Title	Periostin regulates integrin expression in gingival epithelial cells
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Relation	

Periostin regulates integrin expression in gingival epithelial cells

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[1]**Abbreviations:** HDACs, histone deacetylases; HGECs, 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 (HGECs) 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 HGECs. 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 HGECs, assuming periodontal tissue homeostasis.

Methods: Human gingival fibroblasts (HGFs), HGECs, HPL cells, and MSCs were cultured, and the conditioned medium was collected. With or without silencing periostin mRNA, HGECs 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 HGECs. 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 (HGECs) 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 β4, unique among integrin β subunits because of its markedly larger cytoplasmic domain, may link keratin filaments to these structures [7, 8]. Moreover, integrin β4 is expressed in keratinocytes from the human gingiva [9]. The suprabasal distribution of α6 has been proposed owing to its association with β1 in cell-to-cell contacts, whereas the polarized basal expression is associated with β4 in cell-to-extracellular matrix contacts [10]. Integrin β1 and integrin β4 are involved in epidermal growth and differentiation [11]. Furthermore, integrin β1 can be paired with almost all integrin α 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 αVβ3 and αVβ5 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 β1 and integrin β4.

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. HGECs were isolated from the gingivae of three volunteers, as previously described [1]. HGECs 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, HGECs 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 5×10^3 cells/cm^2 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 β1 (Assay No. Hs01127536_m1), integrin β4 (assay no. Hs00236216_m1), periostin (Assay No. Hs00170815_m1), integrin αV (Assay no. Hs00233808_m1), integrin α6 (Assay no.

Hs01041011_m1), integrin α8 (Assay no. Hs00233321_m1), integrin α9 (Assay no. Hs00979865_m1), integrin α11 (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 β1 (Millipore, Burlington, MA, USA) and β4 (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 (Pharma Foods International Co., Ltd, Tokushima, 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 HGECs

HGECs were cultured in a conditioned medium from MSCs or HPL cells for 0–48 h. Integrin β1 and β4 mRNAs were significantly downregulated at 12 h by a conditioned medium from HPL cells and MSCs (Fig. 1A). Integrin β4 mRNA was downregulated but integrin β1 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 β4 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 β1 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 β1 and suppressed β4 mRNA expression at 12 h (Fig. 4A). Periostin downregulated integrin β4 protein and mRNA expression at 24 h. In contrast, integrin β1 protein and mRNA expression levels were significantly enhanced by periostin at 48 h (Fig. 4B). As for integrin α subunits, the mRNA expression levels of integrin α6, α8, α9, and α11 were significantly upregulated by periostin. In contrast, integrin αV 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 β1 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 β1 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 β4 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 α9 and its ligands play regulatory roles in chronic periodontitis [35], and the mutation of the β2 integrin subunit can induce severe periodontal problems [36]. Moreover, the integrin β6 mutation can indirectly affect inflammasome and cytokine expression in periodontitis [37]. Interleukin-1β enhances cell adhesion through β4 integrins in HGECs [38]. In this study, HGECs were positively affected by periostin treatment in terms of mRNA expression of integrin α6, α8, α9, and α11, but negatively affected in terms of mRNA expression of integrin αV (Fig. S2). In other cells, such as MC3T3-E1, periostin positively supported the integrin αV-dependent cell adhesion and motility [20, 21] and the pair of integrin α6 and β1 could regulate keratinocyte function [39] or epithelial cells [40]. Consequently, periostin upregulated integrin β1 and downregulated integrin β4 (Fig. 4A); the pair of integrin β1-α6 and β4-αV 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 β1 [43], and the expression of integrin β1 or β4 is affected by antibacterial agents [44], which means that integrin β1 and β4 can be targets of bacterial infection. Therefore, based on their role in infection advancement and immune response, the regulation of integrins β1 and β4 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 β1 expression, whereas integrin β4 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 β1 or β4. 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 β1 and integrin β4 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.

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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 ± 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 ± 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 0.5×10^4 cells/cm^2 (× 1) or 2.5×10^4 cells/cm^2 (× 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 ± SD) in relation to the cells cultured without periostin (n = 4). Values were normalized by GAPDH and represent % of M1 gated cells (mean ± 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 ± 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 ± SD) in relation to the vehicle (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. 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 0.5×10^4 cells/cm^2 (× 1) or 2.5×10^4 cells/cm^2 (× 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 ± 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 ± SD; n = 4). **:$P < 0.01$, *$P < 0.05$ (Steel–Dwass test). HPL, human periodontal ligament; MSCs, mesenchymal stem cells; HGFs, human gingival fibroblasts.

