

# Occlusal hypofunction mediates alveolar bone apposition *via* relative augmentation of TGF- $\beta$ signaling by decreased Asporin production in rats

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## Abstract

The periodontal ligament (PDL) maintains alveolar bone homeostasis against mastication force. Occlusal hypofunction, which lost mastication force, narrows the PDL by alveolar bone apposition, though the mechanisms remain unclear. Asporin is a secreted extracellular matrix protein and preferentially expressed in the PDL. Asporin binds directly with transforming growth factor-beta (TGF- $\beta$ ) and inhibits TGF- $\beta$ /Smad signaling, resulting in the inhibition of bone formation. In the present study, we hypothesized that Asporin is downregulated by occlusal hypofunction, which results in the increased alveolar bone apposition *via* relative upregulation of TGF- $\beta$  signaling. To clarify the hypothesis, we employed *in vivo* experiments using rats.

Crowns of lower right first molar in Wistar rats were removed to induce occlusal hypofunction in upper right first molar. Alveolar bone apposition was examined by histomorphometric analysis of Calcein/Xylenol-orange vital staining. Asporin, TGF- $\beta$ , and its signaling molecule, Smad3 expression were examined in mRNA and protein levels. Osteoblastic differentiation of the cell in the PDL under the occlusal hypofunction were observed using Bone sialoprotein (BSP), Osteocalcin and Osteopontin expression as osteoblastic differentiation marker.

We discovered that occlusal hypofunction increased the alveolar bone apposition and downregulated Asporin expression in PDL fibroblasts. Furthermore, occlusal hypofunction relative augmented TGF- $\beta$  signaling in PDL judged by phosphorylated (p)-Smad2/3 immunohistochemical staining, and upregulated osteoblastic differentiation in PDL.

In the present study, we firstly reported that occlusal hypofunction mediates alveolar bone apposition via relative augmentation of TGF- $\beta$  signaling by decrease of Asporin production.

## Introduction

The periodontal ligament (PDL) is a fibrous connective tissue that is interposed between the roots of the teeth and the alveolar bone. The PDL plays an important role in supporting and preserving the tooth against external force including mastication force [1]. Optimal mechanical stimuli are essential to maintain healthy alveolar bone by inducing the formation and remodeling of the alveolar bone [2,3]. However, in the situation of loss of the tooth, antagonizing tooth lose the occlusion, (ie occlusal hypofunction), clinically exhibits extrusion of the tooth due to disuse atrophy of PDL [4].

The effects of occlusal hypofunction on the periodontal tissue have been studied [2-8]. These studies indicated that occlusal hypofunction results in atrophic changes of the PDL, narrowing of periodontal width [5-7], and increased alveolar bone formation [2,3,8]. Taken together, occlusal hypofunction narrows the PDL by alveolar bone apposition [2,3]. At this point, the molecules involved in the alveolar bone apposition by occlusal hypofunction are unknown.

TGF- $\beta$  superfamily is known to play an important role in osteogenic cell differentiation and consequently bone formation in PDL [9,10]. Regarding the antagonizing molecule for TGF- $\beta$ , Asporin is one of the well-known inhibitor for TGF- $\beta$  signaling. Asporin binds directly with TGF- $\beta$  and inhibits TGF- $\beta$ /Smad signaling, which is resulting in the inhibition of bone formation, and is thought to be main regulator of alveolar bone homeostasis [11,12]. Asporin is a secreted extracellular

matrix protein that contains 380 amino acids. It is preferentially expressed in the PDL and encodes a novel small leucine-rich repeat proteoglycan protein [13].

In the present study, we hypothesized that Asporin is downregulated by occlusal hypofunction, which results in the increase in alveolar bone apposition *via* upregulation of TGF- $\beta$  signaling. To clarify the hypothesis, we employed *in vivo* experiments.

## Materials and methods

### Animals

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Tsurumi University School of Dental Medicine (approved numbers; 25A062,

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**Key words:** asporin, occlusal hypofunction, tgf- $\beta$ , smad signaling, alveolar bone apposition

**Received:** December 12, 2016; **Accepted:** December 22, 2016; **Published:** December 26, 2016

26A067, 27A029, and 28A024). 12-wk-old male Wistar rats (CLEA Japan Inc., Tokyo, Japan) were used (n=48). The rats were housed 12 h light, 12 h dark cycle. They fed standard rat chow and water ad libitum.

### Experimental design

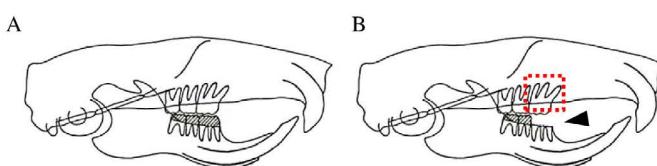
Crowns of lower right first molar were removed to induce occlusal hypofunction [7]. Briefly, the crowns of the lower right molars were cut using small pliers under a combination anesthesia (Medetomidine/Midazolam/Butorphanol:0.15/2.0/2.5 mg/kg body weight, respectively) (Figure 1). Right upper jaws were used as experimental side, and the left were used as control side. Rats were sacrificed by overdose pentobarbital sodium anesthesia, and then they were fixed with perfusion of 10% Formalin Neutral Buffer Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at day-1, 3, 7 and 14 after crown removal.

