Transforming growth factor-beta-induced gene product-h3 inhibits odontoblastic differentiation of dental pulp cells

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Running title: Effect of βig-h3 on dental pulp cells

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Abstract

Objective: The aim of this study was to investigate transforming growth factor-β-induced gene product-h3 (βig-h3) expression in dental pulp tissue and its effects on odontoblastic differentiation of dental pulp cells (DPCs).

Design: A rat direct pulp capping model was prepared using perforated rat upper first molars capped with mineral trioxide aggregate cement. Human DPCs (HDPCs) were isolated from extracted teeth. βig-h3 expression in rat dental pulp tissue and HDPCs was assessed by immunostaining. Mineralization of HDPCs was assessed by Alizarin red-S staining. Odontoblast-related gene expression in HDPCs was analyzed by quantitative RT-PCR.

Results: Expression of βig-h3 was detected in rat dental pulp tissue, and attenuated by direct pulp capping, while expression of interleukin-1β and tumor necrosis factor-α was increased in exposed pulp tissue. βig-h3 expression was also detected in HDPCs, with reduced expression during odontoblastic differentiation. The above cytokines reduced βig-h3 expression in HDPCs, and promoted their mineralization. Recombinant βig-h3 inhibited the expression of odontoblast-related genes and mineralization of HDPCs, while knockdown of βig-h3 gene expression promoted the expression of odontoblast-related genes in HDPCs.

Conclusions: The present findings suggest that βig-h3 in DPCs may be involved in reparative dentin formation and that its expression is likely to negatively regulate this process.

Keywords: dental pulp; transforming growth factor-β-induced gene product-h3; odontoblastic differentiation; reparative dentin; direct pulp capping

1. Introduction

Dental pulp tissue does not become mineralized under physiological conditions. However, reparative dentin is formed on primary dentin in response to stimulation by caries, tooth cutting, or trauma. Dental pulp cells (DPCs) are considered to differentiate into odontoblasts prior to the production of reparative dentin. Several extracellular matrix (ECM) proteins in dentin, such as dentin sialophosphoprotein (DSPP), osteocalcin (OCN), and osteopontin (OPN), are considered to be related to odontoblastic differentiation. Various growth factors or cytokines, such as bone morphogenetic proteins, Wnt, and tumor necrosis factor (TNF)-α, were reported to be involved in odontoblastic differentiation of human DPCs (HDPCs), resulting in reparative dentinogenesis (Peng et al., 2010; Qin et al., 2012; Ueda et al., 2014). Moreover, activation of immunocompetent cells and secretion of inflammatory cytokines were observed in pulp tissue treated with pulp capping agents prior to reparative dentin formation (Goldberg, Njeh & Uzunoglu, 2015), suggesting that these processes may be involved in dentinogenesis. However, the underlying mechanism for odontoblastic differentiation of DPCs has not been revealed.

Transforming growth factor (TGF)-β is a multifunctional growth factor expressed not only in many types of adult tissues, but also in developing cartridge and pituitary gland (Bazina, Vukojevic, Roje & Saraga-Babic, 2009; Moroco, Hinton, Buschang, Milam & Iacopino, 1997). TGF-β is known to regulate cell proliferation, cell migration, ECM synthesis, and inflammatory processes (Cubela et al., 2016; Fujii et al., 2010; ten Dijke, & Arthur, 2007). TGF-β-induced gene product-h3 (βig-h3) is a one of the ECM proteins induced by TGF-β (Skonier et al., 1992). βig-h3 has a signal sequence at the

N-terminus, an RGD sequence in the C-terminus, and a FAS1 domain composed of four repeating sequences. The protein has been detected in various tissues, such as skin, blood vessels, and smooth muscle (Billings et al., 2000; LeBaron et al., 1995; Schorderet et al., 2000), and is involved in cell attachment, cell spreading, angiogenesis, and tumorigenesis (Aitkenhead et al., 2002; Ma et al., 2008; Ohno et al., 1999).

βig-h3 contributes to cell-matrix attachment through interactions of its RGD sequence with integrin subtypes, such as αvβ3 and αvβ5 (Choi et al., 2015; Ma et al., 2008). βig-h3 was also shown to interact with other ECM components, such as collagen I (Hashimoto et al., 1997). βig-h3 expression was observed in osteoblasts and periodontal ligament cells, while recombinant βig-h3 inhibited osteoblastic maturation (Thapa, Kang, & Kim, 2005) and osteoblastic differentiation of periodontal ligament cells (Ohno et al., 2002). However, the expression and functions of βig-h3 in dental pulp tissue have not been examined.

We hypothesized that βig-h3 is involved in reparative dentinogenesis and odontoblastic differentiation of DPCs. To explore this hypothesis, we investigated βig-h3 expression in dental pulp tissue after direct pulp capping, and its effects on odontoblastic differentiation of DPCs.

2. Materials and methods

2.1. Cell culture

Three samples of HDPCs were isolated from healthy teeth of a 24-year-old male (HDPC-3R), 27-year-old female (HDPC-3F), and 24-year-old female (HDPC-5A) with

informed consent as described previously (Yoshida et al., 2016). Briefly, the extracted teeth were split and the dental pulp tissue was removed. The obtained pulp tissue was digested with 0.2% collagenase and 0.25% trypsin (Wako Pure Chemical Industries, Osaka, Japan) for 20 min at 37°C to isolate single-cell populations. HDPCs at passages 3–8 were maintained in control medium (CM), defined as α-minimal essential medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Biowest, Nuaille, France), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. Reagents

The goat anti-rat βig-h3 polyclonal antibody, rabbit anti-rat interleukin (IL)-1β polyclonal antibody, normal goat IgG, and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Alexa Fluor 568-conjugated rabbit anti-goat IgG antibody was purchased from Invitrogen (Carlsbad, CA). The rabbit anti-rat TNF-α polyclonal antibody, recombinant human IL-1β, and recombinant human TNF-α were purchased from PeproTech EC (London, UK). Recombinant human βig-h3 was purchased from R&D Systems (Minneapolis, MN). βig-h3 small-interfering RNA (siRNA) and control siRNA were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Direct pulp capping model

A direct pulp capping model was prepared in rats as described previously (Yoshida et al., 2016). Briefly, the upper first molars of 8-week-old male Wistar rats (Kyudo, Saga, Japan) were perforated through the occlusal surface with a no. 1/2 round steel bur.

Direct pulp capping was performed with ProRoot mineral trioxide aggregate (MTA; Dentsply-Sankin, Tokyo, Japan) and sealed with glass ionomer cement (Fuji IX; GC Corporation, Tokyo, Japan).

2.4. Immunofluorescence analysis

HDPCs cultured in CM for 24 h were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) containing 0.5% dimethyl sulfoxide (Wako Pure Chemical Industries) for 20 min, and washed with phosphate-buffered saline (PBS). After blocking with 2% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) in PBS for 1 h, the cells were incubated with anti-βig-h3 goat antibody or normal goat IgG for 1 h, washed with PBS, and incubated with Alexa Fluor 568-conjugated rabbit anti-goat IgG antibody for 30 min. After washing with PBS, nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Fluorescence images were observed using a Biozero digital microscope (Keyence, Osaka, Japan).