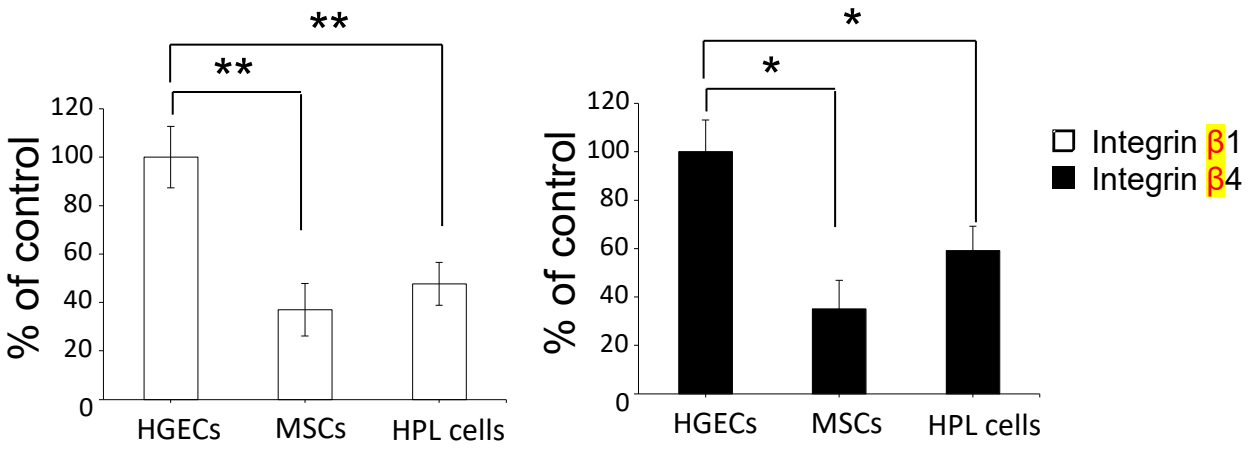
Fig. 2S. Periostin changed integrin α subunit expression in HGECs. HGECs were cultured with periostin (500 ng/mL) for 48 h, total RNA was extracted, and the cells were collected. Integrin αV, α6, α8, α9, and α11 mRNA expression levels were determined using quantitative real-time PCR. Values were normalized using GAPDH and represent mRNA expression (mean ± 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 ± 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 ± 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)


**
**
% of control
120
100
80
60
40
20
0
HGECs
MSCs
HPL cells
*
*
% of control
120
100
80
60
40
20
0
HGECs
MSCs
HPL cells
Integrin β1
Integrin β4

(B)

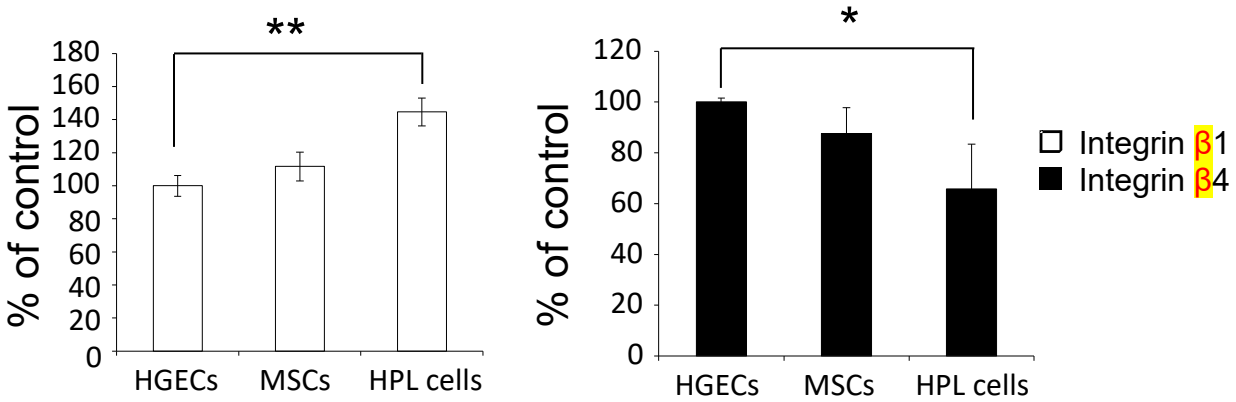

**
% of control
180
160
140
120
100
80
60
40
20
0
HGECs
MSCs
HPL cells
*
% of control
120
100
80
60
40
20
0
HGECs
MSCs
HPL cells
Integrin β1
Integrin β4

Fig.2.