### Calcein/Xylenol-orange labeling

In some rats, fluorescent dyes, calcein (at day-0) (1.6 mg/kg body weight Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and xylenol orange (at day-13) (50 mg/kg body weight Dojindo Molecular Technologies) were subcutaneously injected to evaluate the bone formation rate. On day-14, the rats were sacrificed and prepared undecalcified frozen sections [14].

### Preparation of unfixed undecalcified frozen serial sections

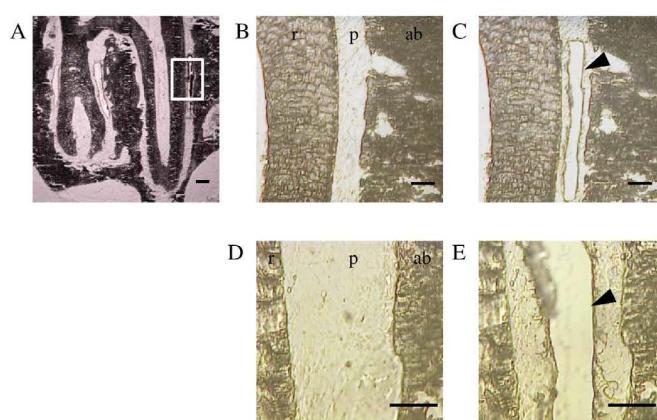
Briefly, the upper jaws were excised out and rapidly immersed in liquid nitrogen, then embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Frozen blocks were sectioned in a



**Figure 1.** Illustration of occlusal hypofunction model.

(A) Control rats with normal occlusion.

(B) Occlusal hypofunction in upper first molar was induced by removal of crowns of lower right first molar. Arrowhead indicates the removed lower first molar. Red box indicates the observed area.



**Figure 2.** LCM of the PDL in the undecalcified frozen sections.

(A) The frontal sections of distobuccal and distopalatal roots of the upper first molar. White box indicates the region of LCM, which are shown in the following enlarged image B.

(B, D) PDL before LCM. ab: alveolar bone, p: PDL, r: root of tooth.

(C, E) PDL after LCM. Arrowheads indicate the excised area of the PDL.

Scale bar: 300 μm (A), 100 μm (B, C, D, and E)

cryostat (Leica Microsystems, Wetzlar, Germany) by using a super-hard tungsten steel knife (Meiwa Shoji Ltd., Tokyo, Japan) [15]. Frozen blocks were cut frontally. 7 μm-thick sections were individually collected using adhesive tape, and then used for the measurement of alveolar bone apposition and RNA extraction.

### Histomorphometry of alveolar bone apposition

The Calcein/Xylenol-orange fluorescent labels in the undecalcified frozen sections were examined under a fluorescent microscope (Keyence BZ-9000 Osaka, Japan). Bone apposition, which is identified by two fluorescent dyes, were measured both in frontal and cross sections. The distance between two fluorescent dyes was measured at the depth of 7-800 μm from interradicular septum in frontal sections. The distance was also measured at buccal and palatal side of distobuccal and distopalatal roots in the cross sections. 4 regions of alveolar bone in each section, three sections in each group, total 12 regions were measured using Image J [16].

### RNA extraction

1/3 of the central portion of the PDL (360 μm<sup>2</sup>) in the distobuccal and distopalatal roots were microdissected from the sections by using laser capture microdissection (LCM) PALM MicroBeam system (PALM Microlaser Technologies AG, Bernried, Germany) (Figure 2) [7]. After LCM, total RNA was extracted using an RNeasy micro kit (Qiagen GmbH, Hilden, Germany) with on-column genomic DNA digestion.

### Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Isolated RNA was reverse transcribed with iScript cDNA-Supermix (Bio-Rad, Hercules, CA), and cDNA stock was diluted (2 $\times$ ) with Tris/ethylenediaminetetraacetic acid (EDTA) buffer. Real-time RT-PCR was performed with SsoFast EvaGreen-Supermix (Bio-Rad). The PCR primers used in the experiments were described in Table 1. Fold changes of gene of interests were calculated with  $\Delta\Delta Ct$  method using  $\beta$ -actin as reference gene.

### Histological examination

Perfusion fixed upper jaws, including the molars, were excised, further fixed with 10% Formalin Neutral Buffer Solution (Wako) overnight, and decalcified with 10% EDTA in PBS for 4 weeks at 4°C. Then the specimen were dehydrated in an ascending ethanol series and embedded in paraffin. Serial paraffin sections (5 μm-thick) of periodontal tissues of the distal root of the upper first molars were prepared, and they were used for hematoxylin and eosin staining or immunohistochemical staining.