2.5. Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously (Hasegawa et al., 2015). Briefly, the upper first molars of direct pulp capping model rats or normal rats were embedded in paraffin and sectioned at 5-μm thickness. After blocking with 2% BSA in PBS for 1 h, the sections were incubated with anti-βig-h3 goat antibody, anti-IL-1β rabbit antibody, anti-TNF-α rabbit antibody, or normal IgG overnight. The

sections were then treated with biotinylated anti-goat IgG antibody or anti-rabbit IgG antibody, followed by avidin-peroxidase conjugate (Nichirei Bioscience, Tokyo, Japan). Positive reactions were visualized using DAB solution (Nichirei Bioscience). Nuclear staining was performed with Mayer's hematoxylin solution (Wako Pure Chemical Industries).

2.6. Odontoblastic differentiation of HDPCs

HDPCs were cultured in CM or CM containing 2 mM $CaCl_2$ as differentiation medium (DM), based on a recent report that this DM promoted osteoblastic differentiation of human periodontal ligament cells (Koori et al., 2014). The expression of odontoblast-related genes (DSPP, OCN, and OPN) in HDPCs was assessed by quantitative RT-PCR. Mineralization of HDPCs was evaluated by Alizarin red-S staining.

2.7. Semi-quantitative RT-PCR

Total cellular RNA was harvested using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription (RT) with an ExScript RT Reagent Kit (Takara Bio, Shiga, Japan). Polymerase chain reaction (PCR) assays were performed with a cycle consisting of heat denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s. The primer sequences, product sizes, annealing temperatures, and cycle numbers for integrin αv, β3, and β5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in

Table 1. The PCR products were electrophoresed in a 2% agarose gel (Seakem ME; BioWhittaker Molecular Applications, Rockland, ME).

2.8. Quantitative RT-PCR

Total cellular RNA was harvested using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized by RT with an ExScript RT Reagent Kit (Takara Bio). Quantitative PCR assays were performed with a SYBR Green RT-PCR Kit (Takara Bio) using a Thermal Cycler Dice Real Time System (Takara Bio) with the following program: 95°C for 10 s; 40 cycles at 95°C for 5 s and 60°C for 30 s; dissociation cycle at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. A human β-actin primer set was used as an internal standard. The expression levels of the target genes were determined by their delta–delta Ct (ΔΔCt) values. The primer sequences, product sizes, annealing temperatures, and cycle numbers for βig-h3, DSPP, OCN, OPN, and β-actin are shown in Table 2.

2.9. Alizarin red-S staining

HDPCs were fixed for 1 h with 10% formalin. After washing with sterile water, the cells were incubated with Alizarin red-S solution (pH 4.1–4.3) (Sigma-Aldrich) for 1 h. Positive areas were quantified using a Biozero digital microscope (Keyence).

2.10. Cell proliferation assay

HDPCs were cultured on 48-well plates for various periods in CM with or without βig-h3 or BSA (10 μg/ml). The proliferation rates were analyzed using a Premix WST-1 Cell Proliferation Assay System (Takara Bio) according to the manufacturer's instructions.

2.11. siRNA transfection

HDPCs were transfected with βig-h3 siRNA or control siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, cells at 80% confluence were cultured in Opti-MEM I (Invitrogen) containing 10% FBS. A siRNA–lipid complex, comprising 10 pmol siRNA and 3 μl of Lipofectamine RNAiMAX in 50 μl of Opti-MEM, was prepared. After incubation for 5 min at room temperature, the complex was added to the cells and incubated for 24 h.

2.12. Statistical analysis

All values are expressed as mean ± standard deviation. Statistical analyses were performed by one-way ANOVA followed by the Benjamini-Hochberg method for multiple comparisons. Student's paired *t*-test was performed for comparisons of two mean values. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Expression of βig-h3 in dental pulp tissue after direct pulp capping treatment

βig-h3 expression in normal dental pulp tissue was examined by immunohistochemistry using rat maxillary first molar sagittal sections. Expression of βig-h3 was detected throughout the dental pulp tissue (Fig. 1A, B), while weak expression was noted in odontoblasts. In negative control sections with normal goat IgG, no positive reactions were detected (Fig. 1C).

Next, βig-h3 expression was investigated during reparative dentin formation after direct pulp capping treatment (Fig. 1D, E). At 14 days after treatment, reparative dentin formation with a tubular structure (surrounded by broken lines in Fig. 1D) was observed in the pulp-exposed region. Expression of βig-h3 was found to be attenuated in DPCs beneath the reparative dentin, and little expression was observed in the odontoblast layer adjacent to the reparative dentin (Fig. 1E).

3.2. Expression of βig-h3 during odontoblastic differentiation of HDPCs

In immunofluorescence analyses, the anti-βig-h3 antibody revealed positive signals in HDPCs, while the negative control IgG showed no signals (Fig. 2A, B). Next, we examined the mineralization and expression of odontoblast-related genes in HDPCs cultured in odontoblastic induction medium containing 2 mM $CaCl_2$. HDPCs exhibited Alizarin red-positive reactions together with increased expression of DSPP, OCN, and OPN (Fig. 2C–F). However, βig-h3 gene expression was decreased under these culture conditions (Fig. 2G).

3.3. Association of IL-1β and TNF-α with βig-h3 expression in rat dental pulp tissue during reparative dentinogenesis

The factors involved in the regulation of βig-h3 expression in HDPCs were examined. Based on our finding that βig-h3 expression was attenuated in the dental pulp tissue in the direct capping model (Fig. 1), the expression of inflammatory cytokines in the exposed pulp tissue was examined. Immunohistochemical analyses revealed increased positive reactions in the pulp tissue beneath the exposed area for both the anti-IL-1β (Fig. 3D, E) and anti-TNF-α (Fig. 3I, J) antibodies, compared with the normal pulp tissue (Fig. 3A, B, F, G). In the negative control experiments using normal rabbit IgG, no positive reactions were detected (Fig. 3C, H).

3.4. Effects of IL-1β and TNF-α on βig-h3 expression and odontoblastic differentiation of HDPCs

Quantitative RT-PCR assays were performed to examine the effects of IL-1β and TNF-α on βig-h3 gene expression in HDPCs. The expression of βig-h3 in HDPCs treated with 10 ng/ml IL-1β or TNF-α was found to be attenuated (Fig. 4A, B).

Moreover, the effects of these cytokines on odontoblastic differentiation of a human dental pulp stem cell line, HDPC-4A-1, observed in our recent report (Yoshida et al., 2016) were examined. IL-1β and TNF-α facilitated Alizarin red-S positive reactions, compared with the control culture in DM (Fig. 4C, D). The cells cultured in CM showed no positive reactions.