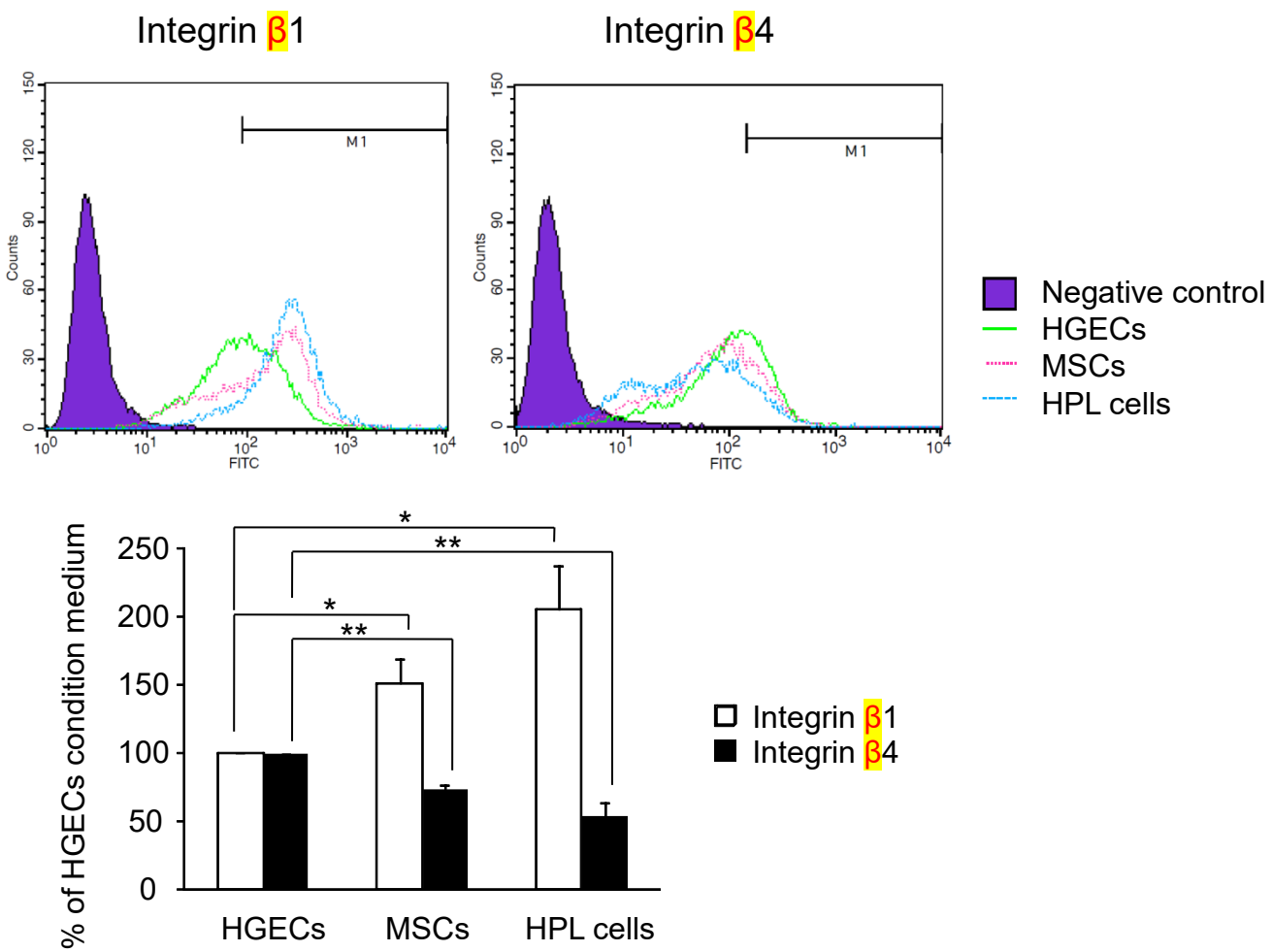

Integrin β1
Integrin β4
M1
Counts
FITC
Negative control
HGECs
MSCs
HPL cells
% of HGECs condition medium
250
200
150
100
50
0
*
**
HGECs
MSCs
HPL cells
Integrin β1
Integrin β4

Fig. 3.

