

## Periostin regulates integrin expression in gingival epithelial cells

Reika Hirata <sup>a</sup>, Tomoyuki Iwata <sup>a\*</sup>, Tsuyoshi Fujita <sup>a</sup>, Takayoshi Nagahara <sup>a</sup>, Shinji Matsuda <sup>a</sup>, Shinya Sasaki <sup>a</sup>, Yuri Taniguchi <sup>a</sup>, Yuta Hamamoto <sup>a</sup>, Kazuhisa Ouhara <sup>a</sup>, Yasusei Kudo <sup>b</sup>, Hidemi Kurihara <sup>a</sup>, Noriyoshi Mizuno <sup>a</sup>

<sup>a</sup> Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima 734-8553, Japan

<sup>b</sup> Department of Oral Bioscience, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770-8504, Japan

\* Corresponding author:

Tomoyuki Iwata

Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical and Health Sciences

1-2-3, Kasumi. Minami-ku, Hiroshima 734-8553, Japan

Phone: +81-82-257-5663; Fax: +81-82-257-5664

Email: [iwatat@hiroshima-u.ac.jp](mailto:iwatat@hiroshima-u.ac.jp) 1

---

<sup>1</sup>**Abbreviations:** HDACs, histone deacetylases; HGEs, human gingival epithelial cells; HGFs, human gingival fibroblasts; hMSCs, human mesenchymal stem cells; HPL, human periodontal ligament; MSCs, mesenchymal stem cells; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline containing 0.01% Tween 20; TBS, Tris-buffered saline

## **Abstract**

**Objective:** Human gingival epithelial cells (HGEs) function as a mechanical barrier against invasion by pathogenic organisms through epithelial cell–cell junction complexes, which are complex components of integrin. Integrins play an important role in the protective functions of HGEs. Human periodontal ligament (HPL) cells regulate periodontal homeostasis. However, periodontitis results in the loss of HPL cells. Therefore, as replenishment, HPL cells or mesenchymal stem cells (MSCs) can be transplanted. Herein, HPL cells and MSCs were used to elucidate the regulatory mechanisms of HGEs, assuming periodontal tissue homeostasis.

**Methods:** Human gingival fibroblasts (HGFs), HGEs, HPL cells, and MSCs were cultured, and the conditioned medium was collected. With or without silencing periostin mRNA, HGEs were cultured under normal conditions or in a conditioned medium. Integrin and periostin mRNA expression was determined using real-time polymerase chain reaction. Integrin protein expression was analyzed using flow cytometry, and periostin protein expression was determined via western blotting.

**Results:** The conditioned medium affected integrin expression in HGEs. Higher expression of periostin was observed in MSCs and HPL cells than in HGFs. The conditioned medium that contained periostin protein regulated integrin expression in

HGECs. After silencing periostin in MSCs and HPL cells, periostin protein was not detected in the conditioned medium, and integrin expression in HGECs remained unaffected.

**Conclusions:** Integrins in HGECs are regulated by periostin secreted from HPL cells and MSCs. This result suggests that periostin maintains gingival cell adhesion and regulates bacterial invasion/infection. Therefore, the functional regulation of periostin-secreting cells is important in preventing periodontitis.

**Keywords:** gingival epithelial cells, mesenchymal stem cells, human periodontal ligament cells, periostin, integrin beta

## 1. Introduction

Human gingival epithelial cells (HGEs) function as a mechanically protective barrier against invasion by pathogenic organisms through epithelial cell–cell junction complexes, such as gap and tight junctions [1, 2]. The junctional epithelium participates in the attachment of epithelial cells to the tooth surface to separate periodontal tissue from the external environment [3, 4]. Therefore, the unique location of the junctional epithelium at the hard-soft tissue interface is key to the initiation and progression of periodontal disease [2].

Integrins are heterodimeric transmembrane proteins that serve as receptors for extracellular matrix components and cell surface proteins [5, 6]. Integrin  $\beta 4$ , unique among integrin  $\beta$  subunits because of its markedly larger cytoplasmic domain, may link keratin filaments to these structures [7, 8]. Moreover, integrin  $\beta 4$  is expressed in keratinocytes from the human gingiva [9]. The suprabasal distribution of  $\alpha 6$  has been proposed owing to its association with  $\beta 1$  in cell-to-cell contacts, whereas the polarized basal expression is associated with  $\beta 4$  in cell-to-extracellular matrix contacts [10]. Integrin  $\beta 1$  and integrin  $\beta 4$  are involved in epidermal growth and differentiation [11]. Furthermore, integrin  $\beta 1$  can be paired with almost all integrin  $\alpha$  subunits [12].

Periostin was originally identified as an 811-amino acid protein secreted by murine osteoblasts and is structurally homologous to the insect axonal guidance protein fasciclin (FasI) [13]. Originally termed osteoblast-specific factor-2, it was renamed as periostin because of its localized expression in the periosteum and periodontal ligament [14]. In humans, the periostin gene is located on chromosome 13 at map position 13q13.3, and the protein is 835 amino acids long. Periostin is a disulfide-linked 90-kDa heparin-binding N terminus-glycosylated protein containing four tandem FasI domains [15]. Periostin is found in the bones [16], skin [17], and periodontal ligament [18]. Furthermore, periostin expression is prominent under fibrotic conditions, including subepithelial fibrosis in bone marrow fibrosis [19]. It supports MC3T3-E1 cell attachment and spreading in addition to  $\alpha$ V $\beta$ 3 and  $\alpha$ V $\beta$ 5 integrin-dependent cell adhesion and motility [20, 21].

The periodontal ligament is a connective tissue between two mineralized tissues: the alveolar bone and cementum. Human periodontal ligament (HPL) cells constitute a heterogeneous cell population containing fibroblasts and progenitor cells that can differentiate into osteoblasts and cementoblasts and have osteoblast-like properties, such as high levels of alkaline phosphatase activity and production of bone-related proteins [22, 23]. Therefore, HPL cells play an important role in maintaining homeostasis in

healthy and inflamed periodontal tissue [24, 25]. However, alveolar bone resorption in severe periodontitis and loss of the periodontal ligament and progenitor cells associated with the absorbed bone are observed. Therefore, transplantation or regeneration is considered to reverse the loss of periodontal ligament [24, 25].

Bone marrow mesenchymal stem cells (MSCs), also known as adherent bone marrow cells or stromal cells, can differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts *in vitro* and *in vivo* [26, 27]. Therefore, similar to HPL cells, MSCs are also candidates for transplantation for periodontal tissue regeneration [28] and maintain periodontal tissue homeostasis.

Since these cells synthesize specific proteins and release them into the conditioned medium in cell culture, they can also regulate HGECs function as a mechanically protective barrier. Therefore, in this study, to elucidate the regulatory role of HGECs in periodontal tissue homeostasis, we focused on the conditioned medium from HPL cells and MSCs and investigated the HGEC function, focusing on integrin  $\beta 1$  and integrin  $\beta 4$ .

## **2. Materials and Methods**

### *2.1. Isolation and culture of HGECs, HPL cells, and MSCs*

Periodontally healthy gingival tissues, surgically dissected through wisdom tooth extraction with no pericoronitis around the extracted wisdom tooth, which are usually discarded, were collected after obtaining informed consent from the patients. HGEs were isolated from the gingivae of three volunteers, as previously described [1]. HGEs were cultured in MCDB153 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 30 µg/mL bovine pituitary extract, 0.1 ng/mL human epidermal growth factor, 5 µg/mL insulin, 0.5 µg/mL hydrocortisone, and 50 µg/mL gentamycin (Kurabo, Osaka, Japan). To elucidate the effect of periostin, HGEs were cultured with 500 ng/mL recombinant Human OSF-2/Periostin (R&D Systems, Minneapolis, MN, USA) for 48 h.

HPL cells were obtained separately from the explant culture of healthy periodontal ligaments from the mid-root of premolars extracted from four patients undergoing orthodontic treatment. Informed consent was obtained, according to a protocol approved by the Ethics Committee of the Hiroshima University Faculty of Dentistry (Hiroshima, Japan; approval no. E-D47-4). Periodontal ligament tissue was cut into small pieces and plated in 35-mm culture dishes (Corning Inc., Corning, NY, USA) with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Cytiva, Marlborough, MA, USA), 100 units/mL of penicillin (Sigma-Aldrich), and 100 µg/mL of streptomycin (Sigma-Aldrich). When the HPL cells formed a 70–80%

confluent monolayer, they were harvested and seeded on a 100-mm culture dish (Corning Inc.) with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Cytiva), 100 units/mL of penicillin (Sigma-Aldrich), and 100 µg/mL of streptomycin (Sigma-Aldrich). HPL cells at the sixth passage were used in the experiments.

All human mesenchymal stem cells (hMSCs) were provided by the RIKEN BioResource Center (Tsukuba, Japan), with approval from the Ethics Committee of Hiroshima University Faculty of Dentistry (Hiroshima, Japan; Approval number: E-422-2). The cells were plated in 35-mm plates and cultured with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Cytiva), 100 units/mL of penicillin (Sigma-Aldrich), and 100 µg/mL of streptomycin (Sigma-Aldrich). Passages were performed when the cells became 70–80% confluent. hMSCs in the fourth passage were used in the experiments.

Human gingival fibroblasts (HGFs) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in a 100-mm culture dish (Corning Inc.) with Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Cytiva), 100 units/mL of penicillin (Sigma-Aldrich), and 100 µg/mL of streptomycin (Sigma-Aldrich).