3.5. Effects of βig-h3 on proliferation and odontoblastic differentiation of HDPCs

Prior to investigation of βig-h3 functions, the gene expression of integrin αv, β3, and β5 subunits, as βig-h3 receptors, in HDPCs was verified by semi-quantitative RT-PCR assays (Fig. 5A). In addition, the effect of βig-h3 on the proliferative capacity of HDPCs was examined by WST-1 cell proliferation assays. After 3 days of incubation, no significant differences were detected between the βig-h3-treated cells and the control cells, with all groups of cells showing similar proliferation ratios (Fig. 5B). To further examine the effects of βig-h3 on odontoblastic differentiation of HDPCs, the cells were cultured in CM and DM in the presence or absence of 1 or 10 μg/ml recombinant human βig-h3. After 7 days of incubation, positive reactions for Alizarin red-S staining were observed in DM-cultured cells (Fig. 5C; Fig. S1). However, 10 μg/ml βig-h3 inhibited the formation of these reactions. Meanwhile, 1 μg/ml βig-h3 and 10 μg/ml BSA showed similar results to the control culture in DM (Fig. 5C, D; Fig. S1). Next, the expression of odontoblast-related genes (DSPP, OCN, and OPN) in HDPCs cultured under the same conditions was examined by quantitative RT-PCR assays. Treatment with 10 μg/ml βig-h3 significantly suppressed the expression of these genes, compared with control cells, including untreated and BSA-treated cells (Fig. 5E–G; Fig. S2).

3.6. Effects of knockdown of βig-h3 gene expression on odontoblastic differentiation of HDPCs

To verify the suppressive effect of βig-h3 on odontoblastic differentiation of HDPCs, the cells were transduced with βig-h3 siRNA or control siRNA for 24 h. First, the

suppression of βig-h3 gene expression in HDPCs was confirmed by quantitative RT-PCR assays (Fig. 6A). The transduced HDPCs were then cultured in DM for 5 days. Quantitative RT-PCR assays revealed that the gene expression of DSPP, OCN, and OPN in βig-h3 siRNA-transduced HDPCs was significantly increased, compared with that in control cells (Fig. 6B–D; Fig. S3).

4. Discussion

A direct pulp capping model was used to observe the dental pulp during reparative dentin formation. Direct pulp capping using MTA cement was reported to promote reparative dentin formation (Okiji & Yoshiba, 2009). MTA was shown to release calcium ions (Maeda et al., 2010), and the resulting elevation of the extracellular Ca^{2+} concentration induced odontoblastic differentiation of HDPCs (Li et al., 2015; Tada et al., 2010). In the present study, we verified that reparative dentin was formed beneath the exposure site capped by MTA, and also that the expression of odontoblast-related genes, namely DSPP, OCN, and OPN, was promoted in HDPCs cultured in the presence of calcium. However, βig-h3 expression was reduced during reparative dentin formation, and showed the opposite expression pattern to the above genes during odontoblastic differentiation of HDPCs stimulated by the presence of calcium. Therefore, the present results suggested that reparative dentin formation associated with MTA pulp capping may be promoted by downregulation of βig-h3 expression via the release of calcium ions.

DSPP is expressed in odontoblasts and dentin matrix and contributes to mineralization (Suzuki, Haruyama, Nishimura, & Kulkarni, 2012). OCN is a late

marker of osteoblasts and also used as a marker of odontoblastic differentiation of HDPCs (Wei, Ling, Wu, Liu, & Xiao, 2007). OPN is a phosphoprotein involved in bone remodeling and expressed in odontoblasts and reparative dentin (Zhang, Fan, Bian, Chen, & Zhu, 2000). These three odontoblast-related ECM proteins are thought to be central proteins for dentinogenesis.

IL-1β was reported to be upregulated in HDPCs from patients diagnosed with pulpitis (Barkhordar, Ghani, Russell, & Hussain, 2002). TNF-α was upregulated in dental pulp after pulp exposure (Tani-Ishii, Wang, & Stashenko, 1995), and promoted odontoblastic differentiation of HDPCs or osteoblastic differentiation of bone marrow cells (Mountziaris, Tzouanas, & Mikos, 2010; Ueda et al., 2014; Wang, Xu, Liu, Zhang, & Lu, 2015). Our results suggested that increased expression of these cytokines in exposed pulp tissue may promote odontoblastic differentiation of immature DPCs. Moreover, we found that these cytokines inhibited βig-h3 gene expression. These findings suggested that the cytokines increased after pulp exposure may trigger reparative dentinogenesis through suppression of βig-h3 expression.

It was previously reported that βig-h3 expressed in human periodontal ligament cells inhibited their osteoblastic differentiation (Ohno et al., 2002), similar to the present results. Periostin, a protein showing high homology with βig-h3, was reported to be expressed in dental pulp tissue, and to inhibit odontoblastic differentiation of DPCs (Zhou et al., 2015). Taken together, βig-h3 and periostin may contain a specific common sequence that works toward inhibition of odontoblastic differentiation. Furthermore, as dental pulp tissue and periodontal ligament tissue are unmineralized under physiological conditions, βig-h3 may be involved in the inhibition of ectopic mineralization in these tissues through such a specific region. Meanwhile, suppression of βig-h3 expression

would play a role to induce reparative dentin formation by promoting the expression of several odontoblast-related genes. In pulp tissue, the expression of βig-h3 may be regulated depending on the microenvironment. Considering our results, it is possible that its expression was downregulated by inflammatory cytokines during reparative dentinogenesis. If a pulp capping agent that can regulate βig-h3 expression is developed, it may be able to prevent rapid and excessive formation of spongy reparative dentin or ectopic dentin formation and allow mature dentin formation with dentinal tubules.

5. Conclusion

In this study, it was revealed for the first time that βig-h3 was expressed in dental pulp tissue and affected odontoblastic differentiation of DPCs. βig-h3 expression was attenuated in the pulp tissue after pulp capping treatment, while the expression of inflammatory cytokines, such as IL-1β and TNF-α, was increased. These cytokines inhibited βig-h3 gene expression, but induced odontoblastic differentiation of HDPCs. Exogenous βig-h3 inhibited odontoblastic differentiation of HDPCs, but not via toxicity because it did not alter the proliferation capacity of HDPCs, while βig-h3 gene silencing promoted cell differentiation. These results allow us to further understand the detailed mechanism of reparative dentinogenesis, and will be useful for the development of more predictable conservative treatments of the dental pulp in the future.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

All procedures were approved by our Animal Ethics Committee and Research Ethics Committee, and conformed to the regulations of our institution.

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Table 1

Primer sequences, product sizes, annealing temperatures, and cycle numbers for semi-quantitative RT-PCR

Target gene	Primer sequence forward/reverse	Annealing temperature (℃)	Cycle numbers	Size of products (bp)	Sequence ID
Integrin αv	5’-GCCCATGCCTGTGCCTGTGT-3’ 5’-TGAGGTGGCCGGACCCGTTT-3’	58	32	120	NM_001144999.2
Integrin β3	5’-CCTACATGACGAAAATACCT-3’ 5’-AATCCCTCCCCACAAATACTG-3’	53	34	517	NM_000212.2
Integrin β5	5’-TGCCATGCAGGTTACATCGG-3’ 5’-ATCATGACGCAGTCCTTGGC-3’	60	34	353	NM_002213.4
GAPDH	5’-ACCACAGTCCATGCCATCCAC-3’ 5’-TCCACCACCCTGTTGCTGTA-3’	60	19	452	NM_001256799.2

Table 2

Primer sequences, product sizes, annealing temperatures, and cycle numbers for quantitative RT-PCR