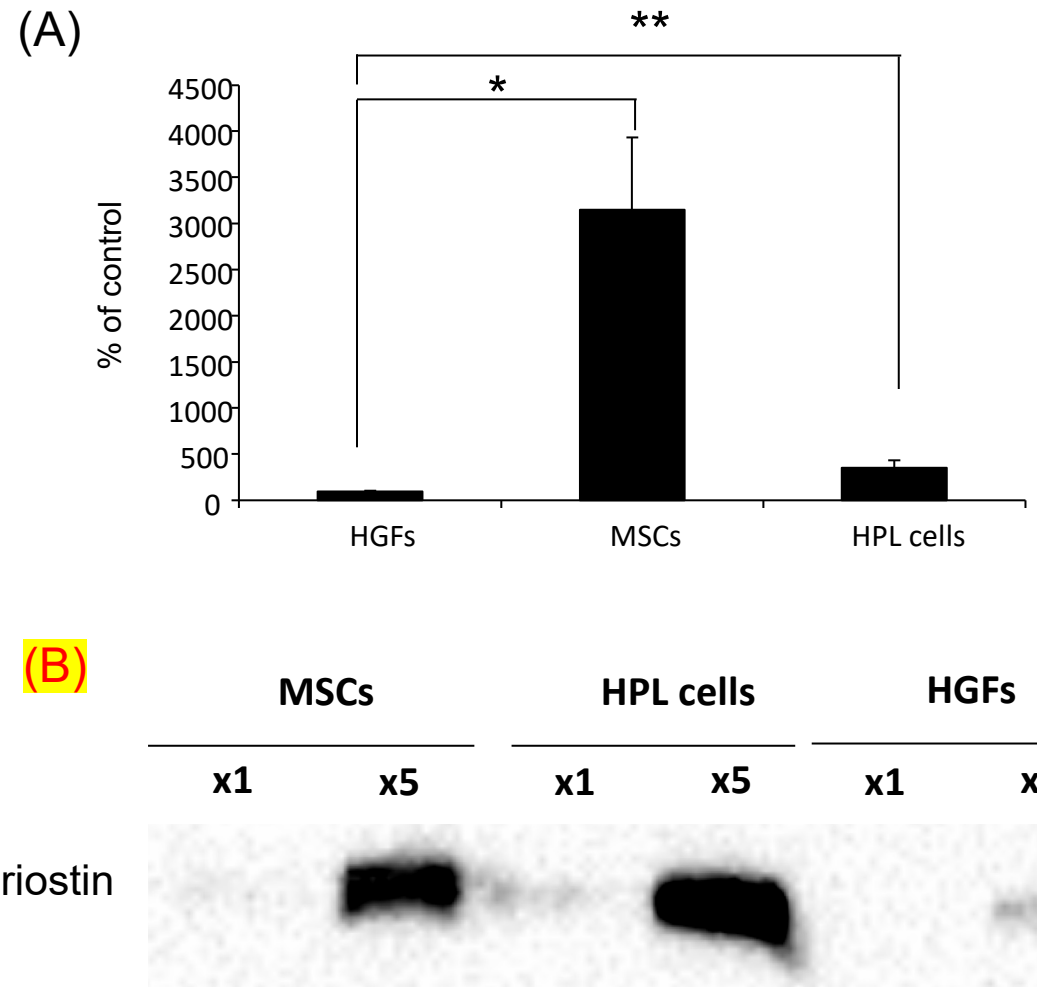
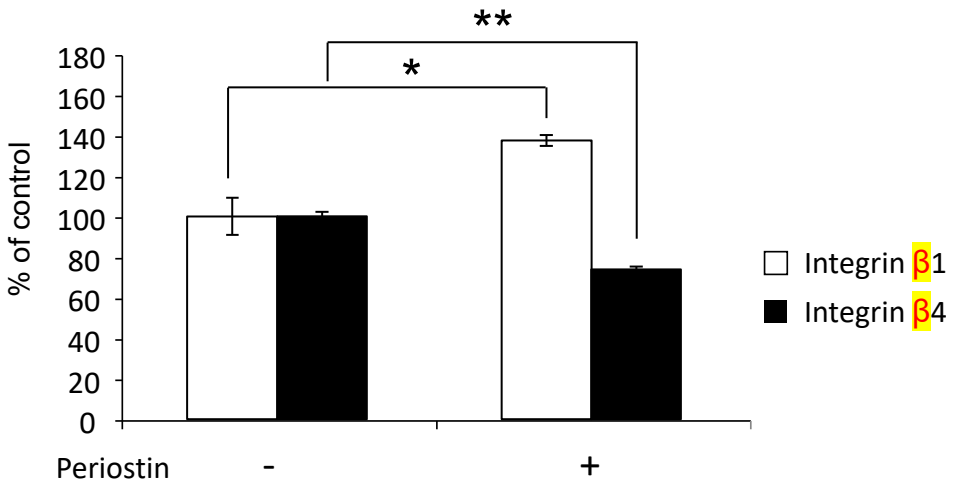

(A)
**
*
4500
4000
3500
3000
2500
2000
1500
1000
500
0
% of control
HGFs
MSCs
HPL cells
(B)
MSCs
HPL cells
HGFs
x1
x5
x1
x5
x1
x5
Periostin

Fig. 4.

(A)


**
*
180
160
140
120
100
80
60
40
20
0
% of control
Periostin
-
+
Integrin β1
Integrin β4

(B)

