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Original Article

Intermittent compressive force regulates dentin matrix protein 1 expression in human periodontal ligament stem cells

Jeeranan Manokawinchoke [a,b], Suconta Chareonvit [b], Vorapat Trachoo [c], Phoonsuk Limraksasin [a,b], Hiroshi Egusa [d,e], Thanaphum Osathanon [a,b*]

[a] *Dental Stem Cell Biology Research Unit, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*
[b] *Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*
[c] *Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand*
[d] *Division of Molecular and Regenerative Prosthodontics, Tohoku University Graduate School of Dentistry, Sendai, Japan*
[e] *Center for Advanced Stem Cell and Regenerative Research, Tohoku University Graduate School of Dentistry, Sendai, Miyagi, Japan*

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Background/purpose: Mechanical force differentially regulates periodontal ligament functions depending on types, magnitudes, and duration of stimulation. Intermittent compressive force (ICF) promotes an *in vitro* mineralization in human periodontal ligament cells. The present study investigated the effect of ICF on dentin matrix protein-1 (DMP1) expression in human periodontal ligament stem cells (hPDLSCs).
Materials and methods: Cells were treated with ICF in a serum-free culture medium for 24 h The mRNA and protein expression were examined using real-time polymerase chain reaction, immunofluorescence staining and Western blot analysis, respectively.
Results: The exposure to ICF in a serum-free condition significantly induced DMP1 expression in both mRNA and protein levels. The effect of ICF-induced *DMP1* expression was inhibited by pretreatment with cycloheximide, indicating the requirement of the intermediated molecule(s). Pretreatment with transforming growth factor β (TGF-β) receptor inhibitor (SB431542) or neutralized antibody against TGF-β1 prior to ICF application abolished the effect of ICF-induced *DMP1* expression. Further, recombinant TGF-β1 treatment stimulated *DMP1* expression.

* Corresponding author. Dental Stem Cell Biology Research Unit, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.
E-mail address: thanaphum.o@chula.ac.th (T. Osathanon).

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Conclusion: The present study illustrated that ICF induces *DMP1* expression in hPDLSCs via the regulation of TGF-β signaling pathway.

Introduction

The periodontal ligament consists of a heterogeneous cell population, collagenous and non-collagenous matrix constituents.[1] Periodontal ligament cells (PDLs) play an important role in periodontal tissue homeostasis, repair, and regeneration.[1,2] PDLs also maintain periodontal space by synthesizing periodontal ligament fibres and play a role in the alveolar bone remodeling.[3,4] Cells isolated from periodontal ligament contain a mesenchymal stem cell population.

During mastication, the periodontal ligament receives various types of mechanical force, including shear, tensile, and compressive force.[5–8] PDLs respond to the force by changing their behaviors to maintain periodontal ligament characteristics and homeostasis and/or differentiate into other lineages depending on many factors, including force types, magnitude, and duration of the force application. Fluid shear stress (FSS) at 6 dyn/cm^2 rearranges the human (h) PDL orientation, reduces proliferation, and induces osteogenic differentiation.[6] Tensile force upregulates scleraxis (Scx) leading to suppression of tension-induced osteogenic differentiation of PDLs both *in vitro* and *in vivo*.[9] Compressive force regulates hPDL homeostasis by modulating osteopontin (OPN), receptor activator of nuclear factor-kappa B ligand (RANKL), adenosine triphosphate (ATP), interleukin-1 beta (IL-1β), sclerostin (SOST), periostin (POSTN) and insulin-like growth factor-1 (IGF-1) expression.[10–14] Our previous study reports that intermittent compressive force (ICF) pretreatment significantly induces osterix (OSX) and promotes osteogenic differentiation in hPDL culture via the transforming growth factor-β signaling pathway.[8]

Dentin matrix protein-1 (DMP1) is a member of the small integrin-binding ligand, N-glycosylated protein, and other glycoproteins with cell attachment activity (SIBLING) family. It is one of the non-collagenous extracellular matrix protein members. The other proteins in the family are bone sialoprotein, osteopontin, dentin sialophosphoproteins, and matrix extracellular phosphoglycoprotein.[15] DMP1 has numerous acidic domains, phosphorylation sites, arginine-glycine-aspartate sequence, and DNA binding domain.[16] DMP1 has been found in the extracellular matrix of dentin and mineralized tissues.[16,17] DMP1 has been shown to play a crucial role in dentin and bone mineralization such as promoting differentiation, mineralization, and nucleate hydroxyapatite.[17–19] DMP1 coated titanium disk promotes human mesenchymal stem cell differentiation toward osteogenic lineage.[20] Recombinant human DMP1 induces osteogenic marker, *BMP2*, *OSX*, and *COL1* mRNA expression in hPDLs, corresponding with the marked increase in mineral deposition.[21] This evidence indicates the crucial role of DMP1 in the osteogenic differentiation of hPDLs

A previous report demonstrated that intermittent but not continuous compressive force significantly promoted osteogenic differentiation in hPDLs as determined by the upregulation of mineral deposition and the osteogenic marker gene expression.[8] However, the role of ICF on the regulation of DMP1 in hPDLs remains unclear. The present study aims to investigate the regulatory mechanism by which ICF modulates DMP1 in human periodontal ligament stem cells (hPDLSCs).