### *2.2.Regulation of periostin expression*

hMSCs and HPL cells were seeded at a density of  $5 \times 10^3$  cells/cm<sup>2</sup> in six-well plates (Corning Inc.) and cultured until 70–80% confluence. Negative control siRNA (siControl) or human periostin siRNA (siPeriostin) was transfected into hMSCs and HPL cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), as previously described [29]. The final concentration of the siRNAs was 135 nM. Silencer Select Negative Control #1 siRNA (Assay. no. 4390843) and periostin siRNA (Assay. no. s20887) were purchased from Ambion (Grand Island, NY, USA).

### *2.3.Real-time polymerase chain reaction*

Total RNA was isolated from cells using the RNAiso Plus (Takara Bio, Shiga, Japan) reagent and cDNA was synthesized using the ReverTra Ace (TOYOBO, Osaka, Japan) kit with an oligo (dT) primer for reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR). RT-qPCR was performed using TaqMan probes and primers (Applied Biosystems, Foster City, CA, USA) for integrin  $\beta$ 1 (Assay No. Hs01127536\_m1), integrin  $\beta$ 4 (assay no. Hs00236216\_m1), periostin (Assay No. Hs00170815\_m1), integrin  $\alpha$ V (Assay no. Hs00233808\_m1), integrin  $\alpha$ 6 (Assay no.

Hs01041011\_m1), integrin  $\alpha$ 8 (Assay no. Hs00233321\_m1), integrin  $\alpha$ 9 (Assay no. Hs00979865\_m1), integrin  $\alpha$ 11 (Assay no. Hs01012939\_m1), and glyceraldehyde-3-phosphate dehydrogenase (Cat No. 4310884E) using an ABI StepOne Plus system (Applied Biosystems).

#### *2.4. Flow cytometry analysis*

The cells were detached using a cell dissociation buffer (Thermo Fisher Scientific, Grand Island, NY, USA). Next, the collected cells were washed twice with phosphate-buffered saline (Sigma-Aldrich) containing 3% fetal bovine serum (HyClone, Cytiva) (FACS buffer), and suspended in FACS buffer. Then, the suspension was incubated with primary antibodies (1:100) for integrin  $\beta$ 1 (Millipore, Burlington, MA, USA) and  $\beta$ 4 (Millipore) for 30 min. After incubation, the cells were washed twice with FACS buffer and resuspended. The suspension was then incubated with a secondary antibody (goat anti-mouse IgG-horseradish peroxidase [HRP] conjugate, 1:200 dilution) for mouse IgG (Vector Laboratories, Burlingame, CA, USA). Finally, the cells were washed twice with FACS buffer and resuspended. The suspension was analyzed using FACScan (Applied Biosystems).

### *2.5. Collection of conditioned media*

HPL cells, MSCs, HGFs, and HGECs were cultured, as described in the section 2.1. After these cells achieved 70–80% confluency, the medium was removed and washed with phosphate-buffered saline (Sigma-Aldrich). Subsequently, they were cultured with serum-free medium for 48 h. siRNA-transfected HPL cells or MSCs were cultured with serum-free medium for 48 h after siRNA transfection. Further, each cell culture supernatant was concentrated with an ultrafiltration filter (Amicon Ultra; Millipore) and collected in a concentrated conditioned medium.

### *2.6. Western blotting*

Western blotting was performed, as previously described [30]. Briefly, each conditioned medium was rapidly lysed in equal volumes of 2× urea buffer [final concentration: 1% (w/v) sodium dodecyl sulfate (SDS), 6.2 M urea, 10% glycerol, 5 mM dithiothreitol, 1% 2-mercaptoethanol, 0.01% (w/v) bromophenol blue, 1% (v/v) protease inhibitor cocktail, and 1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich)] and boiled. Aliquots were separated using SDS-polyacrylamide gel electrophoresis (PAGE) (30 µL/lane) on 10% (v/v) polyacrylamide slab gels and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) at 100 V for 1 h at 4 °C.

Membranes were blocked for 1 h at 20–25 °C in Tris-buffered saline (TBS, pH 7.6) and incubated with primary antibodies (1:500 dilution) against human periostin (Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently washed with TBS containing 0.01% Tween 20 (TBS-T) and then incubated with secondary antibodies (goat anti-rabbit IgG-HRP conjugate, 1:2,000 dilution, Cell Signaling Technology) in TBS-T with 5% skim milk for 1 h at 20–25 °C. HRP activity was visualized using the ECL detection system followed by autoradiography.

For the detection of loaded protein in each lane of SDS-polyacrylamide gel, each PVDF membrane was placed in Coomassie Brilliant Blue (CBB) staining solution (Pharmacia LKB, Tokyo, Japan) and incubated for 1 min at 20–25 °C. Subsequently, the staining solution was discarded, and the PVDF membrane was placed in a decolorization solution [45% methanol (Sigma-Aldrich) and 7 % acetic acid (Sigma-Aldrich)] and incubated for 15 min.

### *2.7. Protein levels in conditioned medium*

The total protein levels were measured using BCA protein assay kits (Thermo Fisher Scientific). In brief, each conditioned medium was mixed with a buffer containing BCA

and incubated at 37 °C for 30 min. Subsequently, the absorbance at 570 nm was measured using a TriSter LB941 (Berthold Technologies, Bad Wildbad, Germany).

The periostin protein levels were determined using the Periostin Human ELISA Kit (Thermo Fisher Scientific). Briefly, conditioned media were added to an antibody-coated plate and incubated for 2.5 h at 20–25 °C. After four washes with a washing buffer, anti-human periostin antibody conjugated with biotin was added to the wells to detect human periostin. This mixture was incubated for 1 h at 20–25 °C. Following the removal of unbound antibodies, streptavidin conjugated with HRP was added to the wells and incubated for 60 min at 20–25 °C. The wells were then washed again, and a tetramethylbenzidine substrate solution was added, followed by a 45-min incubation at 20–25 °C in the dark. The reaction was terminated by adding a stop solution, and the absorbance at 450 nm was measured using a TriSter LB941 (Berthold Technologies).

### *2.8. Statistical analysis*

The statistically significant differences were determined using the Steel–Dwass test.  $P < 0.05$  was considered as statistically significant.

### 3. Results

#### *3.1. Conditioned medium from MSCs and HPL cells affected integrin expression in HGEs*

HGEs were cultured in a conditioned medium from MSCs or HPL cells for 0–48 h. Integrin  $\beta 1$  and  $\beta 4$  mRNAs were significantly downregulated at 12 h by a conditioned medium from HPL cells and MSCs (Fig. 1A). Integrin  $\beta 4$  mRNA was downregulated but integrin  $\beta 1$  mRNA was significantly upregulated by the conditioned medium from HPL cells at 48 h; the same pattern was observed with the conditioned medium from MSCs, but the change was not significant (Fig. 1B). Protein expression of integrin  $\beta 4$  was downregulated by conditioned medium from MSCs and HPL cells at 48 h, and the tendency was the same as that observed for the mRNA expression level; however, the protein expression of integrin  $\beta 1$  was upregulated by conditioned medium from MSCs and HPL cells, in contrast to the mRNA expression levels at 48 h (Fig. 2).

#### *3.2. Comparison of periostin expression between MSCs, HPL cells, and HGFs*

Compared to HGFs, MSCs and HPL cells significantly expressed periostin mRNA (Fig. 3A). Periostin protein was strongly detected in the conditioned medium from the MSC and HPL cell cultures, but weakly detected in the HGF cell culture. However, it was

abundant in highly concentrated cultures of all cell cultures (Fig. 3B). Each loaded protein was confirmed via CBB staining of blotted PVDF membranes and protein assays, and each amount was confirmed to be similar (Figs. S1A and S1B). Moreover, the amount of periostin protein in each conditioned medium was quantified by using ELISA, and the data showed the same pattern with western blotting (Fig. S1C).

### *3.3.Periostin regulated integrin expression in HGECS*

Periostin enhanced the mRNA expression of integrin  $\beta 1$  and suppressed  $\beta 4$  mRNA expression at 12 h (Fig. 4A). Periostin downregulated integrin  $\beta 4$  protein and mRNA expression at 24 h. In contrast, integrin  $\beta 1$  protein and mRNA expression levels were significantly enhanced by periostin at 48 h (Fig. 4B). As for integrin  $\alpha$  subunits, the mRNA expression levels of integrin  $\alpha 6$ ,  $\alpha 8$ ,  $\alpha 9$ , and  $\alpha 11$  were significantly upregulated by periostin. In contrast, integrin  $\alpha V$  mRNA expression was significantly downregulated by periostin stimulation (Fig. S2).

### *3.4.Periostin plays an important role in changing integrin expression by a conditioned medium in HGECS*

HGECs were cultured in a conditioned medium collected from MSCs or HPL cells transfected with siControl or siPeriostin and untransfected MSCs and HPL cells (vehicle). Periostin mRNA expression was suppressed by siPeriostin in MSCs and HPL cells, and their expression levels were 15.03% and 7.99% when compared to the siRNA negative control-transfected cells, respectively (Fig. 5A). However, siRNA negative control-transfected MSCs showed a 250% greater periostin expression level than that in HPL cells (Fig. 5A). In the conditioned medium, periostin expression in MSCs and HPL cells was suppressed by siPeriostin, and the expression in MSCs was lower than that in HPL cells (Fig. 5B). Each loaded protein was confirmed via CBB staining of the blotted PVDF membrane and protein assay, and it was confirmed that the quantity of protein for each treatment was similar (Figs. S3A and S3B). Moreover, the amount of periostin protein in each conditioned medium was quantified using ELISA, and the data showed the same pattern with western blotting (Fig. S3C).