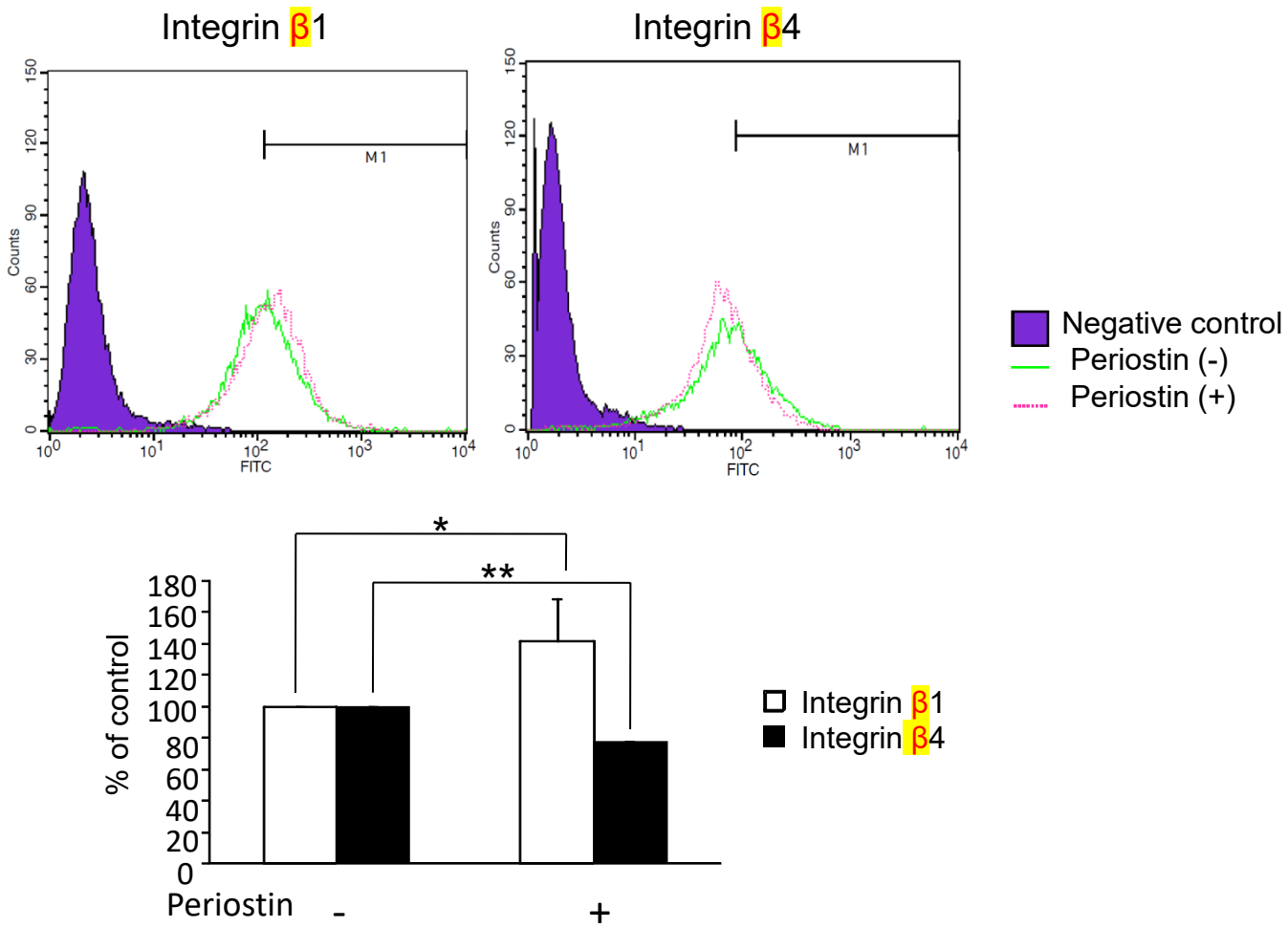
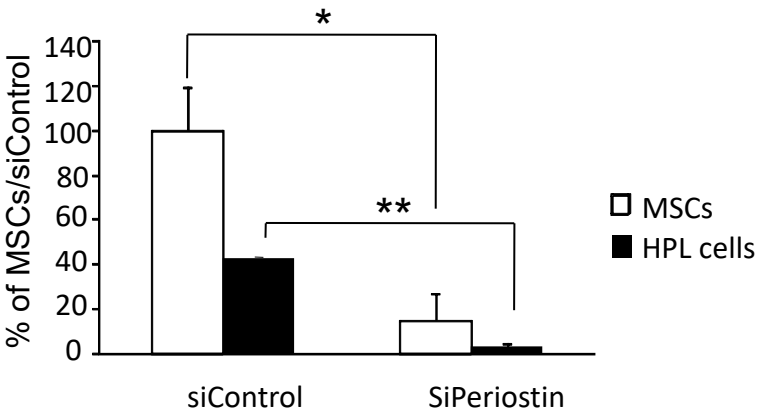
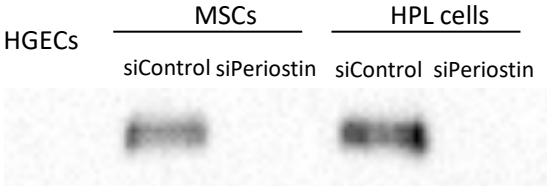

Integrin β1
Integrin β4
M1
M1
Counts
FITC
Negative control
Periostin (-)
Periostin (+)
*
**
% of control
Periostin
-
+
Integrin β1
Integrin β4

Fig. 5.

(A)


*
**
% of MSCs/siControl
140
120
100
80
60
40
20
0
siControl
SiPeriostin
MSCs
HPL cells

(B)


HGECs
MSCs
HPL cells
siControl
siPeriostin
siControl
siPeriostin

(C)