Target gene	Primer sequence forward/reverse	Annealing temperature (℃)	Cycle numbers	Size of products (bp)	Sequence ID
βig-h3	5'-TCCTGAAATACCACATTGGTGATGA-3'	60	40	160	NM_000358.2
	5'- GACATGGACCACGCCATTTG-3'				
DSPP	5'-ATATTGAGGGCTGGAATGGGGA-3'	60	40	136	NM_014208.3
	5'-TTTGTGGCTCCAGCATTGTCA-3'				
OCN	5'-CCCAGGCGCTACCTGTATCAA-3'	60	40	112	NM_199173.5
	5'-GGTCAGCCAACTCGTCACAGTC-3'				
OPN	5'-ACACATATGATGGCCGAGGTGA-3'	60	40	116	NM_000582.2
	5'-TGTGAGGTGATGTCCTCGTCTGT-3'				
β-actin	5'-ATTGCCGACAGGATGCAGA-3'	60	40	89	NM_001101.3
	5'-GAGTACTTGCGCTCAGGAGGA-3'				

Figure legends

Fig. 1. Expression of βig-h3 in rat pulp tissue. (**A–E**) Immunohistochemical staining was performed on sagittal sections of maxillary first molars from normal rats (**A–C**) and direct pulp capping model rats (**D, E**) using an anti-βig-h3 antibody at 14 days after treatment. (**A**) βig-h3 expression in the normal dental pulp tissue (×4). (**B**) Higher magnification of the red rectangle in (**A**) (×40). (**C**) Negative control staining with normal goat IgG. (**D**) βig-h3 expression in the direct pulp capping model (×4). Reparative dentin (surrounded by broken lines) was formed under the pulp capping with MTA. (**E**) Higher magnification of the red rectangle in (**D**) (×40). Positive reactions are shown in brown. Nuclei were stained with hematoxylin. DP, dental pulp; Od, odontoblast; Dn, dentin; RD, reparative dentin; broken line, pulp exposure region; yellow line, MTA filling. Bars: **A, C, D** = 500 μm; **B, E** = 50 μm.

Fig. 2. Expression of βig-h3 and odontoblastic differentiation of HDPCs. (**A**) Immunofluorescence staining with an anti-βig-h3 antibody revealed positive signals in HDPC-3R cells (red). Nuclei were stained with DAPI (blue). Bars: 25 μm. (**B**) Negative control staining with normal goat IgG. (**C**) Alizarin red-S staining of HDPCs cultured in CM or DM for 7 days. Positive signals were observed in the DM-cultured cells. (**D–F**) The expression of odontoblast-related genes, namely DSPP (**D**), OCN (**E**), and OPN (**F**), and βig-h3 (**G**) in HDPCs cultured in CM or DM for 5 days was examined by quantitative RT-PCR assays. Data are shown as mean ± standard deviation ($n = 3$). *$p < 0.05$, **$p < 0.01$.

Fig. 3. Expression of IL-1β and TNF-α in the rat direct pulp capping model. Immunohistochemical staining was performed on sagittal sections of maxillary first molars from normal rats and direct pulp capping model rats using anti-IL-1β (**A, B, D, E**) and anti-TNF-α (**F, G, I, J**) antibodies at 3 days after treatment. (**A**) IL-1β expression in the normal dental pulp tissue (×4). (**B**) Higher magnification of the red rectangle in (**A**) (×20). (**C**) Negative control staining with normal rabbit IgG. (**D**) IL-1β expression in the direct pulp capping model (×4). (**E**) Higher magnification of the red rectangle in (**D**) (×20). (**F**) TNF-α expression in the normal dental pulp tissue (×4). (**G**) Higher magnification of the red rectangle in (**F**) (×20). (**H**) Negative control staining with normal rabbit IgG. (**I**) TNF-α expression in the direct pulp capping model (×4). (**J**) Higher magnification of the red rectangle in (**I**) (×20). Positive reactions are shown in brown. Nuclei were stained with hematoxylin. DP, dental pulp; Dn, dentin; broken line, pulp exposure area; yellow line, MTA filling. Bars: **A, C, D, F, H, I** = 500 μm; **B, E, G, J** = 100 μm.

Fig. 4. Effects of IL-1β and TNF-α on βig-h3 expression and mineralization of HDPCs. (**A**) The expression of βig-h3 in HDPCs treated with 10 ng/ml IL-1β for 12 h was examined by quantitative RT-PCR assays. (**B**) The expression of βig-h3 in HDPCs treated with 10 ng/ml TNF-α for 3 days was examined by quantitative RT-PCR assays. (**C**) Alizarin red-S staining of HDPCs treated with IL-1β or TNF-α in CM or DM for 7 days. The positive reactions were increased in the cytokine-treated cells. (**D**) The positive areas were quantified and shown in a graph. Data are shown as mean ± standard deviation ($n = 3$). $^{**}p < 0.01$.

Fig. 5. Effects of exogenous βig-h3 on odontoblastic differentiation of HDPCs. (**A**) The expression of βig-h3 receptors in HDPC-3R cells was confirmed by semi-quantitative RT-PCR assays. (**B**) The proliferative capacity of HDPCs cultured with βig-h3 was assessed by WST-1 cell proliferation assays. No significant difference was detected between the βig-h3-treated cells and the non-treated or BSA-treated cells. Data are shown as mean ± standard deviation (n = 3). (**C**) Alizarin red-S staining of HDPCs treated with or without βig-h3 or BSA in CM or DM for 7 days. The positive reactions were attenuated in the 10 μg/ml βig-h3-treated cells. (**D**) The Alizarin red-S positive areas were quantified and shown in a graph. (**E–G**) The expression of odontoblast-related genes, namely DSPP (**E**), OCN (**F**) and OPN (**G**), in HDPCs treated with 10 μg/ml βig-h3 or BSA for 5 days was examined by quantitative RT-PCR assays. BSA was used as a negative control. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. 6. Effects of knockdown of βig-h3 gene expression on odontoblastic differentiation of HDPCs. (**A**) HDPCs were transduced with βig-h3 siRNA (siβig-h3) or control siRNA (Cont) for 24 h. Suppression of βig-h3 gene expression was confirmed by quantitative RT-PCR assays. (**B–D**) The expression of odontoblast-related genes, namely DSPP (**B**), OCN (**C**) and OPN (**D**), in the transduced cells cultured in DM for 5 days was examined by quantitative RT-PCR assays. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. S1. Effects of exogenous βig-h3 on mineralization of HDPCs. (**A, C**) Alizarin red-S staining of HDPC-3F (**A**) and HDPC-5A (**C**) cells treated with or without βig-h3 or

BSA in CM or DM for 7 days. The positive reactions were attenuated in the 10 μg/ml βig-h3-treated cells. (**B, D**) The Alizarin red-S positive areas in HDPC-3F (**B**) and HDPC-5A (**D**) cells were quantified and shown in graphs.

Fig. S2. Effects of exogenous βig-h3 on odontoblast-related gene expression in HDPCs. (**A–F**) The expression of odontoblast-related genes, namely DSPP (**A, D**), OCN (**B, E**), and OPN (**C, F**), in HDPC-3F (**A–C**) and HDPC-5A (**D–F**) cells treated with 10 μg/ml βig-h3 or BSA for 5 days was examined by quantitative RT-PCR assays. Data are shown as mean ± standard deviation ($n = 3$). *$p < 0.05$, **$p < 0.01$.