Integrin  $\beta$ 1 expression in HGECs was enhanced by siControl in a conditioned medium from transfected MSCs when compared to that observed with the vehicle at 48 h. However, the conditioned medium from siPeriostin-transfected MSCs suppressed this enhancement (Fig. 5C). In addition to the conditioned medium from MSCs, the conditioned medium from HPL cells also increased integrin  $\beta$ 1 expression when



compared to the vehicle, and this increase approached the baseline following siPeriostin transfection (Fig. 5C).

Conditioned medium from siControl-transfected MSCs decreased integrin  $\beta 4$  expression at 48 h. However, the conditioned medium from siPeriostin-transfected MSCs exerted less potent effects than that from siControl-transfected MSCs. A similar phenomenon was also observed in HPL cells (Fig. 5C).

#### **4. Discussion**

The gingival epithelium functions as the primary mechanical barrier to protect against bacterial infection and invasion or other inflammatory stimuli. During the onset of periodontitis, the gingival epithelium is first exposed and attacked by inflammatory stimuli, followed by the stimulation of the gingival connective tissue and immune cells [31].

Therefore, the defense mechanism of the gingival epithelium constitutes an important frontier barrier. Gingival epithelial cells form cell–cell junction complexes, such as gap or tight junctions [1, 32].

The junctional epithelium participates in the attachment of epithelial cells to the tooth surface with hemidesmosome [33] to separate the periodontal tissue from the external environment [3, 4]. This connection is based on integrin molecules [34], which are involved in the progression or onset of periodontitis. Integrin  $\alpha 9$  and its ligands play regulatory roles in chronic periodontitis [35], and the mutation of the  $\beta 2$  integrin subunit can induce severe periodontal problems [36]. Moreover, the integrin  $\beta 6$  mutation can indirectly affect inflammasome and cytokine expression in periodontitis [37]. Interleukin- $1\beta$  enhances cell adhesion through  $\beta 4$  integrins in HGEs [38]. In this study, HGEs were positively affected by periostin treatment in terms of mRNA expression of integrin  $\alpha 6$ ,  $\alpha 8$ ,  $\alpha 9$ , and  $\alpha 11$ , but negatively affected in terms of mRNA expression of integrin  $\alpha V$  (Fig. S2). In other cells, such as MC3T3-E1, periostin positively supported the integrin  $\alpha V$ -dependent cell adhesion and motility [20, 21] and the pair of integrin  $\alpha 6$  and  $\beta 1$  could regulate keratinocyte function [39] or epithelial cells [40]. Consequently, periostin upregulated integrin  $\beta 1$  and downregulated integrin  $\beta 4$  (Fig. 4A); the pair of integrin  $\beta 1$ - $\alpha 6$  and  $\beta 4$ - $\alpha V$  may play an important role in periostin-regulated gingival epithelium function.

Previous studies on immune cell function revealed that the regulation of neutrophil migration and monocyte adhesion is regulated by integrin expression [41, 42].

Furthermore, the studies on inflammatory stimulation showed that *Porphyromonas gingivalis* degrades integrin  $\beta 1$  [43], and the expression of integrin  $\beta 1$  or  $\beta 4$  is affected by antibacterial agents [44], which means that integrin  $\beta 1$  and  $\beta 4$  can be targets of bacterial infection. Therefore, based on their role in infection advancement and immune response, the regulation of integrins  $\beta 1$  and  $\beta 4$  in HGECS is essential. Moreover, other integrins or adhesion molecules related to the gingival epithelium [34, 45] and claudin 1 are the main components of tight junctions in gingival epithelial cells [46].

In this study, conditioned medium from HPL cells and MSCs enhanced integrin  $\beta 1$  expression, whereas integrin  $\beta 4$  was suppressed at the mRNA/protein level (Figs. 1 and 2). Thus, the presence of Humoral factors in HPL cells and MSCs have been reported previously. HPL cells are a target of progenitor cells, and hence, the humoral response may be involved in cell differentiation/regeneration [47, 48]. In our previous study, we reported that the humoral factors from HPL cells regulate the expression and activity of histone deacetylase (HDAC) 1 and 2 [49]. HDACs regulate the transcription of each gene. Furthermore, regulation of integrin transcription is desirable. Therefore, further studies on integrin regulatory HDACs are necessary. In addition, the humoral factors regulate inflammation [50].

Periostin supports integrin-dependent cell adhesion and motility [21, 51] and regulates tumor invasion [52]. Thus, periostin may be involved in integrin regulation. Periostin is associated with inflammation and allergic reactions [53, 54]. Therefore, the functional regulation of gingival cell adhesion by integrins can be regulated by periostin included in humoral factors from periodontal cells. Integrin regulation by periostin is important for providing a mechanical barrier for bacterial invasion. Thus, periostin can help in the prevention or reversal of the progress of periodontitis and maintain homeostasis in periodontal tissue. Additionally, periostin can regulate cell function in periodontal tissue, especially multiple potential cells. Periostin from HPL cells regulates MSCs [47] and stem cell function via integrins [55]. Moreover, MSCs and HPL cells are multipotent [25, 27] and induce periodontal regeneration by cell transplantation [25, 28, 56]. Therefore, periostin-producing and multipotent cells in periodontal tissue are important. Even after their reduction or elimination due to the progression of periodontitis, HPL cell or MSC transplantation is valuable for tissue regeneration and regulating inflammation and multipotent cell function.

However, these results implicate only the interaction between periostin and integrin  $\beta 1$  or  $\beta 4$ . Other integrins or adhesion molecules related to the gingival epithelium [34, 45] or claudin 1 [46] should be considered for their role in HGEC function. The results of this

study shed light on the mechanism of action of the gingival barrier system in periodontitis.

Further studies are required to investigate the effect of MSCs or HPL cells on other molecular mechanisms.

## **5. Conclusions**

Integrin  $\beta 1$  and integrin  $\beta 4$  in gingival epithelial cells are regulated by periostin, included in the humoral factor from periodontal ligament cells and MSCs. This result suggests that periostin is one of the key humoral regulatory factors from MSCs or HPL cells for regulating adhesion of HGECs. Therefore, to maintain a mechanical barrier in the gingiva, periostin expression must be regulated, and periostin-expressing/releasing cells or periostin itself should be used when its expression is downregulated. The mechanical barrier in the gingiva can also be regulated by other molecules targeting different adhesion molecules. Therefore, in future, the interactions between various adhesion molecules and humoral factors generated by periodontal cells should be investigated. Based on these results, cell transplantation therapy for periodontitis can be formulated.

### **Ethical approval and informed consent**

Informed consent was obtained according to a protocol approved by the Ethics Committee of the Hiroshima University Faculty of Dentistry (Hiroshima, Japan; Approval no. E-D47-4). All human mesenchymal stem cells (hMSCs) were provided by the RIKEN BioResource Center (Tsukuba, Japan), with approval from the Ethics Committee of Hiroshima University Faculty of Dentistry (Hiroshima, Japan; Approval number: E-422-2).

### **Author contributions**

Dr. Reika Hirata designed and performed the experiments; collected, analyzed, and interpreted the data; and wrote and edited the manuscript; Dr. Tsuyoshi Fujita and Dr. Tomoyuki Iwata wrote and edited the manuscript; Dr. Takayoshi Nagahara, Dr. Shinji Matsuda, Dr. Yasusei Kudo, Dr. Shinya Sasaki, Dr. Yuri Taniguchi, Dr. Yuta Hamamoto, and Dr. Kazuhisa Ouhara performed the experiments. Dr. Hidemi Kurihara and Dr. Noriyoshi Mizuno supervised all aspects of the study as senior investigators and directors of the laboratory.

### **Conflict of interest disclosure**

The authors have no conflicts of interest to declare.

### **Acknowledgments**

This study did not receive any grants from public, commercial, or non-profit funding agencies. Part of this work was carried out with material support from the Research Facility of the Hiroshima University Faculty of Dentistry.

### **References**

- [1] Fujita T, Ashikaga A, Shiba H, Uchida Y, Hirono C, Iwata T, Takeda K, Kishimoto A, Hirata R, Kawaguchi H, Shiba Y, Kurihara H. Regulation of IL-8 by Irsogladine maleate is involved in abolishment of *Actinobacillus actinomycetemcomitans*-induced reduction of gap-junctional intercellular communication. *Cytokine*. 2006;34:271-7.
- [2] Hormia M, Sahlberg C, Thesleff I, Airenne T. The epithelium-tooth interface--a basal lamina rich in laminin-5 and lacking other known laminin isoforms. *Journal of dental research*. 1998;77:1479-85.
- [3] Sawada T, Inoue S. Mineralization of basement membrane mediates dentogingival adhesion in mammalian and nonmammalian vertebrates. *Calcified tissue international*. 2003;73:186-95.
- [4] Schroeder HE. [Healing and regeneration following periodontal treatment]. *Deutsche zahnärztliche Zeitschrift*. 1986;41:536-8.