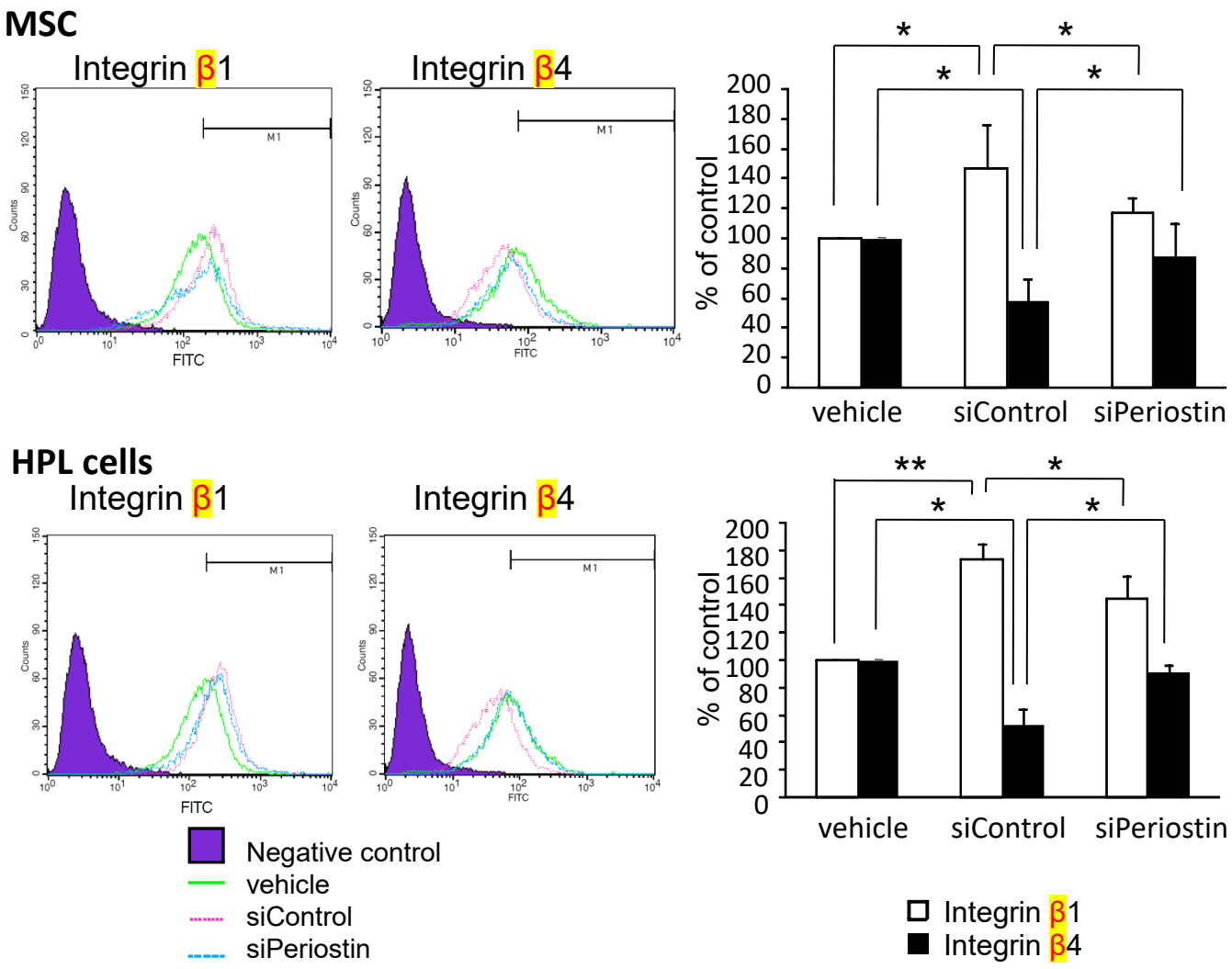
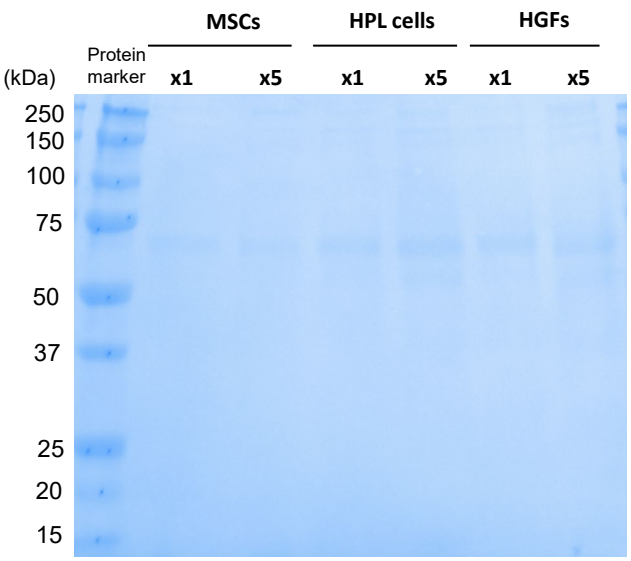
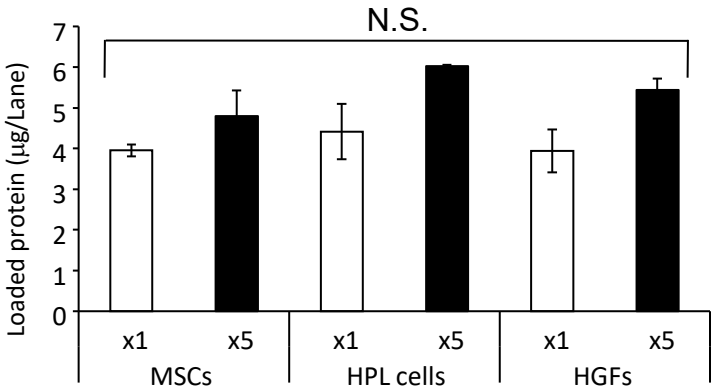

MSC
Integrin β1
Integrin β4
M1
Counts
FITC
% of control
vehicle
siControl
siPeriostin
HPL cells
Integrin β1
Integrin β4
Negative control
vehicle
siControl
siPeriostin
Integrin β1
Integrin β4

Fig. S1.