Fig. S3. Effects of knockdown of βig-h3 gene expression on odontoblastic differentiation of HDPCs. (**A–F**) The expression of odontoblast-related genes, namely DSPP (**A, D**), OCN (**B, E**), and OPN (**C, F**), in transduced HDPC-3F (**A–C**) and HDPC-5A (**D–F**) cells cultured in DM for 3–7 days was examined by quantitative RT-PCR assays. Data are shown as mean ± standard deviation ($n = 3$). *$p < 0.05$, **$p < 0.01$.

Fig. 1

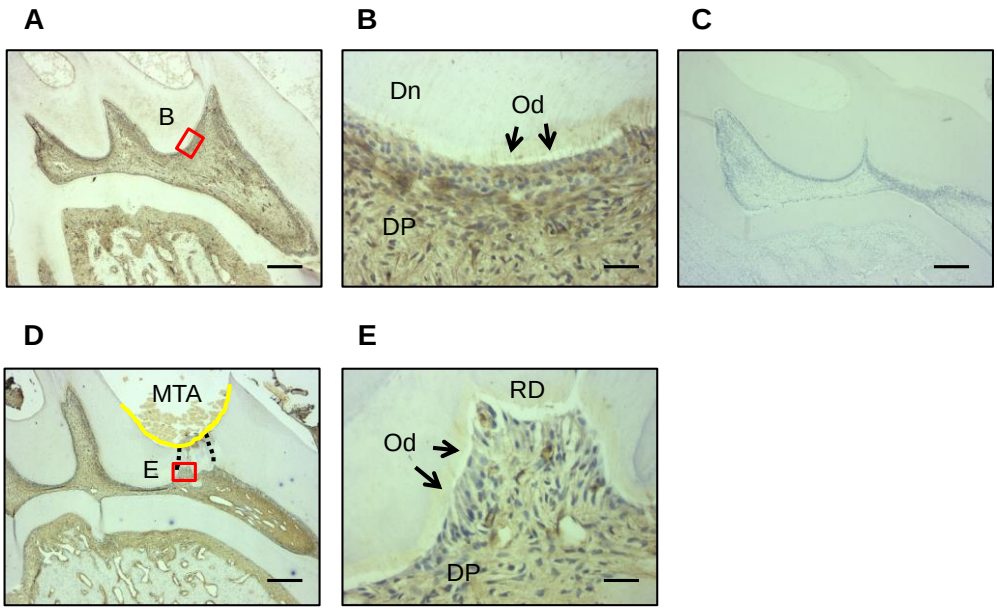


Fig. 2

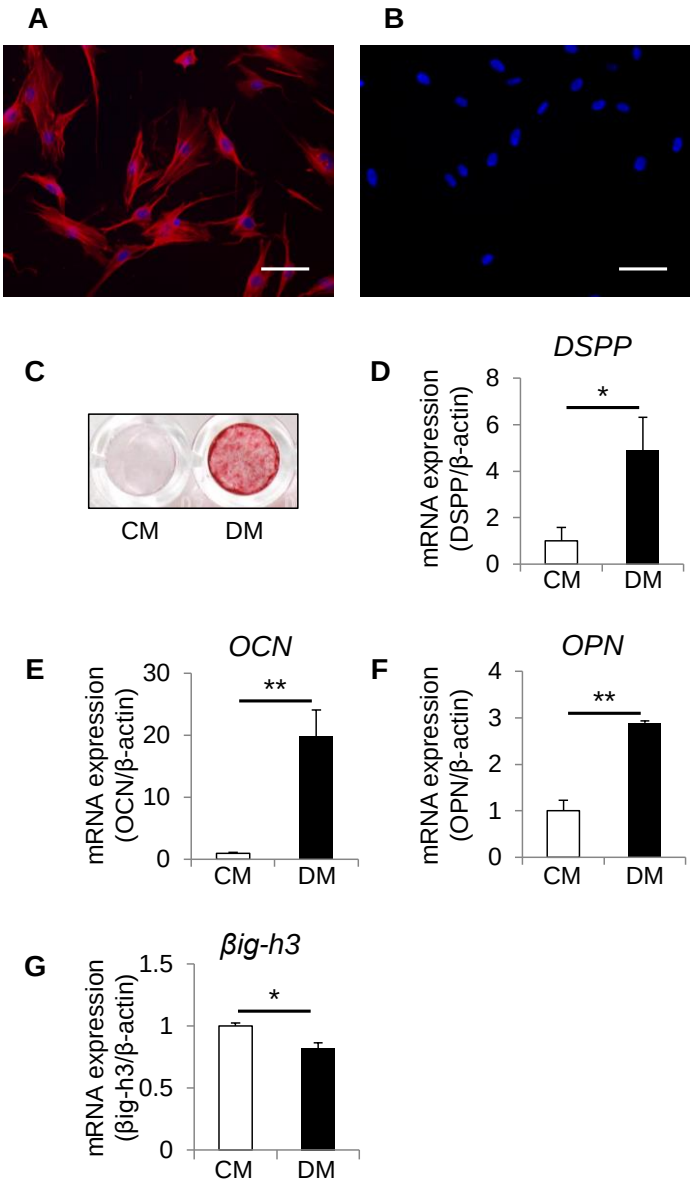

A
B
C
CM
DM
D
DSPP
mRNA expression (DSPP/β-actin)
8
6
4
2
0
*
CM
DM
E
OCN
mRNA expression (OCN/β-actin)
30
20
10
0
**
CM
DM
F
OPN
mRNA expression (OPN/β-actin)
4
3
2
1
0
**
CM
DM
G
βig-h3
mRNA expression (βig-h3/β-actin)
1.5
1
0.5
0
*
CM
DM