### Immunohistochemistry

The sections were deparaffinized, and inactivated endogenous peroxidase activity for 30min (3% hydrogen peroxide). Then they were preincubated in 2.5% normal horse serum for 1h (Vector Laboratories, Burlingame, CA), and were subsequently incubated with primary antibodies overnight. The primary antibodies we used were anti-Asporin antibody (Acris Antibodies, Inc. Herford Germany), anti-TGF- $\beta$  antibody (R&D systems, Inc. Minneapolis, MN), anti-phosphorylated (p)-Smad2/3 antibody (Santa Cruz Biotechnology, Inc. Dallas, TX), anti-Osteocalcin antibody (R&D systems, Inc. Minneapolis, MN) and anti-Osteopontin antibody (Proteintech Group, Rosemont, IL). Antigen retrieval procedure were performed in the sections for p-Smad2/3 and Osteopontin staining with L.A.B Solution (Polysciences, Inc. Warrington, PA) prior to the blocking step.

**Table 1.** Primers used for Real-time RT-PCR.

Primer	Accession number		Sequence(5'-3')
<i>Asporin</i>	NM_001014008	Forward	TCCCGAAAGGACTACCACCA
		Reverse	TGTTTCCAAGACCCAGCCTC
<i>Smad3</i>	NM_013095	Forward	CAGGGCTTGAGGCTGTCTACC
		Reverse	GTGCTGGTCAGTGTCTCCT
<i>Bone sialoprotein</i>	NM_012587	Forward	TTCTGAACGGGTTTCAGCAGAC
		Reverse	CTGGTGGTAGTAATAATCCT
<i>Osteocalcin</i>	NM_013414	Forward	AAGCCCAGCGACTCTGAGTC
		Reverse	GCTCCAAGTCCATTGTGAGG
<i>Osteopontin</i>	NM_012881	Forward	GAAACTCTGGACTTTGACC
		Reverse	GCCACTTGGCTGAAGCCTG
$\beta$ -actin	NM_031144	Forward	AGCCATGTACGTAGCCATCC
		Reverse	TCTCAGCTGTGGTGGTGAAG

After being rinsed, the sections were incubated with the peroxidase micropolymer-conjugated secondary antibody (Vector Laboratories). To visualize immunoreactivity, the sections were flooded with a diaminobenzidine solution (Vector Laboratories). Counterstaining was carried out with hematoxylin (Merck Japan, Tokyo, Japan). Sections were mounted with Entellan (Merck) and observed with a microscope. Intensity levels were measured using Image J (colour deconvolution) [16].

### Statistical analysis

The results were presented as the mean  $\pm$  standard deviation. The Student's t-test was used for comparisons between the control and each experimental group, and the one-way analysis of variance (ANOVA) and Dunnett analysis was used for multiple comparisons using SPSS@ 11.0 (SPSS Inc., Chicago, IL).  $p<0.05$  and  $p<0.01$  were considered to be statistically significant.

## Results

### Occlusal hypofunction increased the alveolar bone apposition

Histological observation demonstrated that the PDL width in the occlusal hypofunction side (experimental side) was narrowed as compared to that in control side. A considerable amount of new bone was formed on the old bone in the occlusal hypofunction side (Figure 3B and 3C), which resulted in the decrease in PDL width as compared to these in control sections (Figure 3B and 3E). This was observed not only in the frontal section but also in the cross section (Figure 3C and 3F). These histological results confirmed by the measurement of PDL width (Figure 3G and 3I) and alveolar bone width (Figure 3H and 3J). The periodontal width in the occlusal hypofunction side was significantly smaller than that in the control side.

To further clarify the mechanism of the decrease in PDL width, alveolar bone apposition during 2 weeks after the experiment was examined in the several areas in the PDL (Figure 4A). Fluorescent labeling clearly demonstrated that augmented alveolar bone apposition was occurred during the experimental period. A thin layer of bone apposition occurred in the control side (Figure 4B and 4C). On the other hand, the bone apposition was enhanced and a considerable thick layer of bone was formed at 14 day in the experimental side (Figure 4D and 4E).

To evaluate the difference in the amount of bone apposition between experimental and control sides, measurement of the distance between two fluorescent dyes was performed. Occlusal hypofunction induced a significant thick layer of the alveolar bone, as compared with that in the control at all the points of frontal sections (Figure 4F). The 14day/control ratio of alveolar bone apposition were 1 to 6-folds in frontal sections, showing increased bone apposition at whole measured points (Figure 4G). These results indicate that the occlusal hypofunction increases the alveolar bone apposition.