- [5] Frank DE, Carter WG. Laminin 5 deposition regulates keratinocyte polarization and persistent migration. *Journal of cell science*. 2004;117:1351-63.
- [6] Fukushima Y, Ohnishi T, Arita N, Hayakawa T, Sekiguchi K. Integrin alpha3beta1-mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. *International journal of cancer*. 1998;76:63-72.
- [7] Hormia M, Owaribe K, Virtanen I. The dento-epithelial junction: cell adhesion by type I hemidesmosomes in the absence of a true basal lamina. *Journal of periodontology*. 2001;72:788-97.
- [8] Salonen JI, Kautsky MB, Dale BA. Changes in cell phenotype during regeneration of junctional epithelium of human gingiva in vitro. *Journal of periodontal research*. 1989;24:370-7.
- [9] Calenic B, Ishkitiev N, Yaegaki K, Imai T, Kumazawa Y, Nasu M, Hirata T. Magnetic separation and characterization of keratinocyte stem cells from human gingiva. *Journal of periodontal research*. 2010;45:703-8.
- [10] Ishikawa H, Hashimoto S, Tanno M, Ishikawa T, Tanaka T, Shimono M. Cytoskeleton and surface structures of cells directly attached to the tooth in the rat junctional epithelium. *Journal of periodontal research*. 2005;40:354-63.
- [11] Fuchs E, Dowling J, Segre J, Lo SH, Yu QC. Integrators of epidermal growth and differentiation: distinct functions for beta 1 and beta 4 integrins. *Current opinion in genetics & development*. 1997;7:672-82.
- [12] Takada Y, Ye X, Simon S. The integrins. *Genome biology*. 2007;8:215.
- [13] Takeshita S, Kikuno R, Tezuka K, Amann E. Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. *The Biochemical journal*. 1993;294 ( Pt 1):271-8.
- [14] Kruzynska-Frejtag A, Wang J, Maeda M, Rogers R, Krug E, Hoffman S, Markwald RR, Conway SJ. Periostin is expressed within the developing teeth at the sites of epithelial-mesenchymal interaction. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2004;229:857-68.
- [15] Kudo Y, Siriwardena BS, Hatano H, Ogawa I, Takata T. Periostin: novel diagnostic and therapeutic target for cancer. *Histology and histopathology*. 2007;22:1167-74.
- [16] Oshima A, Tanabe H, Yan T, Lowe GN, Glackin CA, Kudo A. A novel mechanism for the regulation of osteoblast differentiation: transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, twist. *Journal of cellular biochemistry*. 2002;86:792-804.
- [17] Yamaguchi Y. Periostin in Skin Tissue Skin-Related Diseases. *Allergology international : official journal of the Japanese Society of Allergology*. 2014;63:161-70.



- [18] Xu HY, Nie EM, Deng G, Lai LZ, Sun FY, Tian H, Fang FC, Zou YG, Wu BL, Ou-Yang J. Periostin is essential for periodontal ligament remodeling during orthodontic treatment. *Molecular medicine reports*. 2017;15:1800-6.
- [19] Oku E, Kanaji T, Takata Y, Oshima K, Seki R, Morishige S, Imamura R, Ohtsubo K, Hashiguchi M, Osaki K, Yakushiji K, Yoshimoto K, Ogata H, Hamada H, Izuhara K, Sata M, Okamura T. Periostin and bone marrow fibrosis. *International journal of hematology*. 2008;88:57-63.
- [20] Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY, Chang DD. Periostin secreted by epithelial ovarian carcinoma is a ligand for  $\alpha(V)\beta(3)$  and  $\alpha(V)\beta(5)$  integrins and promotes cell motility. *Cancer research*. 2002;62:5358-64.
- [21] Khurana S, Schouteden S, Manesia JK, Santamaria-Martínez A, Huelsken J, Lacy-Hulbert A, Verfaillie CM. Outside-in integrin signalling regulates haematopoietic stem cell function via Periostin-Itgav axis. *Nature communications*. 2016;7:13500.
- [22] Gould TR, Melcher AH, Brunette DM. Location of progenitor cells in periodontal ligament of mouse molar stimulated by wounding. *The Anatomical record*. 1977;188:133-41.
- [23] Nohutcu RM, McCauley LK, Koh AJ, Somerman MJ. Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization in vitro. *Journal of periodontology*. 1997;68:320-7.
- [24] Komaki M. Pericytes in the Periodontal Ligament. *Advances in experimental medicine and biology*. 2019;1122:169-86.
- [25] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet (London, England)*. 2004;364:149-55.
- [26] Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92:4857-61.
- [27] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science (New York, NY)*. 1999;284:143-7.
- [28] Kawaguchi H, Hirachi A, Hasegawa N, Iwata T, Hamaguchi H, Shiba H, Takata T, Kato Y, Kurihara H. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *Journal of periodontology*. 2004;75:1281-7.
- [29] Iwata T, Kawamoto T, Sasabe E, Miyazaki K, Fujimoto K, Noshiro M, Kurihara H, Kato Y. Effects of overexpression of basic helix-loop-helix transcription factor Dec1 on osteogenic and adipogenic differentiation of mesenchymal stem cells. *European journal of cell biology*. 2006;85:423-31.

- [30] Iwata T, Kantarci A, Yagi M, Jackson T, Hasturk H, Kurihara H, Van Dyke TE. Ceruloplasmin induces polymorphonuclear leukocyte priming in localized aggressive periodontitis. *Journal of periodontology*. 2009;80:1300-6.
- [31] Bosshardt DD. The periodontal pocket: pathogenesis, histopathology and consequences. *Periodontology 2000*. 2018;76:43-50.
- [32] Groeger SE, Meyle J. Epithelial barrier and oral bacterial infection. *Periodontology 2000*. 2015;69:46-67.
- [33] Fischer NG, Aparicio C. Junctional epithelium and hemidesmosomes: Tape and rivets for solving the "percutaneous device dilemma" in dental and other permanent implants. *Bioactive materials*. 2022;18:178-98.
- [34] Tanno M, Hashimoto S, Muramatsu T, Matsuki M, Yamada S, Shimono M. Differential localization of laminin gamma and integrin beta in primary cultures of the rat gingival epithelium. *Journal of periodontal research*. 2006;41:15-22.
- [35] Xu S, Jiang C, Liu H, Zhang H, Liao H, Wang X, Yao S, Ma L, Guo Y, Cao Z. Integrin- $\alpha$ 9 and Its Corresponding Ligands Play Regulatory Roles in Chronic Periodontitis. *Inflammation*. 2020;43:1488-97.
- [36] Larjava H, Koivisto L, Heino J, Häkkinen L. Integrins in periodontal disease. *Experimental cell research*. 2014;325:104-10.
- [37] Bi J, Dai J, Koivisto L, Larjava M, Bi L, Häkkinen L, Larjava H. Inflammasome and cytokine expression profiling in experimental periodontitis in the integrin  $\beta$ 6 null mouse. *Cytokine*. 2019;114:135-42.
- [38] Mezawa M, Tsuruya Y, Yamazaki-Takai M, Takai H, Nakayama Y, McCulloch CA, Ogata Y. IL-1 $\beta$  enhances cell adhesion through laminin 5 and  $\beta$ 4 integrin in gingival epithelial cells. *Journal of oral science*. 2019;61:491-7.
- [39] Ibrahim MR, Medhat W, El-Fakahany H, Abdel-Raouf H, Snyder EY. Deriving Keratinocyte Progenitor Cells and Keratinocytes from Human-Induced Pluripotent Stem Cells. *Current protocols in stem cell biology*. 2020;54:e119.
- [40] Park HJ, Park JE, Lee H, Kim SJ, Yun JI, Kim M, Park KH, Lee ST. Integrins functioning in uterine endometrial stromal and epithelial cells in estrus. *Reproduction (Cambridge, England)*. 2017;153:351-60.
- [41] Gonzalez AL, El-Bjeirami W, West JL, McIntire LV, Smith CW. Transendothelial migration enhances integrin-dependent human neutrophil chemokinesis. *Journal of leukocyte biology*. 2007;81:686-95.
- [42] Graves DT, Milovanova TN. Mucosal Immunity and the FOXO1 Transcription Factors. *Frontiers in immunology*. 2019;10:2530.

- [43] Li Q, Zhou J, Lin L, Zhao H, Miao L, Pan Y. Porphyromonas gingivalis degrades integrin  $\beta$ 1 and induces AIF-mediated apoptosis of epithelial cells. Infectious diseases (London, England). 2019;51:793-801.
- [44] Inoue K, Kumakura S, Uchida M, Tsutsui T. Effects of eight antibacterial agents on cell survival and expression of epithelial-cell- or cell-adhesion-related genes in human gingival epithelial cells. Journal of periodontal research. 2004;39:50-8.
- [45] Yoshimoto T, Fujita T, Kajiya M, Ouhara K, Matsuda S, Komatsuzawa H, Shiba H, Kurihara H. Aggregatibacter actinomycetemcomitans outer membrane protein 29 (Omp29) induces TGF- $\beta$ -regulated apoptosis signal in human gingival epithelial cells via fibronectin/integrin $\beta$ 1/FAK cascade. Cellular microbiology. 2016;18:1723-38.
- [46] Fujita T, Hayashida K, Shiba H, Kishimoto A, Matsuda S, Takeda K, Kawaguchi H, Kurihara H. The expressions of claudin-1 and E-cadherin in junctional epithelium. Journal of periodontal research. 2010;45:579-82.
- [47] Kaneda-Ikeda E, Iwata T, Mizuno N, Nagahara T, Kajiya M, Takeda K, Hirata R, Ishida S, Yoshioka M, Fujita T, Kawaguchi H, Kurihara H. Periodontal ligament cells regulate osteogenesis via miR-299-5p in mesenchymal stem cells. Differentiation; research in biological diversity. 2020;112:47-57.
- [48] Mizuno N, Ozeki Y, Shiba H, Kajiya M, Nagahara T, Takeda K, Kawaguchi H, Abiko Y, Kurihara H. Humoral factors released from human periodontal ligament cells influence calcification and proliferation in human bone marrow mesenchymal stem cells. Journal of periodontology. 2008;79:2361-70.
- [49] Iwata T, Kaneda-Ikeda E, Takahashi K, Takeda K, Nagahara T, Kajiya M, Sasaki S, Ishida S, Yoshioka M, Matsuda S, Ouhara K, Fujita T, Kurihara H, Mizuno N. Regulation of osteogenesis in bone marrow-derived mesenchymal stem cells via histone deacetylase 1 and 2 co-cultured with human gingival fibroblasts and periodontal ligament cells. Journal of periodontal research. 2023;58:83-96.
- [50] Ishiuchi N, Nakashima A, Doi S, Yoshida K, Maeda S, Kanai R, Yamada Y, Ike T, Doi T, Kato Y, Masaki T. Hypoxia-preconditioned mesenchymal stem cells prevent renal fibrosis and inflammation in ischemia-reperfusion rats. Stem cell research & therapy. 2020;11:130.
- [51] Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, Ozawa H, Toyama Y, Bonewald LF, Kudo A. Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1999;14:1239-49.
- [52] Dorafshan S, Razmi M, Safaei S, Gentilin E, Madjd Z, Ghods R. Periostin: biology and function in cancer. Cancer cell international. 2022;22:315.