Fig. 3

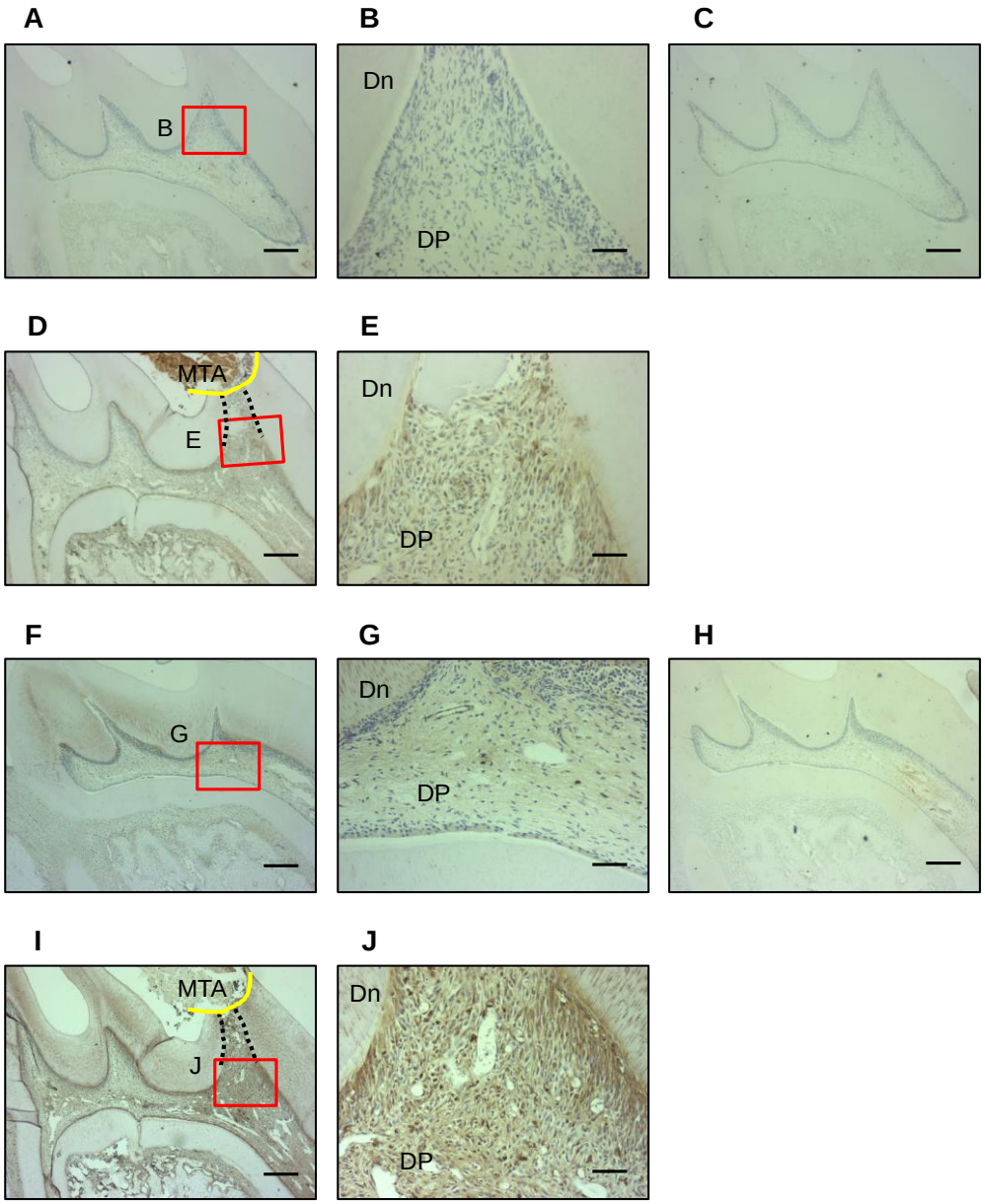


Fig. 4

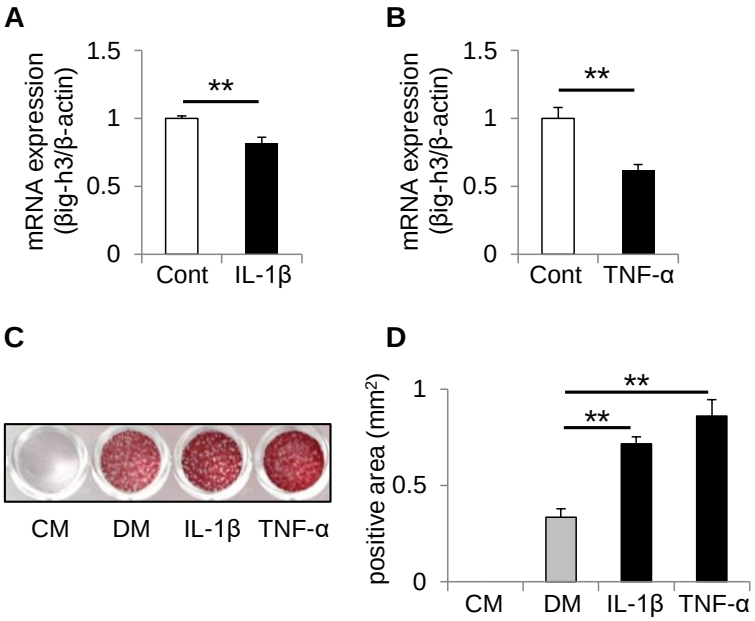

A
mRNA expression (βig-h3/β-actin)
1.5
1
0.5
0
**
Cont
IL-1β
B
mRNA expression (βig-h3/β-actin)
1.5
1
0.5
0
**
Cont
TNF-α
C
CM
DM
IL-1β
TNF-α
D
positive area (mm²)
1
0.5
0
**
**
CM
DM
IL-1β
TNF-α