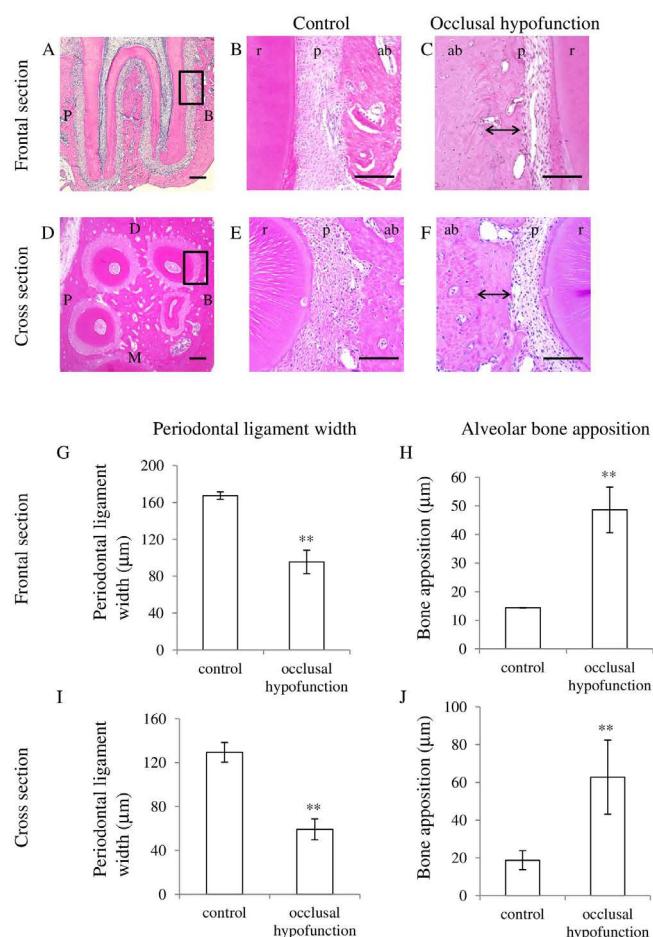
### Occlusal hypofunction downregulated asporin expression

It was reported that Asporin negatively regulates osteoblastic bone formation [11], therefore we examined the Asporin expression in the PDL under occlusal hypofunction. The expression of Asporin was significantly downregulated after 1, 3, and 7 day in the PDL in the experimental group as compared to that in the control group (Figure 5A-5C).

Then we immunohistochemically examined the Asporin expression in the PDL. In the control group, Asporin was located in the most of fibroblasts in the PDL. However, consistent with the results of real-time RT-PCR, the number of Asporin positive fibroblasts was gradually decreased with experimental periods (Figure 5D-5I). Significantly small number of Asporin positive fibroblasts was observed at experimental group (Figure 5C). These results suggest that the occlusal hypofunction downregulates Asporin expression in the PDL.

### Occlusal hypofunction upregulated TGF- $\beta$ signaling

Since Asporin binds directly to TGF- $\beta$  and inhibits TGF- $\beta$  signaling [11,12,17], expression of TGF- $\beta$  in the PDL was immunohistochemically examined in the PDL. TGF- $\beta$  was located in the most of fibroblasts in the PDL irrespective of control and experiment (Figure 6A). Considering the reduction of Asporin in the PDL, TGF- $\beta$  has chance to escape from Asporin and works as stimulating factor for osteoblastic differentiation. The localization in the osteoblast in experimental group was somewhat different from that in control group. TGF- $\beta$  was located in the osteoblasts in the PDL in control group. In the experimental group it was also located in the osteoblasts at day-1 but its localization in osteoblast was gradually reduced in the PDL on day-7 and 14.



**Figure 3.** Hematoxylin and eosin staining of PDL.

(A) The frontal section of the upper first molar in control. Higher magnified image of the area indicated by black box is shown in Fig.3B.

(B) Higher magnified image of frontal section of control.

(C) Higher magnified image of frontal section of day-21 after occlusal hypofunction. Corresponding area indicated by the black box in Fig.3A is shown. Black double arrow indicates the osteoid which can be recognized by the staining properties.

(D) The cross section of the upper first molar in control. Higher magnified image of the area indicated by black box is shown in Fig.3D.

(E) Higher magnified image of cross section of control.

(F) Higher magnified image of cross section of day-21 after occlusal hypofunction. Corresponding area indicated by the black box in Fig.3D is shown. Black double arrow indicates osteoid.

B: buccal side, P: palatal side, M: mesial side, D: distal side. ab: alveolar bone, p : PDL, r: tooth root. Scale bar: 300  $\mu\text{m}$  (A, D), 100  $\mu\text{m}$  (B, C, E, and F).

(G) The width of the PDL in frontal section.

(H) The width of osteoid in frontal section.

(I) The width of the PDL in cross section.