- [53] Sonnenberg-Riethmacher E, Miehle M, Riethmacher D. Periostin in Allergy and Inflammation. *Frontiers in immunology*. 2021;12:722170.
- [54] Nakajima M, Honda T, Miyauchi S, Yamazaki K. Th2 cytokines efficiently stimulate periostin production in gingival fibroblasts but periostin does not induce an inflammatory response in gingival epithelial cells. *Archives of oral biology*. 2014;59:93-101.
- [55] Suresh A, Biswas A, Perumal S, Khurana S. Periostin and Integrin Signaling in Stem Cell Regulation. *Advances in experimental medicine and biology*. 2019;1132:163-76.
- [56] Hasegawa N, Kawaguchi H, Hirachi A, Takeda K, Mizuno N, Nishimura M, Koike C, Tsuji K, Iba H, Kato Y, Kurihara H. Behavior of transplanted bone marrow-derived mesenchymal stem cells in periodontal defects. *Journal of periodontology*. 2006;77:1003-7.

## Figure legends

**Fig. 1. Conditioned medium from MSCs and HPL cells changed integrin mRNA expression in HGECS.** HGECS were cultured in a conditioned medium from MSCs and HPL cells for 12 h (A) and 48 h (B), and total RNA was extracted. Integrin beta 1 and beta 4 mRNA levels were determined using quantitative real-time PCR. Values were normalized by GAPDH and represent mRNA expression (mean  $\pm$  SD) in relation to HGECS medium (n = 4). \*\*:P < 0.01, \*P < 0.05 (Steel–Dwass test). HGECS, human gingival epithelial cells; HPL, human periodontal ligament; MSCs, mesenchymal stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SD, standard deviation

**Fig. 2. Conditioned medium from MSCs and HPL cells changed integrin expression in HGECS.** HGECS were cultured in a conditioned medium from MSCs and HPL cells for 48 h, after which the cells were collected. Integrin beta 1 and beta 4 protein levels were determined using flow cytometry. Histogram shows the intensity of FITC in the conditioned medium from HGECS, MSCs, and HPL cells. Values represent the % of M1 gated cells (mean  $\pm$  SD) in relation to HGECS medium (n = 4). \*\*:P < 0.01, \*P < 0.05 (Steel–Dwass test). HGECS, human gingival epithelial cells; HPL, human periodontal

ligament; MSCs, mesenchymal stem cells; SD, standard deviation; FITC, fluorescein isothiocyanate

**Fig. 3. Periostin mRNA expression in MSCs and HPL cells, and MSCs and HPL cells released periostin in a conditioned medium.** (A) MSCs, HPL cells, and HGFs were cultured for two days. The seeding concentrations of the cells were  $0.5 \times 10^4$  cells/cm<sup>2</sup> ( $\times 1$ ) or  $2.5 \times 10^4$  cells/cm<sup>2</sup> ( $\times 5$ ). Total RNA was extracted from the cells. cDNA was synthesized from each total RNA sample, and periostin mRNA expression level was determined using quantitative real-time PCR. Values were normalized by GAPDH and represented in relation to HGFs ( $n = 5$ ). \*\* $P < 0.01$ , \* $P < 0.05$  (Steel–Dwass test). (B) MSCs, HPL cells, and HGFs were cultured and collected in a conditioned medium. Periostin protein levels in each medium were determined using western blotting. HPL, human periodontal ligament; MSCs, mesenchymal stem cells; HGFs, human gingival fibroblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction

**Fig. 4. Periostin changed integrin expression in HGECS.** HGECS were cultured with periostin (500 ng/mL) for 48 h, total RNA was extracted, and the cells were collected.

Integrin beta 1 and beta 4 mRNA expression levels were determined using quantitative real-time PCR (A) and protein using flow cytometry (B). Values represent mRNA expression (mean  $\pm$  SD) in relation to the cells cultured without periostin (n = 4). Values were normalized by GAPDH and represent % of M1 gated cells (mean  $\pm$  SD) in relation to the cells cultured without periostin (n = 4). \*\*:P < 0.01, \*P < 0.05 (Steel–Dwass test). HGECS, human gingival epithelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SD, standard deviation

**Fig. 5. Periostin regulated the effect of conditioned medium on integrin expression in HGECS.** MSCs and HPL cells were transfected with negative control siRNA (siControl), or siRNA for periostin (siPeriostin), or untransfected MSCs and HPL cells (vehicle). (A) Total RNA was extracted from siRNA-transfected cells, and periostin mRNA expression levels were determined using quantitative real-time PCR. Values were normalized by GAPDH and represent mRNA expression (mean  $\pm$  SD) in relation to each siControl (n = 4). (B) The siRNA-transfected cells were collected from each conditioned medium. Periostin protein levels in each medium were determined by western blotting. (C) HGECS were cultured in a conditioned medium from MSCs and HPL cells transfected with vehicle, siControl, or siPeriostin for 48 h, after which the cells were collected.

Integrin beta 1 and beta 4 protein levels were determined using flow cytometry. Values represent % of M1 gated cells (mean  $\pm$  SD) in relation to the vehicle (n = 4). \*\*:P < 0.01, \*P < 0.05 (Steel–Dwass test). HGEs, human gingival epithelial cells; HPL, human periodontal ligament; MSCs, mesenchymal stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SD, standard deviation

**Fig. S1. Total protein levels and periostin in each conditioned medium secreted from**

**MSCs, HPL cells, and HGFs.** MSCs, HPL cells, and HGFs were cultured for two days.

The seeding concentrations of the cells were  $0.5 \times 10^4$  cells/cm<sup>2</sup> ( $\times 1$ ) or  $2.5 \times 10^4$  cells/cm<sup>2</sup> ( $\times 5$ ). MSCs, HPL cells, and HGFs were cultured and collected from each

conditioned medium. (A) The amounts of loaded whole protein expression were determined using CBB staining. (B) The amounts of loaded protein were determined

using the BCA protein assay. Values are the amounts of loaded protein (mean  $\pm$  SD; n =

4) N.S.: not significant (the Steel–Dwass test) (C) The amounts of periostin protein in

each conditioned medium were determined using ELISA. Values indicate the amounts of

periostin protein in each conditioned medium (mean  $\pm$  SD; n = 4). \*\*:P < 0.01, \*P < 0.05

(Steel–Dwass test). HPL, human periodontal ligament; MSCs, mesenchymal stem cells;

HGFs, human gingival fibroblasts.



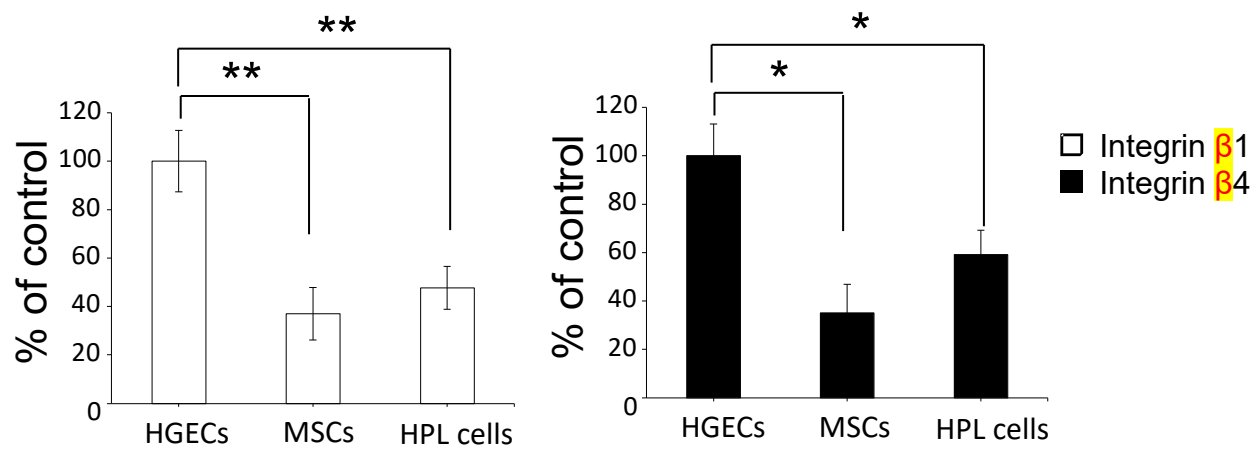
**Fig. 2S. Periostin changed integrin  $\alpha$  subunit expression in HGECS.** HGECS were cultured with periostin (500 ng/mL) for 48 h, total RNA was extracted, and the cells were collected. Integrin  $\alpha$ V,  $\alpha$ 6,  $\alpha$ 8,  $\alpha$ 9, and  $\alpha$ 11 mRNA expression levels were determined using quantitative real-time PCR. Values were normalized using GAPDH and represent mRNA expression (mean  $\pm$  SD) in relation to HGECS cultured without periostin (n = 5). \*\*:P < 0.01, \*P < 0.05 (the Steel–Dwass test). HGECS, human gingival epithelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SD, standard deviation