(A)


MSCs
HPL cells
HGFs
Protein marker
(kDa)
x1
x5
x1
x5
x1
x5
250
150
100
75
50
37
25
20
15

(B)


N.S.
Loaded protein (μg/Lane)
7
6
5
4
3
2
1
0
x1
x5
x1
x5
x1
x5
MSCs
HPL cells
HGFs

(C)

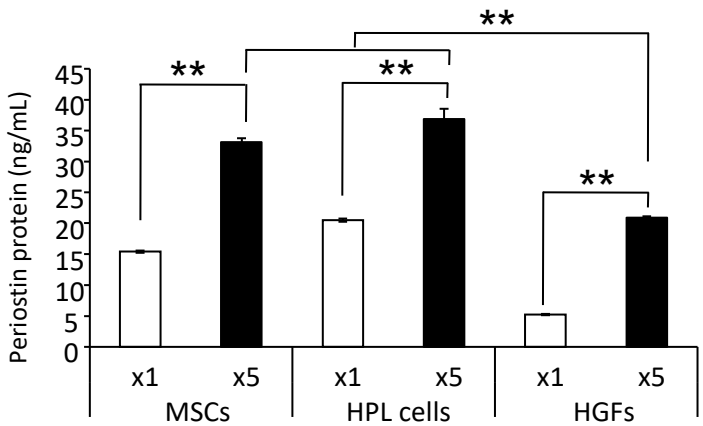

**
**
**
**
Periostin protein (ng/mL)
45
40
35
30
25
20
15
10
5
0
x1
x5
x1
x5
x1
x5
MSCs
HPL cells
HGFs

Fig. S2.