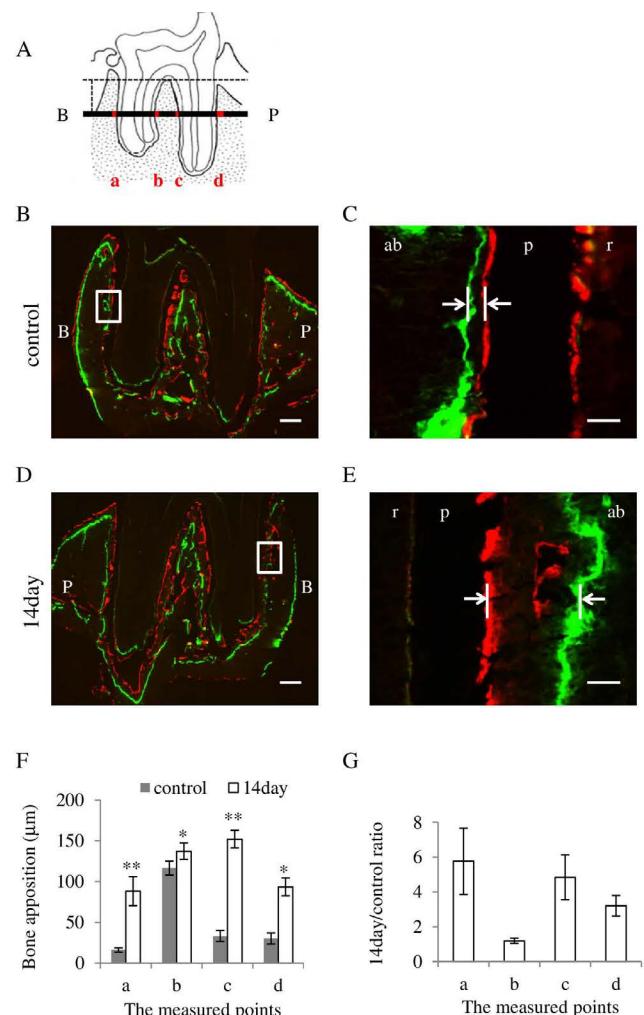
(J) The width of osteoid in cross section.

\*\*\*:  $p<0.01$  between the samples. n=3.

Then we examined the major signaling molecule for TGF- $\beta$  superfamily signaling, Smad3 [9,18]. Real-time RT-PCR revealed that Smad3 was significantly upregulated after day-7 of the occlusal hypofunction (Figure 6B). Immunohistochemistry demonstrated that p-Smad2/3 was found in several fibroblasts in the control group. p-Smad2/3 was found in considerable number of fibroblast in the PDL in experimental group at day-7 (Figure 6C). These results suggest that the occlusal hypofunction relatively augments TGF- $\beta$  signaling in PDL.

### Occlusal hypofunction upregulated osteoblastic differentiation

It is well known that TGF- $\beta$  superfamily signaling promotes



**Figure 4.** Occlusal hypofunction increased the alveolar bone apposition.

(A) The measured points of distobuccal and distopalatal roots in frontal section are illustrated. 4 regions marked as red, a: buccal side of buccal root, b: palatal side of buccal root, c: buccal side of palatal root, and d: palatal side of palatal root, were measured.

(B) Representative photographs of the fluorescent labeling in frontal section of control. Higher magnified image of the area indicated by white box is shown in Figure 4C. Green indicates Calcein label, and red indicates Xylenol-orange label.

(C) Higher magnified image of frontal section of control. The distance of two fluorescent dye indicated by white arrow were measured.

(D) Representative photographs of the fluorescent labeling in frontal section of 14 day after occlusal hypofunction. Higher magnified image of the area indicated by white box is shown in Figure 4E. Green indicates Calcein label, and red indicates Xylenol-orange label.

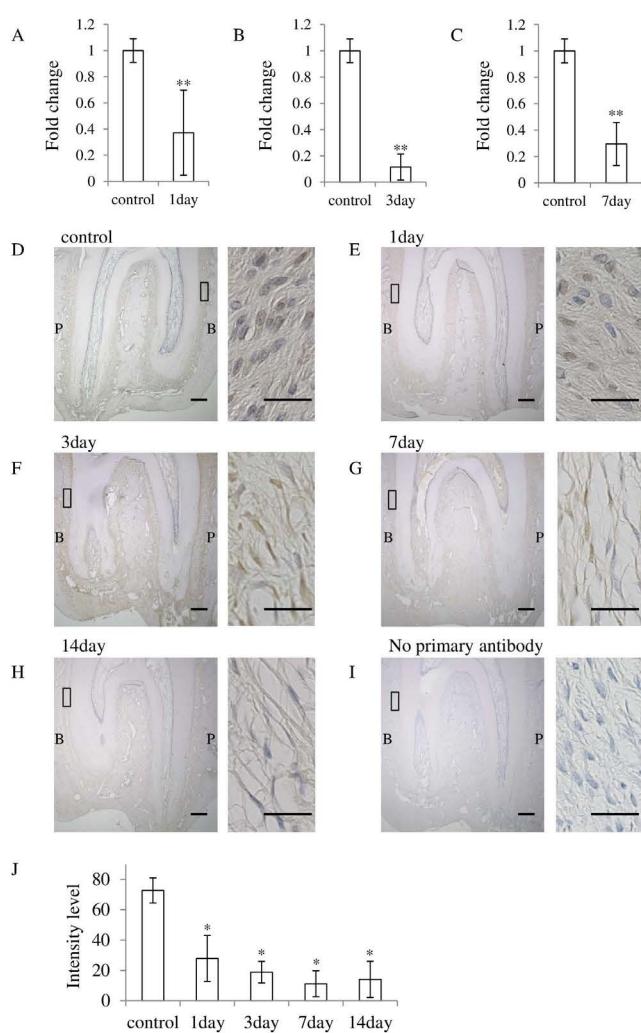
(E) Higher magnified image of frontal section of 14day after occlusal hypofunction. The distance of two fluorescent dye indicated by white arrow were measured.