**Fig. S3. Amounts of total protein and periostin in each conditioned medium secreted from siRNA-transfected MSCs, HPL cells, and HGECS.** MSCs and HPL cells were transfected with negative control siRNA (siControl), or siRNA for periostin (siPeriostin), or HGECS. The siRNA-transfected cells and HGECS were collected from each conditioned medium. (A) The amounts of loaded whole protein expression were determined using CBB staining. (B) The amounts of loaded protein were determined using protein assays. Values indicate the amounts of loaded protein (mean  $\pm$  SD; n = 4) N.S.: not significant (Steel–Dwass test). (C) The amounts of periostin protein in each

conditioned medium were determined using ELISA. Values indicate the amounts of periostin protein in each conditioned medium (mean  $\pm$  SD; n = 4). \*\*:P < 0.01, \*P < 0.05 (Steel–Dwass test). HGECs, human gingival epithelial cells; HPL, human periodontal ligament; MSCs, mesenchymal stem cells.

Fig. 1.

(A)



(B)

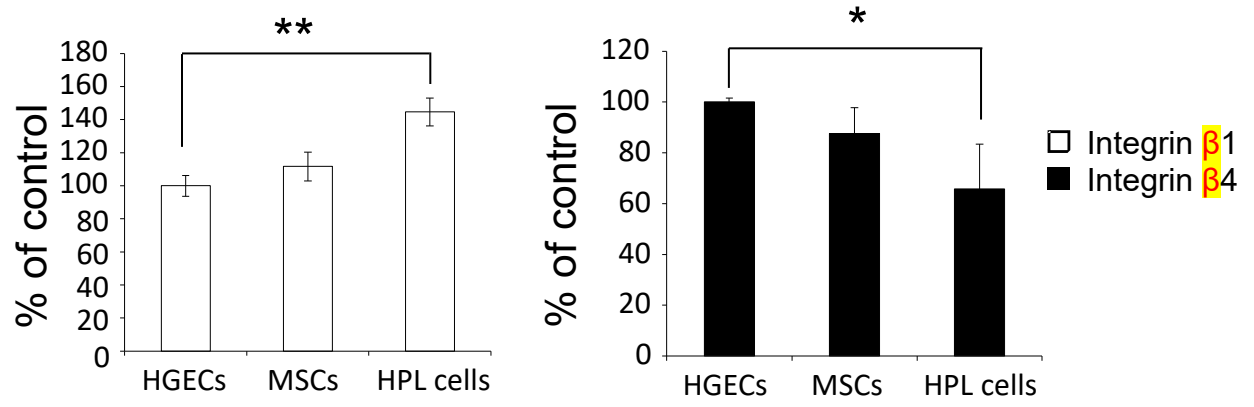
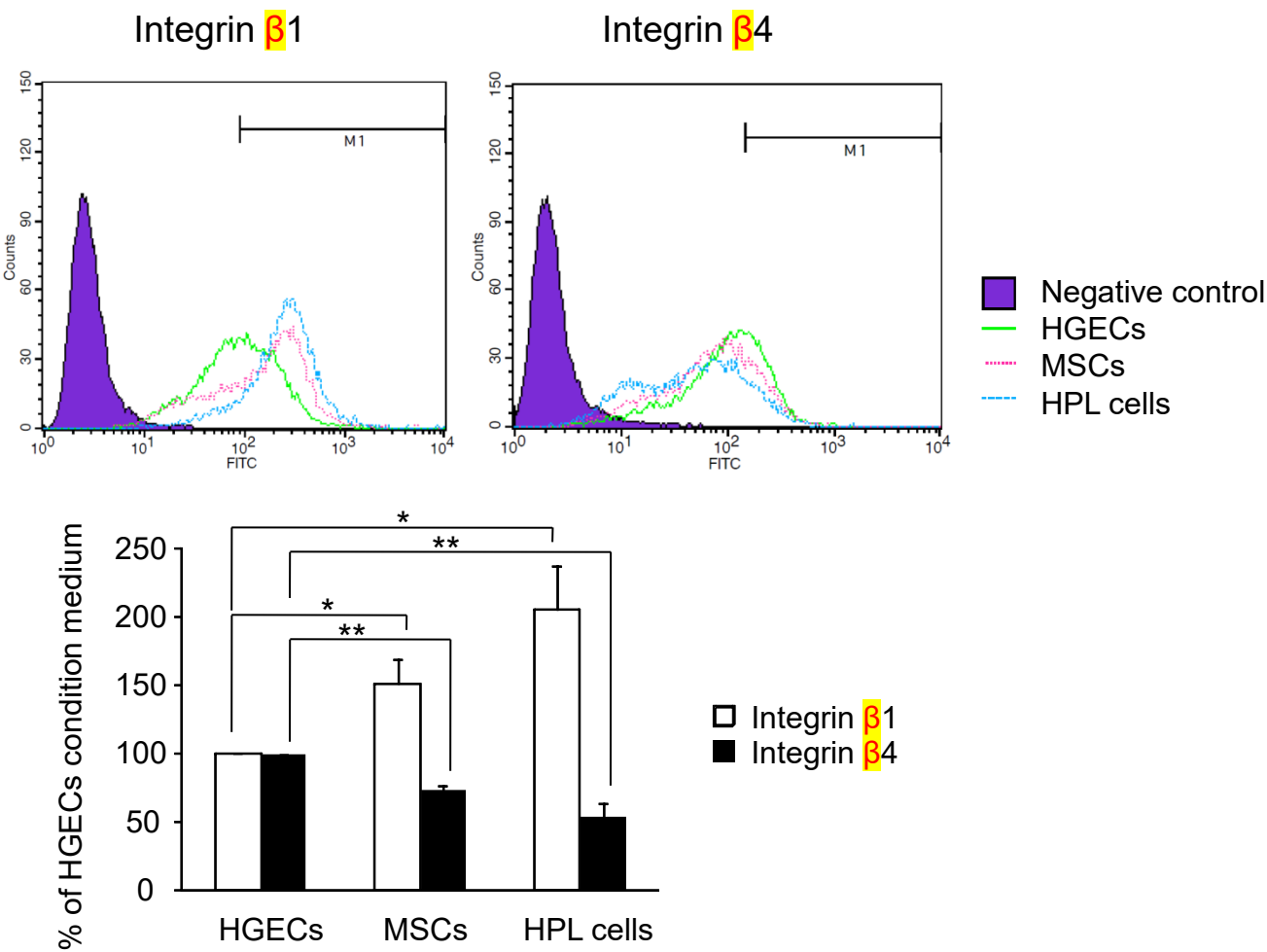
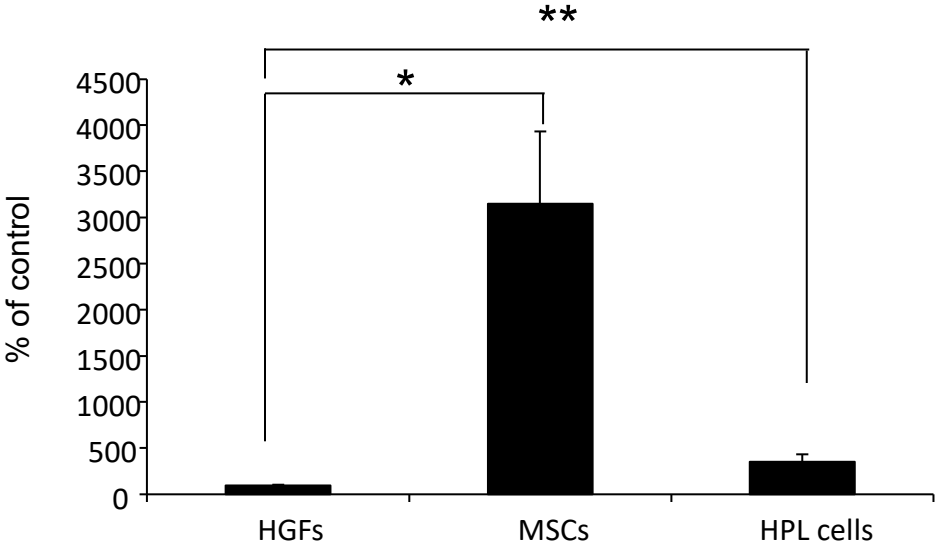


Fig.2.