Fig. 5

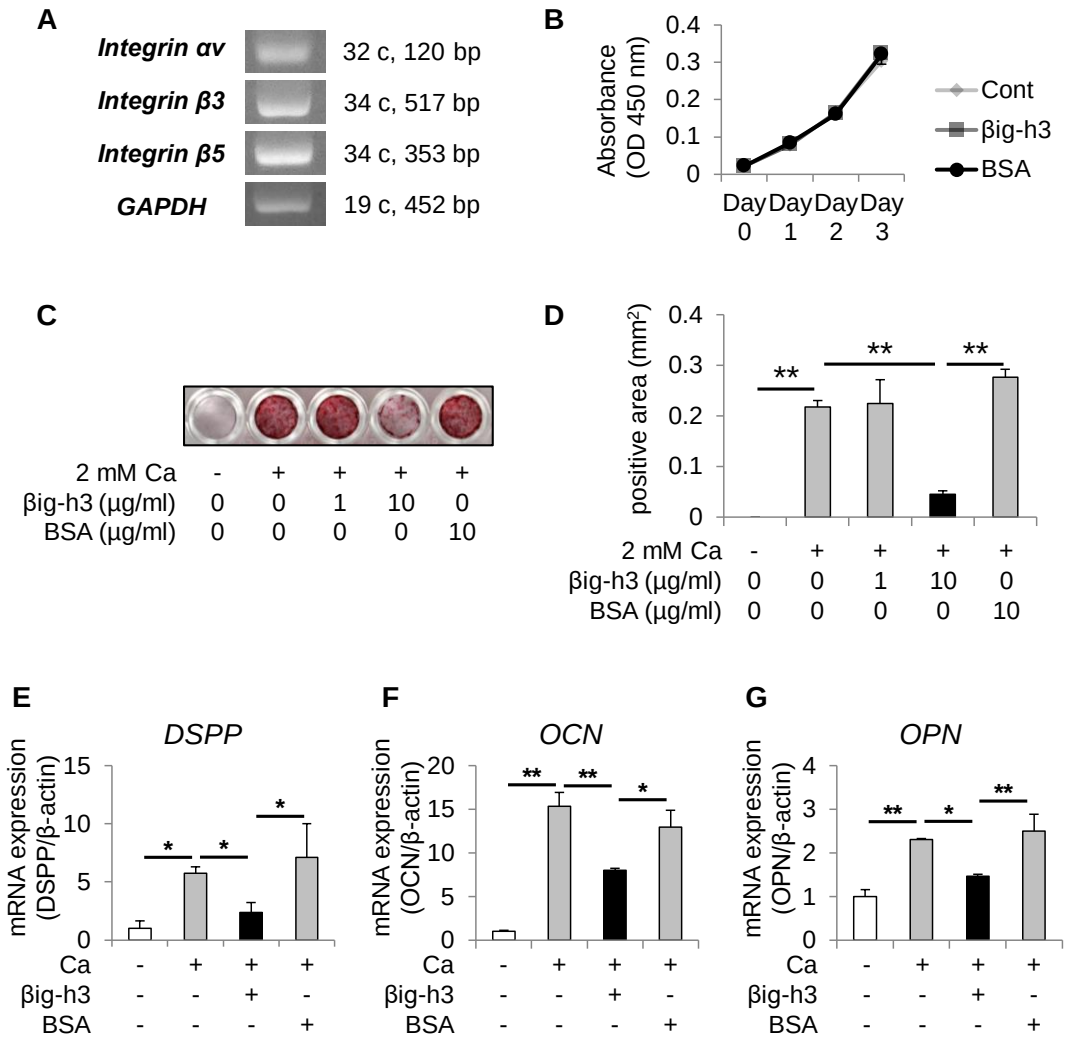

A
Integrin αv
Integrin β3
Integrin β5
GAPDH
32 c, 120 bp
34 c, 517 bp
34 c, 353 bp
19 c, 452 bp
B
Absorbance (OD 450 nm)
0.4
0.3
0.2
0.1
0
Day 0
Day 1
Day 2
Day 3
Cont
βig-h3
BSA
C
2 mM Ca - + + + +
βig-h3 (μg/ml) 0 0 1 10 0
BSA (μg/ml) 0 0 0 0 10
D
positive area (mm²)
0.4
0.3
0.2
0.1
0
**
**
**
2 mM Ca - + + + +
βig-h3 (μg/ml) 0 0 1 10 0
BSA (μg/ml) 0 0 0 0 10
E
DSPP
mRNA expression (DSPP/β-actin)
15
10
5
0
*
*
*
Ca - + + +
βig-h3 - - + -
BSA - - - +
F
OCN
mRNA expression (OCN/β-actin)
20
15
10
5
0
**
**
*
Ca - + + +
βig-h3 - - + -
BSA - - - +
G
OPN
mRNA expression (OPN/β-actin)
4
3
2
1
0
**
*
**
Ca - + + +
βig-h3 - - + -
BSA - - - +

Fig. 6

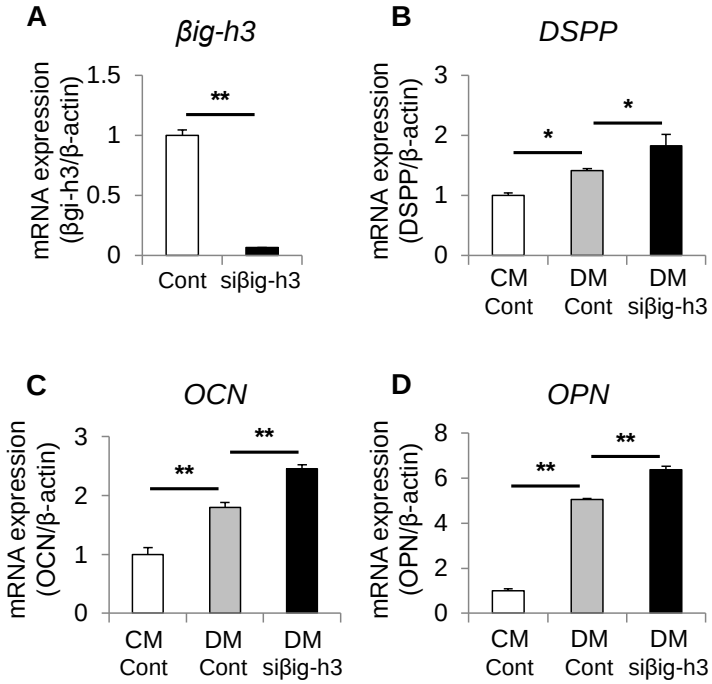

A
βig-h3
mRNA expression (βgi-h3/β-actin)
1.5
1
0.5
0
**
Cont
siβig-h3
B
DSPP
mRNA expression (DSPP/β-actin)
3
2
1
0
*
*
CM Cont
DM Cont
DM siβig-h3
C
OCN
mRNA expression (OCN/β-actin)
3
2
1
0
**
**
CM Cont
DM Cont
DM siβig-h3
D
OPN
mRNA expression (OPN/β-actin)
8
6
4
2
0
**
**
CM Cont
DM Cont
DM siβig-h3

Fig. S1

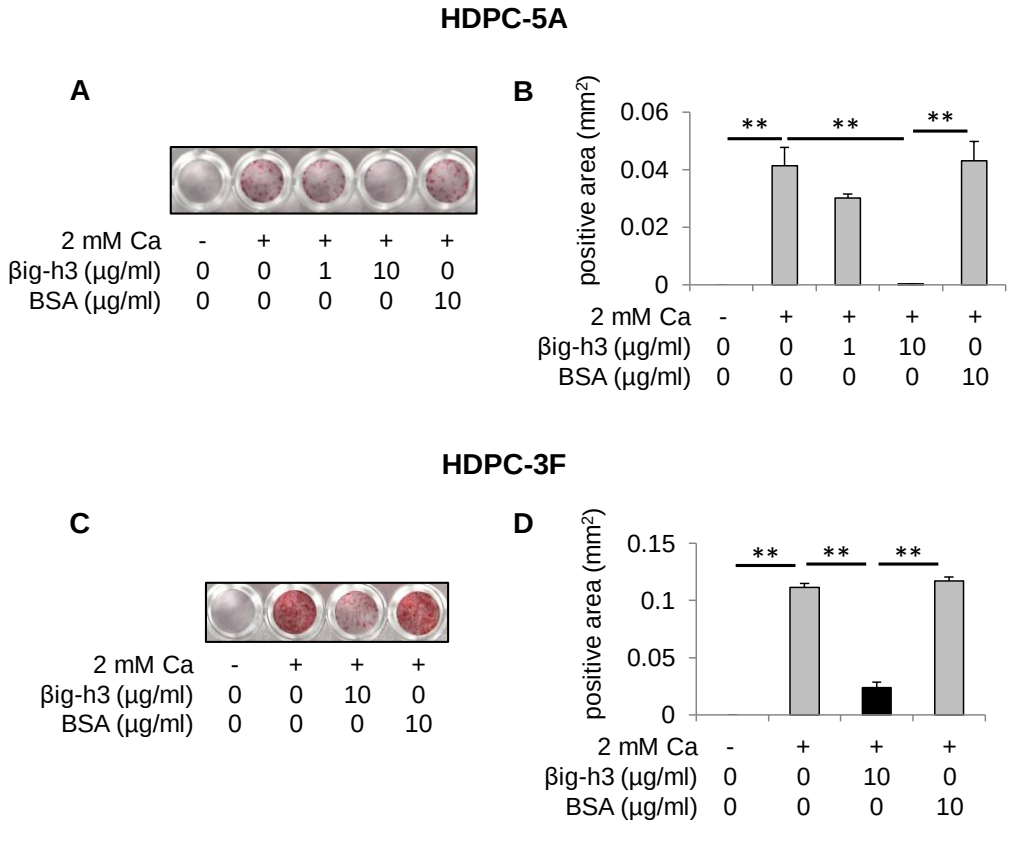

HDPC-5A
A
2 mM Ca - + + + +
βig-h3 (μg/ml) 0 0 1 10 0
BSA (μg/ml) 0 0 0 0 10
B
positive area (mm²)
0.06
0.04
0.02
0
**
**
**
2 mM Ca - + + + +
βig-h3 (μg/ml) 0 0 1 10 0
BSA (μg/ml) 0 0 0 0 10
HDPC-3F
C
2 mM Ca - + + +
βig-h3 (μg/ml) 0 0 10 0
BSA (μg/ml) 0 0 0 10
D
positive area (mm²)
0.15
0.1
0.05
0
**
**
**
2 mM Ca - + + +
βig-h3 (μg/ml) 0 0 10 0
BSA (μg/ml) 0 0 0 10