(F) The distance of two fluorescent dye in frontal sections.

(G) The 14day/control ratio of alveolar bone apposition in frontal sections.

B: buccal side, P: palatal side. ab: alveolar bone, p: PDL, r: tooth root. Scale bar: 300  $\mu\text{m}$  (B, D), 50  $\mu\text{m}$  (C, E). \*:  $p<0.05$ , \*\*:  $p<0.01$  between the samples. n=3.

osteoblastic differentiation [9,19], therefore we examined osteoblastic differentiation marker expression in the PDL under occlusal hypofunction. Osteopontin mRNA was significantly upregulated after occlusal hypofunction (Figure 7C), though bone sialoprotein (BSP) and Osteocalcin exhibited tendency toward upregulation (Figure 7A and 7B). Then localization of the Osteocalcin and Osteopontin in the PDL was immunohistochemically examined. Osteocalcin and Osteopontin were detected in the osteoblast on the bone surface and fibroblast in the PDL even in control. Osteocalcin and Osteopontin were observed



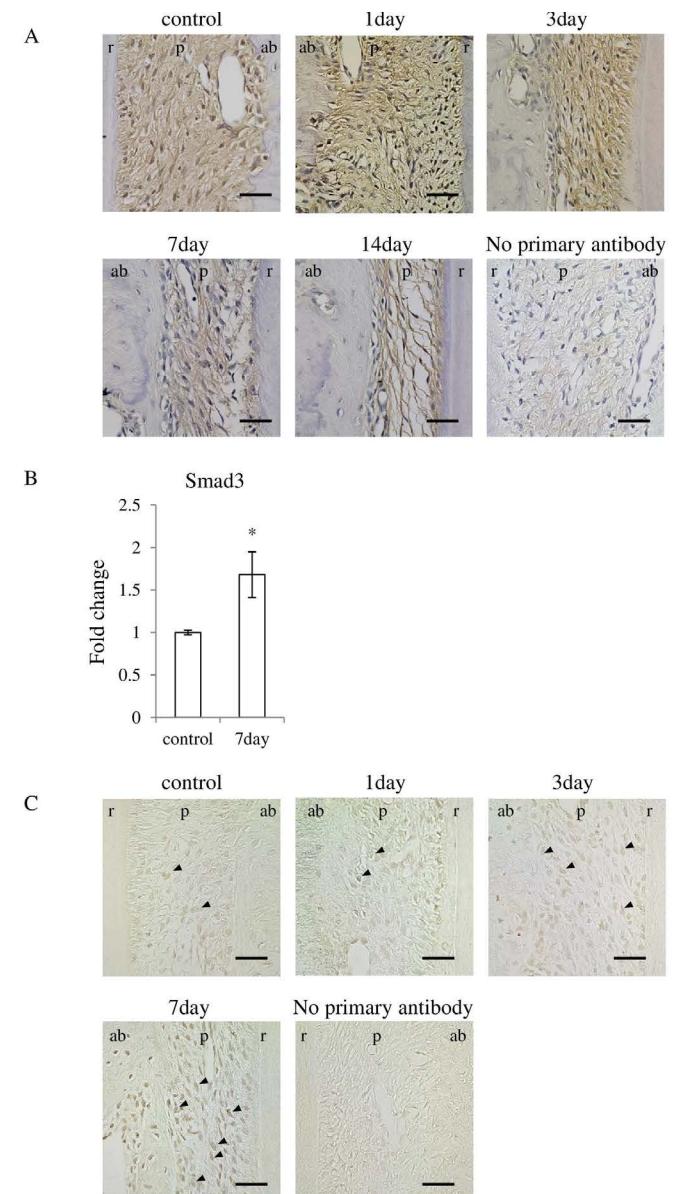
**Figure 5.** Occlusal hypofunction downregulated Asporin expression. Real-time RT-PCR analysis for the Asporin at day-1 (A), day-3 (B), and day-7(C) mRNA expression in PDL tissue. \*\*:  $p<0.01$  between the samples. n=5. (D-I) Immunohistochemical staining of Asporin in PDL tissue. n=3. Whole image of upper molar in lower magnification are shown in each left side (Scale bar : 300  $\mu$ m). The right side images are higher magnified images of the boxed area in the left side images. (1/3 of the central portion of the PDL tissue) (Scale bar : 25  $\mu$ m). Expression of the Asporin protein in PDL of control (D), at day-1 (E), at day-3 (F), at day-7 (G), at day-14 (H), no primary antibody (I). B: buccal side, P: palatal side. (J) The Intensity of the immuno-reactivity for Asporin. Mean value of 3 sections are shown. \*:  $p<0.05$  versus control.

in the most of fibroblast in the PDL and osteoblast on the bone surface expression at day-7 (Figure 7D and 7E). These results suggest that occlusal hypofunction upregulates osteoblastic differentiation in PDL.

