG-3′                |
| CTGF   | 5′-CAGCATGAGATGCTGCTG-3′                 |
|        | 5′-AACCAGGTTTGGTCCCTTG-3′               |
| GAPDH  | 5′-TGAAGGTCTGGAGTCAACGAT-3′              |
|        | 5′-TCACACCCATGACAAATG-3′                 |

**Western blotting**

Cells were lysed in sample buffer supplemented with protease and phosphatase inhibitor cocktails (both from Naclalai 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 knockdown; 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 90g did not cause different degrees of cytotoxicity, consistent with previous findings. 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).
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-β1 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-β1/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-β1 supplementation in HPDLFs upon knockdown of FBN1 during exposure to mechanical force.

To explore the possibility that TGF-β1 affects the expression of POSTN, the medium was treated with 0.001, 0.01, 0.1, 1.0, or 10 ng/ml TGF-β1. The expression of POSTN was significantly increased upon supplementation with ≥0.1 ng/ml TGF-β1 (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-β1 (p < 0.01) (Fig. 6B).
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. 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. 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, CTGF, and TGF-β1. 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; it also activates osteoclasts. The increased expression of BMP2—in response to tension force in the previous study—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. 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-β1 signaling pathway, was decreased after exposure to mechanical force, regardless of FBN1 knockdown (Fig. 5). A previous study showed that TGF-β1 was induced by intermittent compressive force on human periodontal ligament cells and the expression of POSTN was induced by TGF-β1, 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-β1 signaling pathway.
Furthermore, in a study by Panchamanon et al., TGF-β1 and POSTN expression levels increased when human periodontal ligament cells were subjected to a compression force of 1.0 g/cm². These results indicate an important role for FBN1 in the expression of POSTN under mechanical force via Smad2 phosphorylation and TGF-β1 signaling.

To further clarify the involvement of TGF-β1 in POSTN expression, we evaluated changes in POSTN expression upon TGF-β1 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 active TGF-β1 release from latency-associated propeptide. A previous study reported that FBN1 binds to TGF-β1 via latency-associated propeptide and LTBP1; TGF-β1 is activated upon release from LTBP1. FBN1 is important for the storage of TGF-β1. 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-β1 release from latency-associated propeptide. FBN1 presumably binds to integrins present on the cell membrane and participates in cell signaling; the integrin expression levels are increased with 24 hours of...
application of orthodontic force. In conclusion, we demonstrated that \textit{FBN1} contributes to the maintenance of \textit{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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![Figure 6](image-url) Changes in the expression of \textit{POSTN} upon treatment with TGF-\(\beta\)-1, evaluated by quantitative reverse transcription-polymerase chain reaction. (A) Expression of \textit{POSTN} was increased by supplementation with 0.1, 1.0, or 10 ng/ml TGF-\(\beta\)-1 (\(p < 0.01\)) (\(n = 3\)). (B) Expression of \textit{POSTN} in Si-90 g was rescued by supplementation with 0.1 ng/ml TGF-\(\beta\)-1 (\(p < 0.01\)) (\(n = 3\)). (A) **\(p < 0.01\), versus control. (B) **\(p < 0.01\), versus Scr-0 g and Si-0 g (without TGF-\(\beta\)-1 supplementation). \textit{FBN1}: fibrillin-1, \textit{GAPDH}: glyceraldehyde 3-phosphate dehydrogenase, HPDLFs: human periodontal ligament fibroblasts, \textit{POSTN}: periostin, Scr: scrambled siRNA, Si: siRNA against \textit{FBN1}.
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