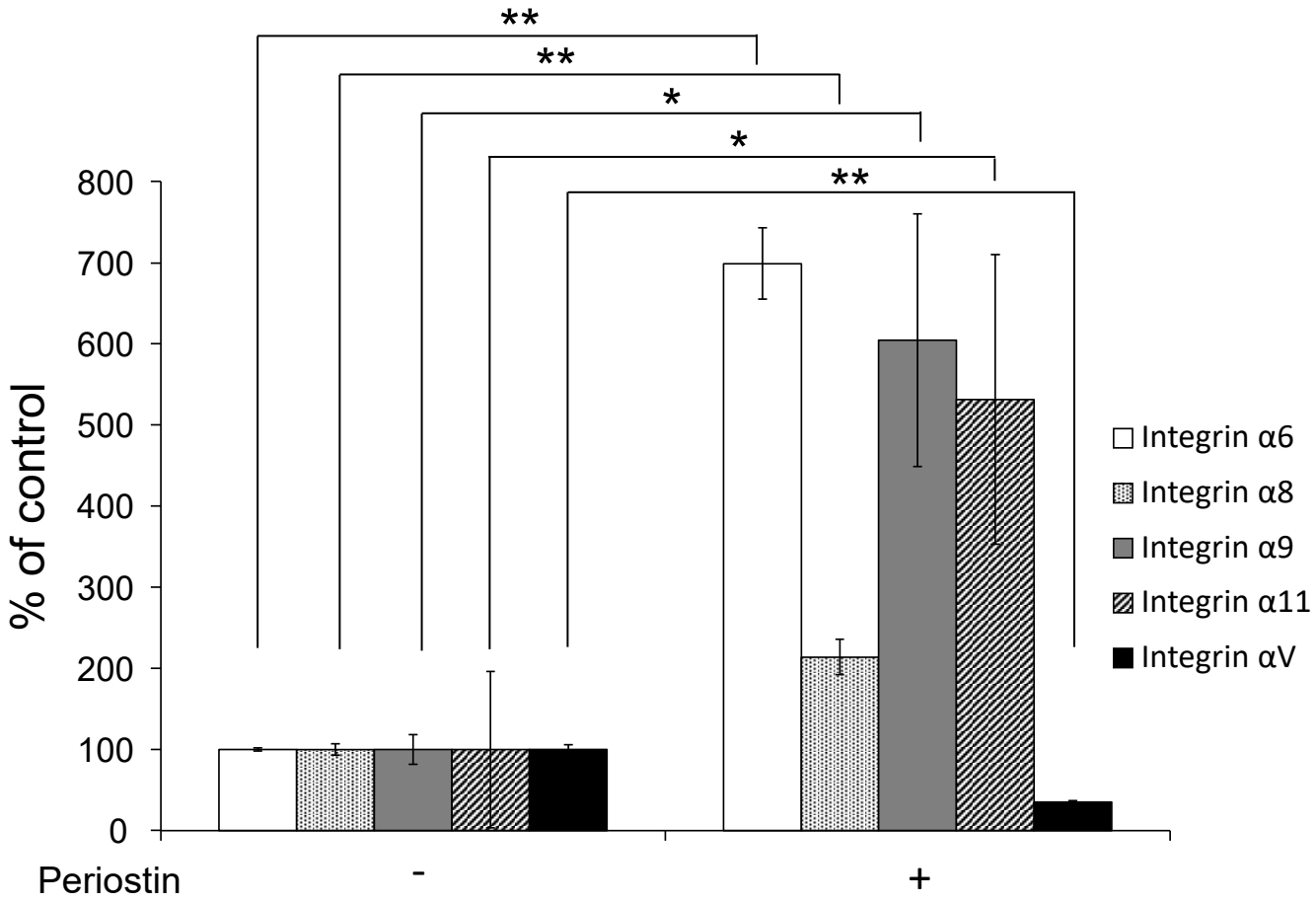
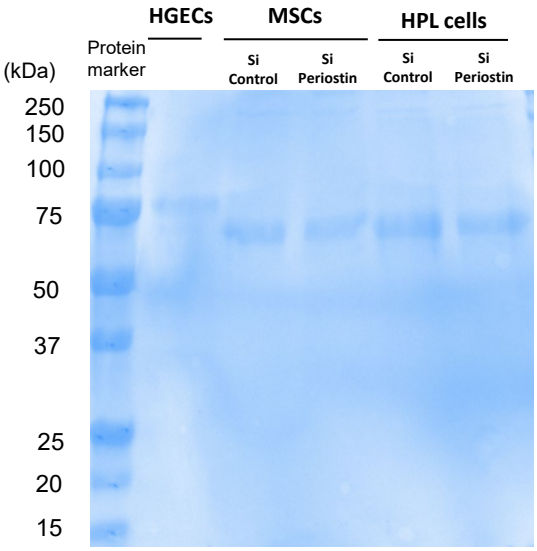
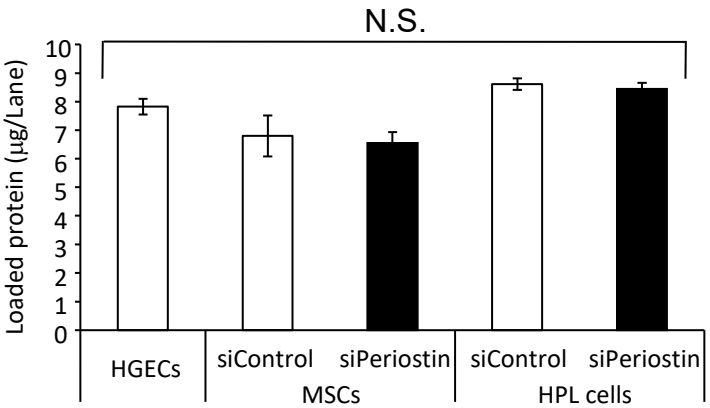

**
**
*
*
**
800
700
600
500
400
300
200
100
0
% of control
Periostin
-
+
Integrin α6
Integrin α8
Integrin α9
Integrin α11
Integrin αV

Fig. S3.

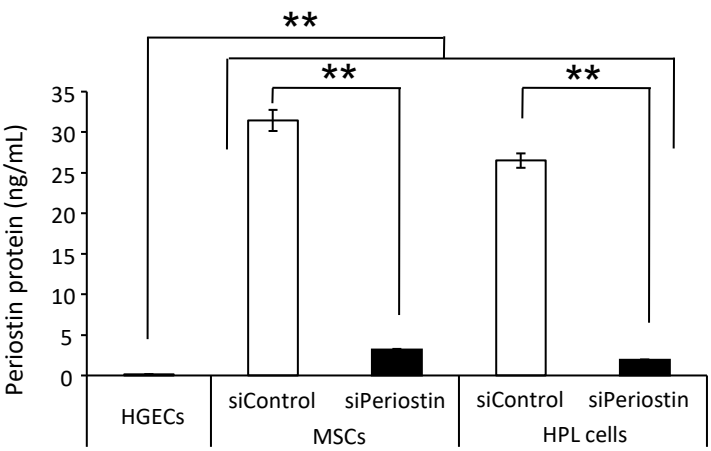
(A)


HGECs
MSCs
HPL cells
Protein marker
Si Control
Si Periostin
Si Control
Si Periostin
(kDa)
250
150
100
75
50
37
25
20
15

(B)


N.S.
Loaded protein (μg/Lane)
10
9
8
7
6
5
4
3
2
1
0
HGECs
siControl
siPeriostin
siControl
siPeriostin
MSCs
HPL cells

(C)


**
**
**
Periostin protein (ng/mL)
35
30
25
20
15
10
5
0
HGECs
siControl
siPeriostin
siControl
siPeriostin
MSCs
HPL cells