## Discussion

In the present study, we discovered that the occlusal hypofunction induce alveolar bone apposition through relative augmentation of TGF- $\beta$  signaling by decrease of Asporin production. It has been well-known clinically and in basic science that occlusal hypofunction caused the narrowing the width of PDL by alveolar bone apposition [2,3], though the mechanism has remained unclear.

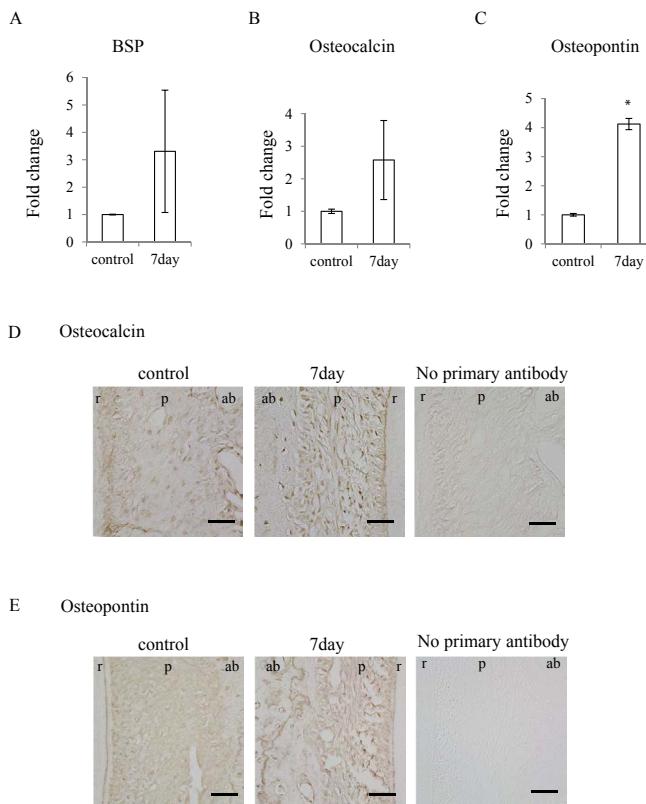
Consistent with previous reports, the middle of the distobuccal and distopalatal roots were examined in this study. Since physiological distal movement of the molars usually takes place in the 12-13 week old



**Figure 6.** Occlusal hypofunction upregulated TGF- $\beta$  signaling. (A) Immunohistochemical staining for TGF- $\beta$  in PDL tissue. Representative photographs are shown. Scale bar: 50  $\mu$ m. n=3. (B) Real-time RT-PCR analysis for Smad3 mRNA expression in PDL tissue. \*:  $p<0.05$  between the samples. n=3. (C) Immunohistochemical staining for p-Smad2/3 in PDL tissue. Arrowhead indicates p-Smad2/3 positive cells. Scale bar: 50  $\mu$ m. n=3. ab: alveolar bone, p : PDL, r: tooth root.

rat [20], bone apposition was observed on the alveolar bone at mesial side of the roots. Therefore, we mainly observed the PDL on the buccal and palatal side of roots to eliminate the effect of physiological distal movement of molars.

Occlusal hypofunction induced a significant amount of bone apposition at the most of the alveolar bone surface on buccal and palatal side, and consequently the PDL width was about a half of the control. Choi, *et al.* reported that the width of the PDL space in the rats at day-7, narrowed to less than half as compared to the control, and after that width of the PDL space was stably narrow [7]. Levy, *et al.* reported that alveolar bone formation was increased during 15



**Figure 7.** Occlusal hypofunction upregulated osteoblastic differentiation markers. Real-time RT-PCR analysis for the BSP (A), Osteocalcin (B), and Osteopontin (C) mRNA expression in PDL tissue. \*:  $p < 0.05$  versus control. n=3. Immunohistochemical staining for Osteocalcin (D) and Osteopontin (E), in PDL tissue. The photographs under the same color development period after DAB step are shown. Scale bar: 50  $\mu$ m. ab: alveolar bone, p: PDL, r: tooth root. n=3.

days after occlusal hypofunction, and after that bone formation was slightly increased [3]. The results in this study indicate that occlusal hypofunction considerably increases the alveolar bone apposition, as compared with those in control group. This also causes the narrowing of the width of PDL.