**Fig. 3.**

**(A)**



**(B)**

**MSCs**

**HPL cells**

**HGFs**

**x1**

**x5**

**x1**

**x5**

**x1**

**x5**

**Periostin**

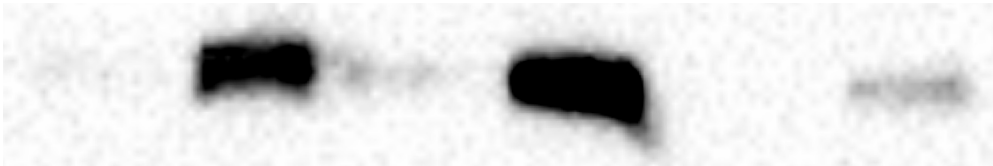
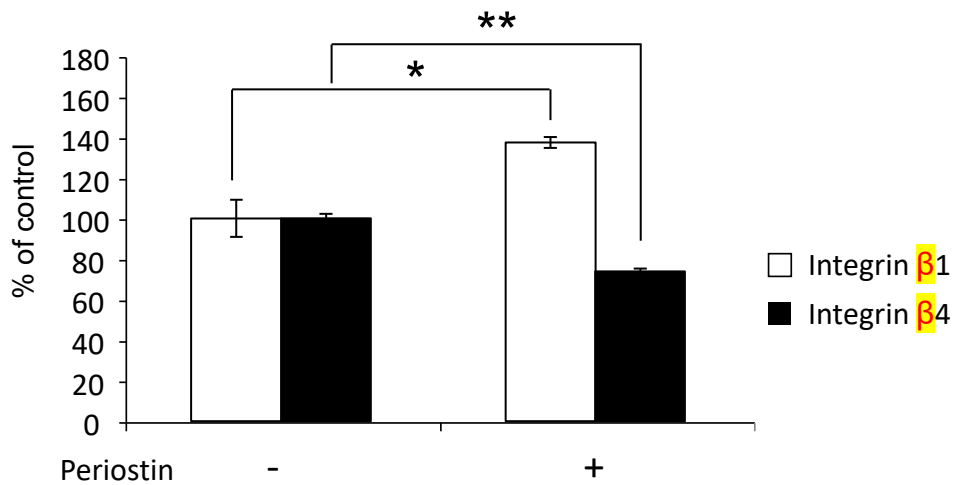
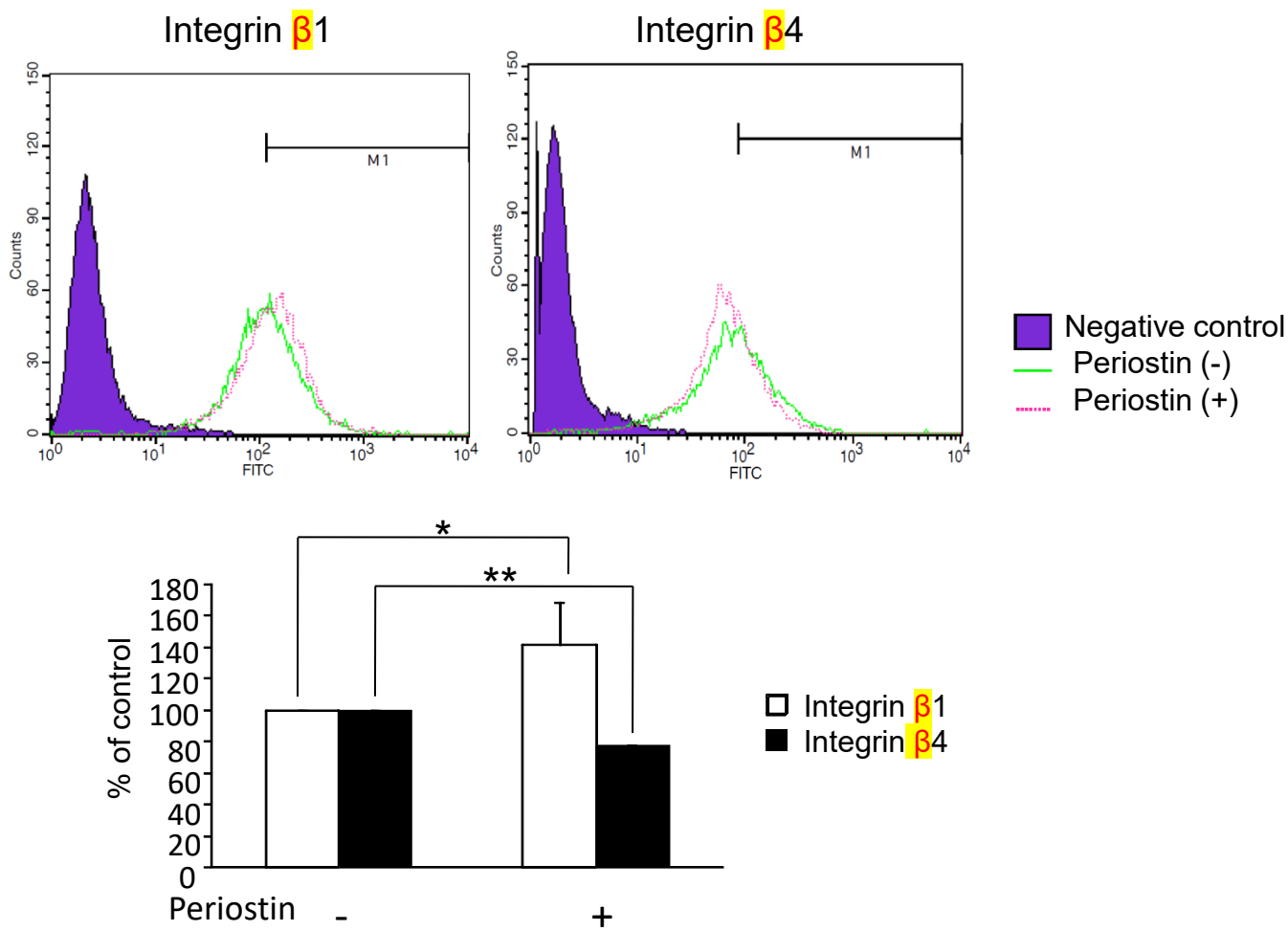


Fig. 4.

(A)

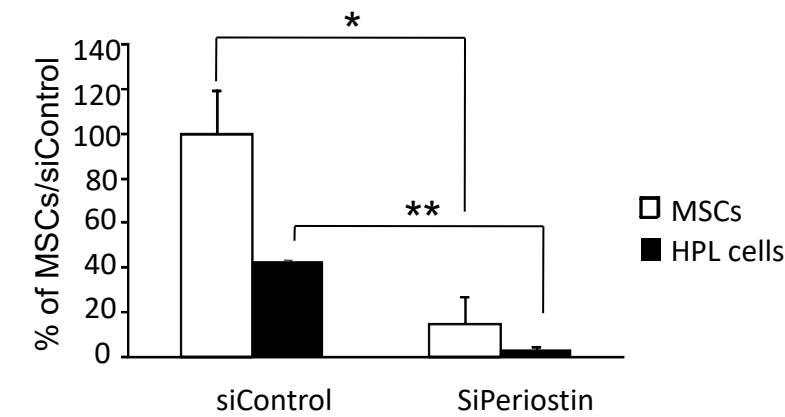


(B)