Materials and methods

Human periodontal ligament stem cell culture

The study protocol was approved by the Ethical Committee and Institutional Biosafety Committee, Faculty of Dentistry, Chulalongkorn University (Approval number 103/2021 and DENT CU-IBC 032/2021, respectively). hPDLSCs (from at least 4 donors) were obtained from healthy periodontal ligament tissue of non-carious, freshly extracted third molars removed for orthodontic reasons. Cells isolated from each donor were tested individually. Periodontal ligament tissue was scraped from the middle third of the root and placed on 35-mm tissue culture dishes for hPDLSC explantation. The cell explant and isolated hPDLSCs were maintained in growth medium (GM), Dulbecco's modified Eagle's medium (DMEM cat. No. 11960, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (cat. No.10270, Gibco), 2 mM L-glutamine (GlutaMAX™$^{-1}$, cat. No. 35050, Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Antibiotic-Antimycotic, cat. No. 15240, Gibco) in a humidified atmosphere of 95% air, 5% CO^2 at 37 °C. The medium was changed every 2 days until confluence.

Human periodontal ligament stem cell characterization

To characterize stem cell surface marker expression, CD45 (a hematopoietic cell marker), CD44, CD73, CD90, and CD105 (mesenchymal stem cell surface marker), hPDLSCs were stained with the following antibodies: PerCP-conjugated anti-CD45 antibody (BD Biosciences Pharmingen, San Diego, CA, USA), FITC-conjugated anti-human CD44 mAb (BD Biosciences Pharmingen), FITC-conjugated anti-human CD73 antibody (BD Biosciences Pharmingen), APC-conjugated anti-CD90 antibody (BD Biosciences Pharmingen), and PE-conjugated anti-human CD105 mAb (BD Biosciences Pharmingen) and determined using flow cytometry (FACSCalibur, BD Bioscience).

To evaluate osteogenic differentiation lineage ability, hPDLSCs were maintained in an osteogenic induction

medium (OM) with is GM supplemented with, 250 nM dexamethasone (cat. No. D8893, Sigma—Aldrich, St. Louis, MO, USA), 5 mM β-glycerophosphate (cat. No. G9422, Sigma—Aldrich) and 50 μg/mL ascorbic acid (cat. No. A-4034, Sigma—Aldrich) for 14 and 21 days. An *in vitro* mineralization was determined using Alizarin Red S staining.

Intermittent compressive force application

hPDLSCs were seeded in 6-well tissue culture plates at a density of 300,000 cells/well. The ICF at 0.23 Hz and 1.5 g/cm^2 was applied to the cells using a computer-controlled loading apparatus as previously described[12] for 24 h in serum-free condition. In some experiments, cells were exposed to inhibitors or recombinant protein in serum-free culture medium 30 min prior to being subject to ICF treatment. The reagents used were 4 μM of SB431542 (a TGF-β inhibitor; cat. No. S4317, Sigma—Aldrich), 5 μg/mL neutralizing TGF-β1 antibody (cat. No. MAB240, R&D Systems, Minneapolis, MN, USA), cycloheximide (1 μg/mL, cat. No. C-0934, Sigma—Aldrich), and 1—10 ng/mL recombinant human TGF-β1 protein (cat. No. 616455, Calbiochem®, La Jolla, CA, USA).

Ribonucleic acid (RNA) preparation and quantitative real-time polymerase chain reaction

RiboEx™ solution (cat. No. 301—001, GeneAll®, Seoul, South Korea) was used to extract total RNA according to the manufacturer's instructions. One microgram of each total RNA was converted to complementary DNA (cDNA) using Reverse Transcription System (ImProm-II™, cat. No. A3800, Promega, Madison, WI, USA). The cDNA was employed for quantitative real-time polymerase chain reaction (qPCR) using the FastStart Essential DNA Green Master kit (cat. No. 06402712001, Roche Diagnostics, Mannheim, Germany). The qPCR was performed in a LightCycler® 96 real-time polymerase chain reaction system (Roche Diagnostics). The reaction amplification was performed as follows: denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, extension at 72 °C for 20 s, and repeated for 40 cycles. The expression level was normalized with *GAPDH* as the reference gene. The details of primers were indicated as follows, *DMP1* (NM_004407.3) forward- CAG GAG CAC AGG AAA AGG AG, *DMP1* reverse- CTG GTG GTA TCT TGG GCA CT and *GAPDH* (NM_002046.3) forward- TCA TGG GTG TGA ACC ATG AGA A, *GAPDH* reverse- GGC ATG GAC TGT GGT CAT GAG.