Fig. S2

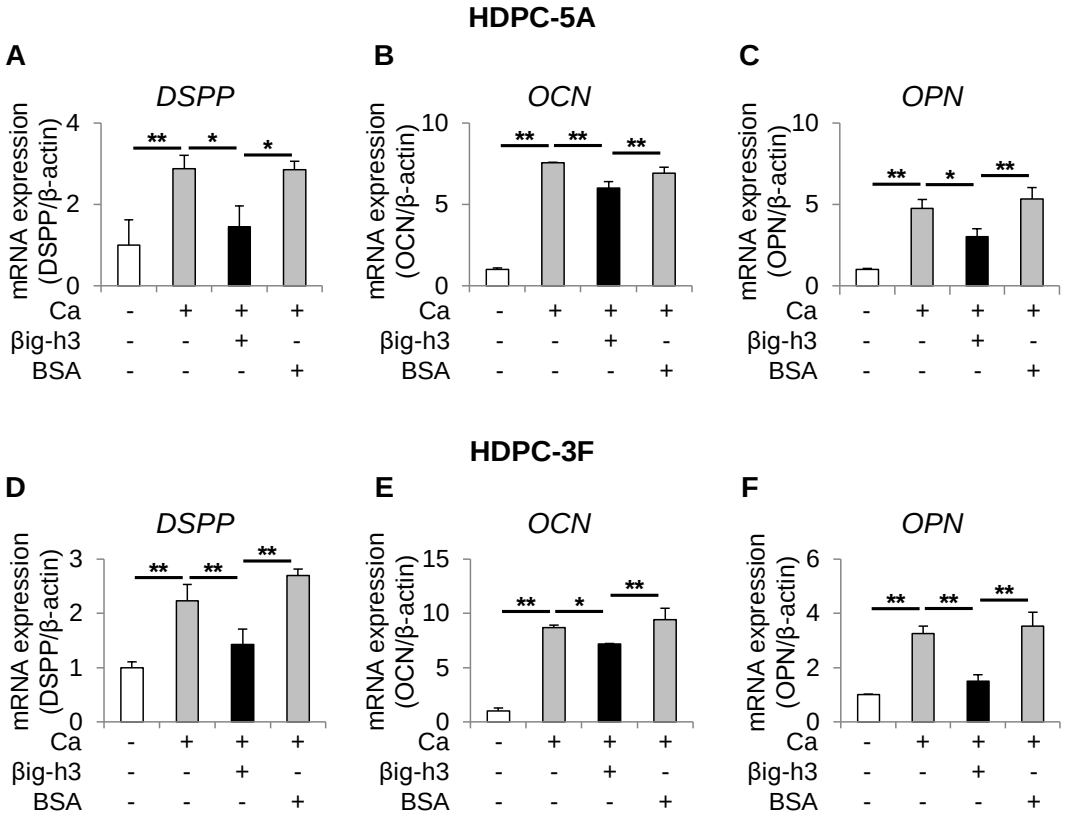
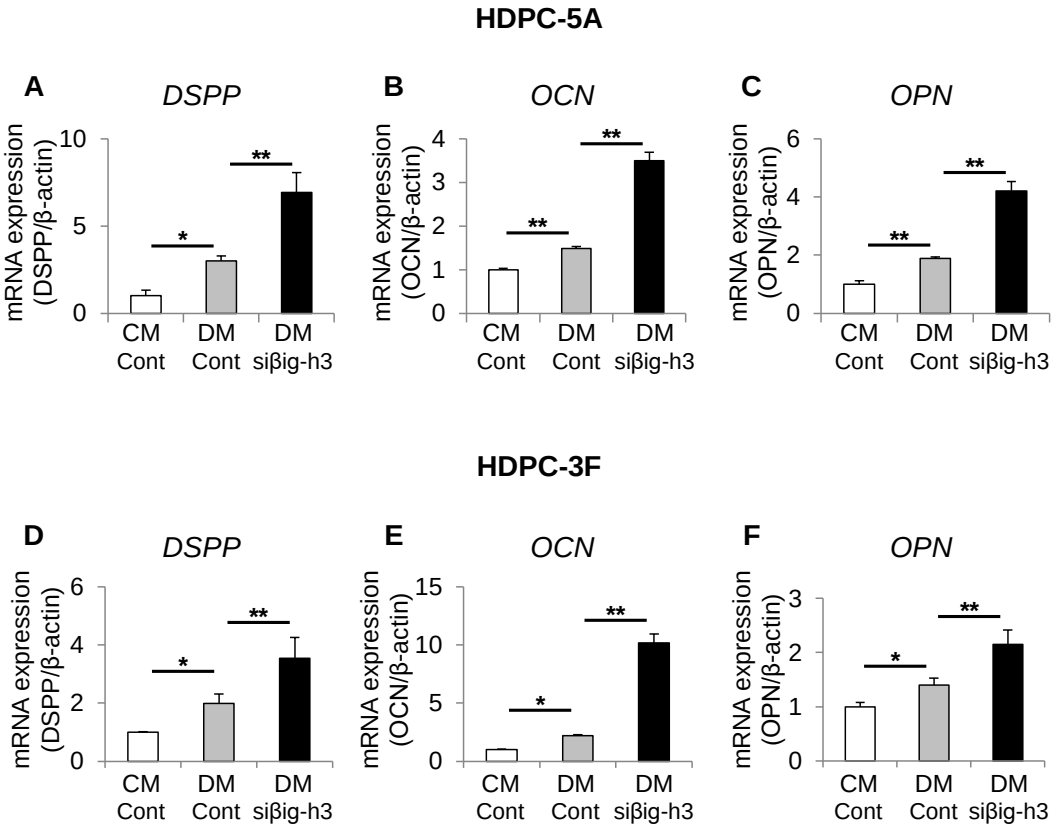

HDPC-5A
A
DSPP
mRNA expression (DSPP/β-actin)
B
OCN
mRNA expression (OCN/β-actin)
C
OPN
mRNA expression (OPN/β-actin)
Ca
βig-h3
BSA
HDPC-3F
D
DSPP
E
OCN
F
OPN

Fig. S3


HDPC-5A
A
DSPP
mRNA expression (DSPP/β-actin)
10
5
0
*
**
CM Cont
DM Cont
DM siβig-h3
B
OCN
mRNA expression (OCN/β-actin)
4
3
2
1
0
**
**
CM Cont
DM Cont
DM siβig-h3
C
OPN
mRNA expression (OPN/β-actin)
6
4
2
0
**
**
CM Cont
DM Cont
DM siβig-h3
HDPC-3F
D
DSPP
mRNA expression (DSPP/β-actin)
6
4
2
0
*
**
CM Cont
DM Cont
DM siβig-h3
E
OCN
mRNA expression (OCN/β-actin)
15
10
5
0
*
**
CM Cont
DM Cont
DM siβig-h3
F
OPN
mRNA expression (OPN/β-actin)
3
2
1
0
*
**
CM Cont
DM Cont
DM siβig-h3