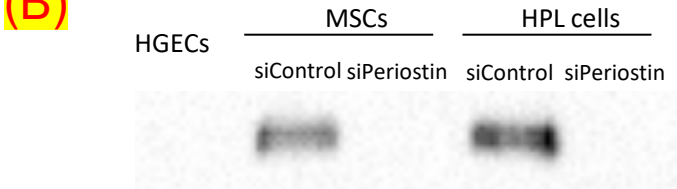


**Fig. 5.**

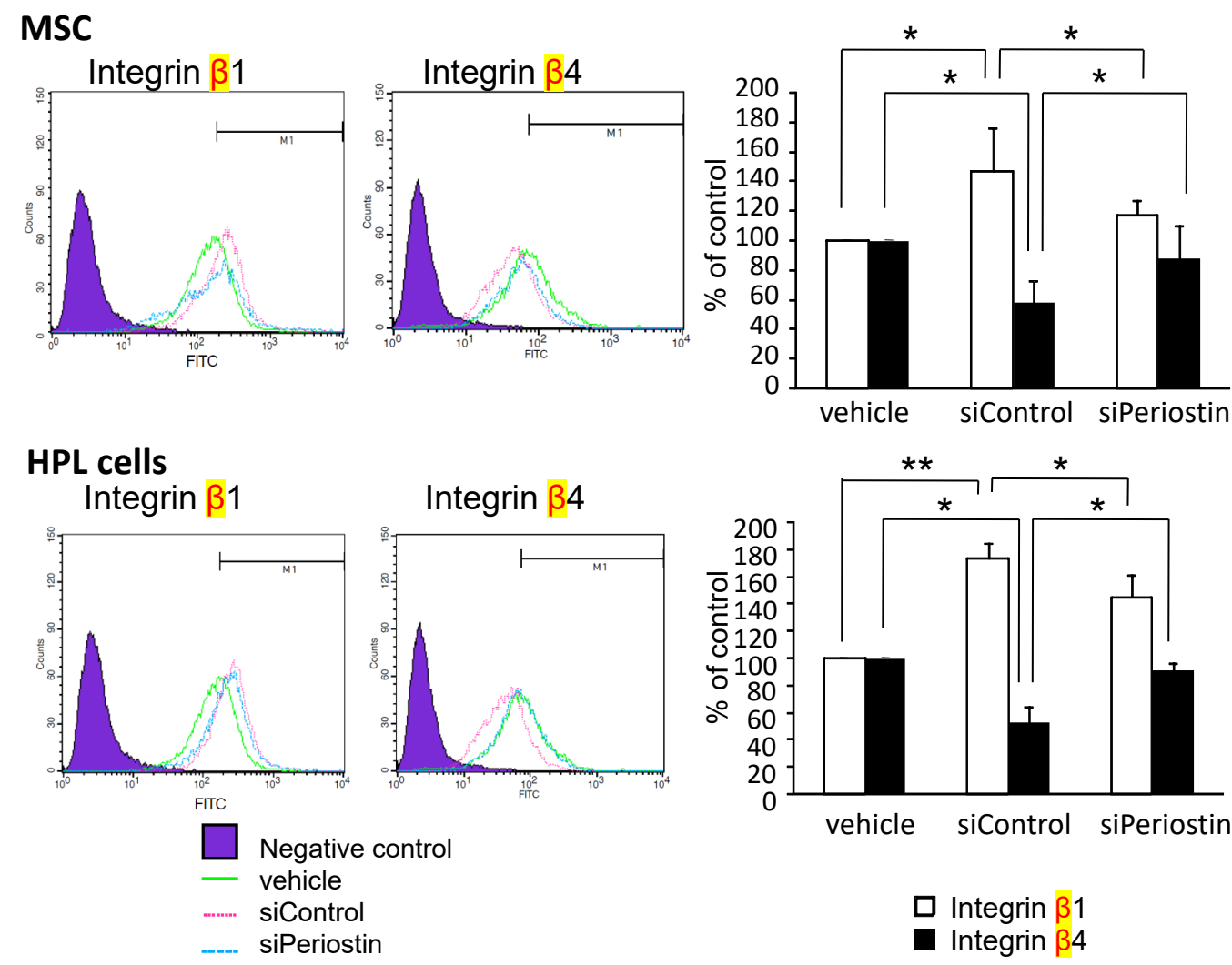
**(A)**



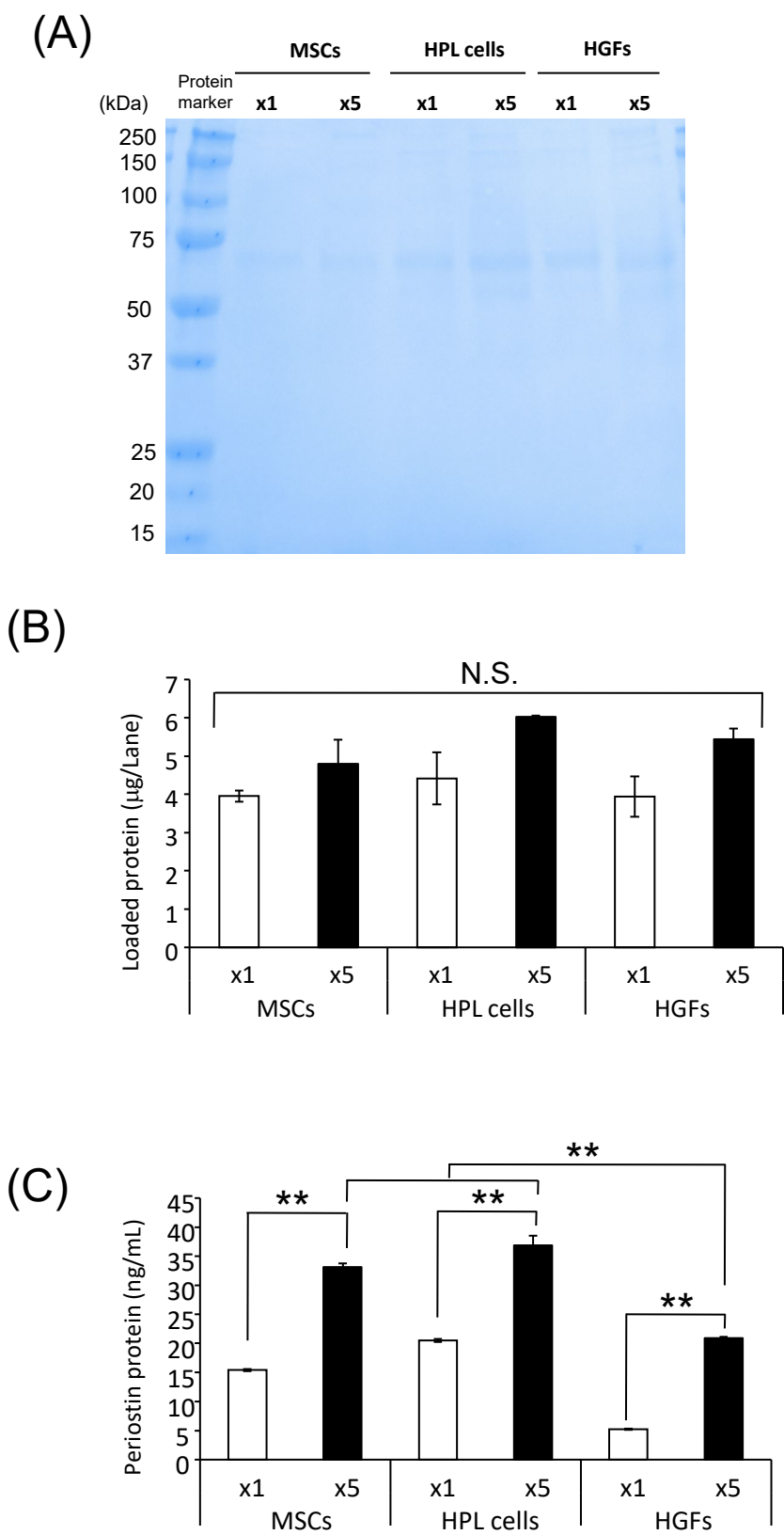
**(B)**



**(C)**



**Fig. S1.**





**Fig. S2.**

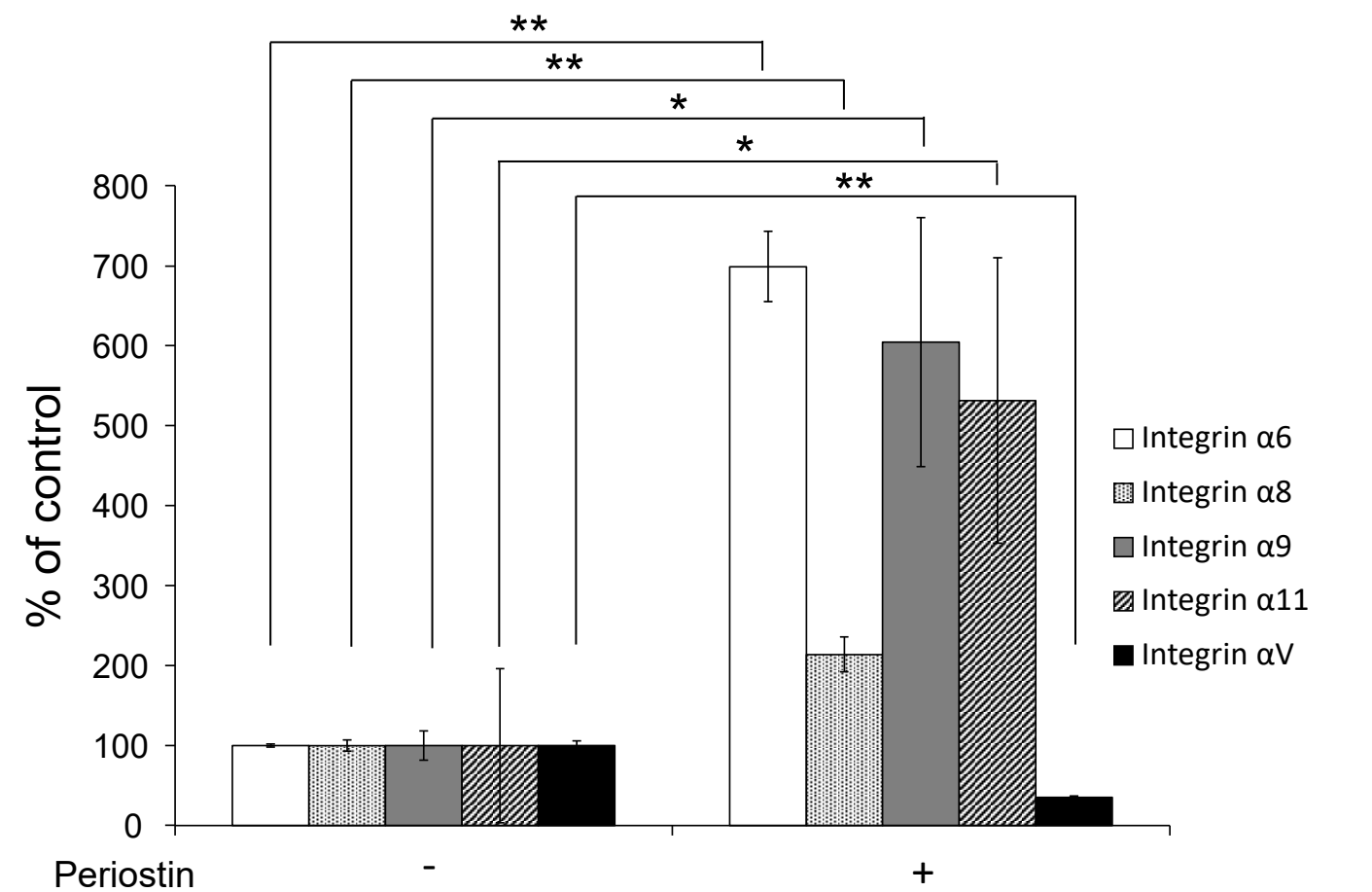


Fig. S3.