Alizarin red s staining

hPDLSCs were washed with phosphate buffer saline (PBS) and fixed with cold methanol for 10 min on day 14 and 21 after being cultured in OM, then were washed twice with deionized water and stained with 2% Alizarin Red S solution for 3 min at room temperature.

Immunofluorescence staining

hPDLSCs were washed with phosphate buffer saline (PBS) and fixed with cold methanol for 10 min. Non-specific binding protein was blocked using 2% horse serum (cat. No. SH30074, Hyclone, South Logan, UT, USA). The cells were stained with DMP1 primary antibodies (cat. No. Ab81985, Abcam, Cambridge, UK) at 4 °C overnight, after that, the specimen was labeled with fluorescence by incubating in secondary antibodies and streptavidin-FITC (cat. No. S3762, Sigma—Aldrich) for 40 min each. DAPI (cat. No. 5748, TOCRIS bioscience, Bristol, UK) was used to counterstain the nuclei. The specimen was visualized under an Apotome.2 (Carl Zeiss, Jena, Germany) fluorescent microscope.

Western blot analysis

RIPA buffer with a protease inhibitor cocktail (Sigma—Aldrich) was used to extract cellular proteins. The protein extractions were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were then incubated with DMP1 and GAPDH (cat. No. MAB374, MILLIPORE, Temecula, CA, USA) primary antibody overnight. Next, the membranes were incubated with secondary antibody and peroxidase-labelled streptavidin respectively and examined the signal by using chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, ThermoFisher Scientific, Rockford, IL, USA).

Statistical analysis

All data were analyzed using Prism 9 (GraphPad Software, San Diego, CA, USA). The data were presented as mean ± standard error of the mean (SEM). The Mann—Whitney U test was used to determine two-group comparison statistical differences and the Kruskal Wallis test followed by pairwise comparison was employed for more than two-group comparisons. The data differences at $P < 0.05$ were considered to be significant.

Results

Human periodontal ligament stem cell characteristics

The characterization of hPDLSCs was identified. The cells exhibited a fibroblast-like shape (Fig. 1A). The cells expressed the mesenchymal stem cell markers, CD44, CD73, CD90, and CD105, while CD45 expression, a hematopoietic cell surface marker, was lacking (Fig. 1B). Co-expression of CD44, CD73, CD90, and CD105 was also illustrated (Fig. 1C—F). After maintaining hPDLSCs in an osteogenic induction medium, an *in vitro* mineralization was increased at day 14 and 21 compared to those cultured in normal growth medium conditions (Fig. 1G). Taken all results together, these isolated cells exhibited the mesenchymal stem cell characteristics.

Intermittent compressive force induced dentin matrix protein 1 (DMP1) expression in human periodontal ligament stem cells

After ICF application for 2, 4, 8, 24 and 48 h in a serum-free culture medium, *DMP1* gene expression was investigated by