TGF- $\beta$  expression and localization was observed in the PDL throughout the experimental period in the control and experimental groups, and the results were consistent with previous reports about TGF- $\beta$  in the PDL [9,21,22]. TGF- $\beta$  superfamily is known to play an important role in osteogenic cell differentiation and consequently bone formation in the PDL [9,19].

In this study the localization in the PDL in experimental group was somewhat different from that in control group. TGF- $\beta$  was located in the fibroblasts and osteoblasts in the PDL in control group. On the other hand, in the experimental group it was located in the fibroblast and osteoblast at day-1 but its localization in osteoblast on the bone surface was gradually reduced in the PDL. Previous studies reported that TGF- $\beta$  regulated osteoblastic differentiation not only positively but also negatively in the PDL cells [10,23]. The expression of TGF- $\beta$  mRNA continued to increase at confluent cell stage and decreased at mineralization stage in PDL cells [10]. In this context, the reduction of TGF- $\beta$  in the osteoblast at the alveolar bone surface in experimental group might indicate that the osteoblast in this area has already differentiated into osteoblast and engaged in mineralization of bone in the rapid bone formation [24] in response to occlusal hypofunction.

We discovered that Asporin was downregulated in the PDL under occlusal hypofunction. Asporin is expressed predominantly in the PDL and negatively regulates the cytodifferentiation of PDL cells to osteoblast [11,13] and mineralization. It is known that Asporin binds directly with TGF- $\beta$  and inhibits TGF- $\beta$ /Smad signaling, resulting in the inhibition of bone formation [11,12]. Furthermore Asporin inhibits TGF- $\beta$  signaling without affecting TGF- $\beta$  expression [17]. Asporin overexpression suppressed the differentiation of rat bone marrow stromal cells into osteoblasts [25]. Awata, *et al.* reported that mouse embryonic fibroblasts from Asporin knockout mice were cultured in mineralization inducing medium, calcified nodule formation was more enhanced [26]. Orthodontic compressive force induces the expression and release of Asporin, which inhibits bone formation on the compressed zone of the PDL during orthodontic tooth movement [27]. Taken together, occlusal hypofunction reduces the expression of Asporin, which is advantageous for the cytodifferentiation PDL cells to osteoblasts through TGF- $\beta$  signaling in PDL. Consequently this causes the rapid bone formation in the PDL. However, it still remains unclear that the downregulatory mechanism for Asporin expression by occlusal hypofunction. Therefore, further experiments are necessary to elucidate the regulatory mechanism of Asporin expression under occlusal hypofunction.

The expression of Smad3 was significantly upregulated in the PDL and p-smad2/3 positive cell was evidently increased in the PDL under occlusal hypofunction. Smad3 is known as an intracellular signaling molecule for TGF- $\beta$  signaling [9,18,19]. Positive relation between Smad3 and osteoblastic differentiation was also reported [28]. Consisted with the reports, occlusal hypofunction induced osteoblastic differentiation in our experiments.

This is supported by the results that the expressions of BSP, Osteocalcin, and Osteopontin were upregulated in the PDL after occlusal hypofunction. BSP, Osteocalcin, and Osteopontin are osteoblast-specific proteins and have been used for osteoblastic differentiation marker [29-37]. Occlusal hypofunction evidently stimulate the differentiation of PDL cells to osteoblasts in the PDL. It is interesting in this study that BSP, Osteocalcin and Osteopontin were located not only in osteoblast at alveolar bone surface, but also in the most of fibroblast, as compared to that in the control. These might indicate that the fibroblast in the PDL also contribute to the bone formation in the PDL under hypofunction.

As to the source of osteoblastic differentiated cells in PDL, we presumed that one of them are mesenchymal stem cell, because mesenchymal stem cells are present in the PDL and are able to differentiate into multiple types of cells by responding to the mechanical forces [38]. Another possibility is direct osteoblastic differentiation of PDL cells because many researchers reported that PDL cells can differentiate into mature osteoblasts by suitable stimulation [39-42].

In the present study, we demonstrated that occlusal hypofunction induced rapid alveolar bone apposition through advantage relative augmentation of TGF- $\beta$  signaling by decrease of Asporin production. Attenuation of Asporin expression would be therapeutic target for alveolar bone augmentation in periodontal disease on pharmacological retention after orthodontic treatment.

## Disclosure

The authors have no conflicts of interest.

## Acknowledgement

This research was supported in part by Grants-in-Aid for Scientific

Research from the Japan Society for the Promotion of Science (23689081, 15K11376, 15K11356, and 23593052).

## Contributions

Kanako Itohiya: study design, data collection, analysis, and writing. Hiroyuki Kanzaki: study design instruction, editing assistance. Misao Ishikawa: animal experiments design instruction. Satoshi Wada: immunohistochemical staining technique instruction. Yutaka Miyamoto: LCM technique instruction. Tsuyoshi Narimiya: data collection. Yoshiki Nakamura: editing assistance.

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