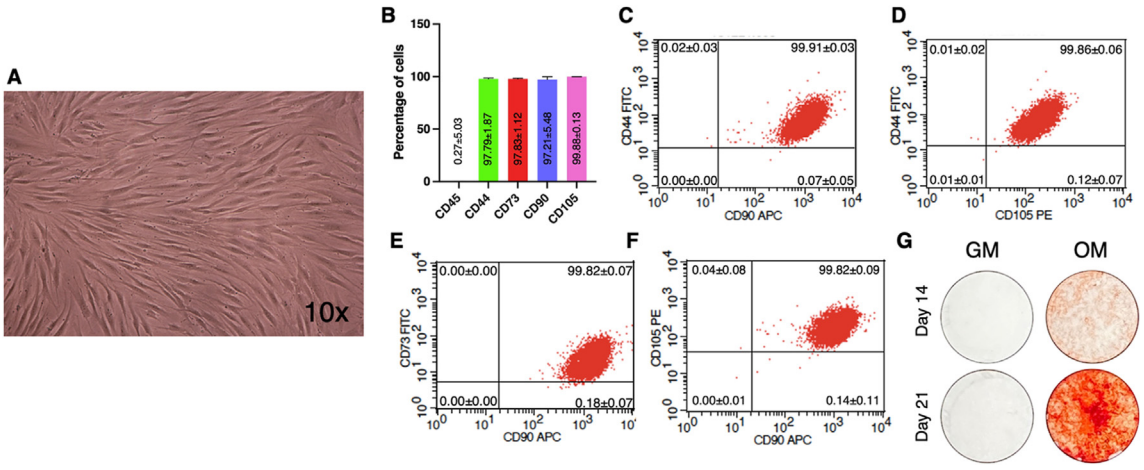

Figure 1 Characterization of hPDLSCs. hPDLSC morphology was shown (A). The percentage of mesenchymal stem cell surface marker expression, CD44, CD73, CD90, and CD105, and hematopoietic cell marker expression, CD45 in hPDLSCs was examined using flow cytometry analysis (B). Co-expression of CD44, CD73, CD90, and CD105 in hPDLSCs was illustrated (C—F). hPDLSCs were maintained in an osteogenic induction medium and an *in vitro* mineralization was determined on day 14 and 21 using alizarin red s staining (G).

qPCR. For protein expression, immunofluorescence staining and Western blot analysis were performed at 24 and 48 h (Fig. 2A). ICF did not affect cell morphology compared to the control (Fig. 2B). ICF treatment markedly upregulated both DMP1 mRNA and protein levels at 24 and 48 h after treatment (Fig. 2C—E).

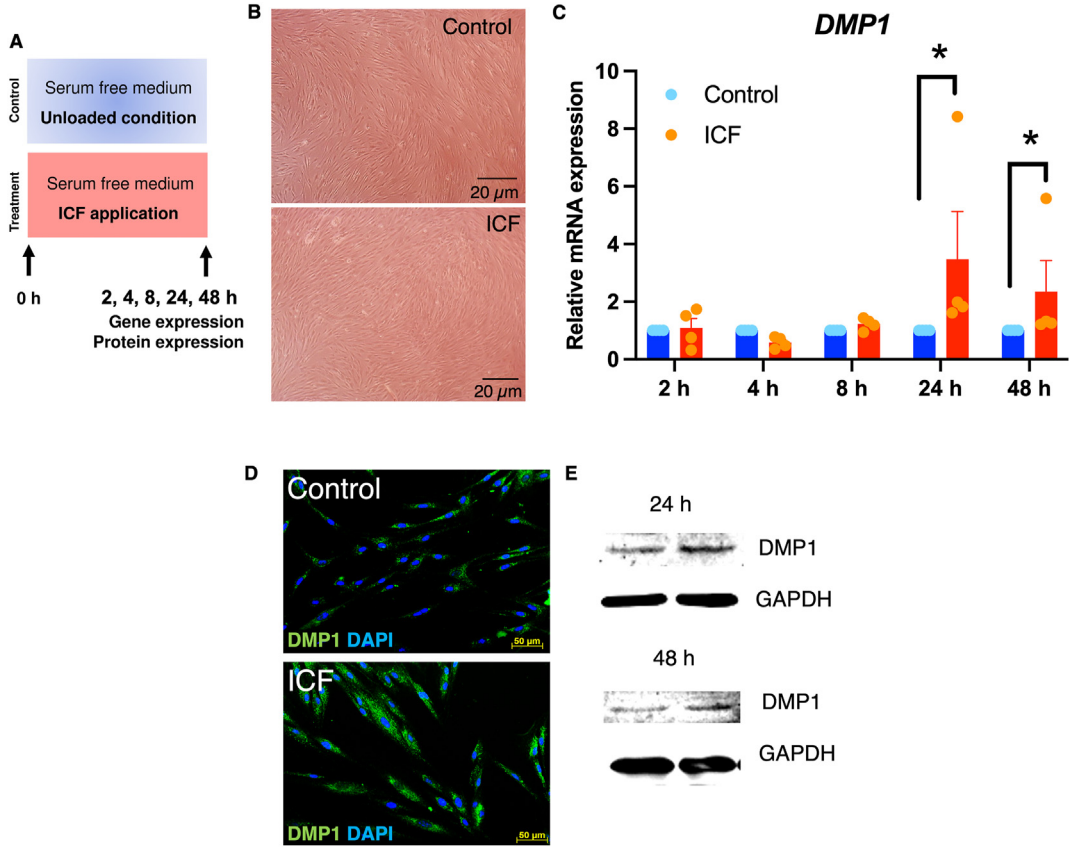

Figure 2 ICF induced DMP1 expression in hPDLSCs. The experimental scheme was illustrated (A). hPDLSCs were exposed to ICF for 2, 4, 8, 24, and 48 h in serum-free condition. hPDLSC morphology was demonstrated using a phase-contrast microscope (B). *DMP1* mRNA (C) and protein (D, E) expression were investigated by qPCR, immunofluorescence staining, and Western blot analysis, respectively. Bars indicated a significant difference between conditions (*$P < 0.05$).

Transforming growth factor β (TGF-β) pathway regulated intermittent compressive force-induced dentin matrix protein 1 (DMP1) expression in human periodontal ligament stem cells

Cells were pretreated with each inhibitor for 30 min prior to force treatment (Fig. 3A). Subsequently, cells were exposed to ICF for 24 h in a serum-free culture medium. Results showed that ICF-induced *DMP1* mRNA expression can be inhibited by cycloheximide (CHX) (Fig. 3B), suggesting the involvement of intermediate molecules. Our previous reports demonstrate that the ICF at this magnitude regulates hPDL and induced pluripotent stem cell (iPSC) behaviours via the TGF-β pathway.[8,22] We hypothesized that the ICF-induced DMP1 expression would occur in the same fashion. To clarify this hypothesis, SB431542 (a TGF-β inhibitor) or neutralizing TGF-β1 antibody was employed to block signal transduction of the TGF-β pathway. The results showed that both TGF-β inhibitor and neutralizing TGF-β1 antibody attenuated the effect of ICF on *DMP1* expression (Fig. 3C and D).

Recombinant transforming growth factor β1 (TGF-β1) protein promoted dentin matrix protein 1 (DMP1) expression in human periodontal ligament stem cells

To confirm the influence of TGF-β1 on *DMP1* expression of hPDLSCs. Cells were exposed to recombinant human TGF-β1 protein at 1 and 10 ng/mL in serum-free culture condition for 24 h and then examined the *DMP1* mRNA expression (Fig. 4A). The result demonstrated that recombinant human TGF-β1 protein increased *DMP1* expression in a dose-dependent manner (Fig. 4B).

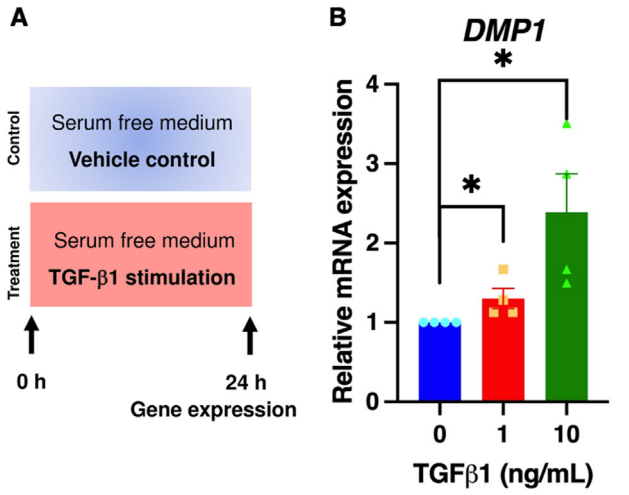

Figure 4 Recombinant TGF-β1 protein promoted DMP1 expression in hPDLSCs. The experimental scheme was illustrated (A). hPDLSCs were exposed to recombinant human TGF-β1 protein at 1 and 10 ng/mL in serum-free culture condition for 24 h and then examined the *DMP1* mRNA expression (B). Bars indicated a significant difference between conditions (*$P < 0.05$).

Discussion

DMP1 is detected in PDL and found to be localized with periostin indicating the biological function in the periodontal ligament homeostasis.[23] Addition of recombinant DMP1 results in the upregulation of *ALP* and *TGFB1* mRNA expression in hPDLSCs.[24] In addition, DMP1 treatment promotes various osteogenic related genes in hPDLs, including osteogenic transcription factor (CBFA1 and OSX), bone matrix protein (COL1 and OPN) as well as osteogenic inductive protein (BMP2 and WNT3A).[21] DMP1 treatment promotes an *in vitro* mineralization in the dose-dependent manner[21] and extracellular matrix organization in hPDLSCs, potentially via the activation of ERK signaling.[24] It has also been shown that DMP1 interacts with glucose-regulated protein-78 at the cell membrane and subsequently is internalized and translocated to the nucleus to promote osteogenic differentiation in hPDLs.[25] Taken together, this evidence implicates the crucial role of DMP1 on osteogenic differentiation in PDLs. As periodontal ligaments are subject to mechanical force during both normal and para-normal functions. Different force application leads to the distinct biological regulation of PDLs. Our previous report demonstrated that intermittent but not continuous compressive force significantly promoted osteogenic differentiation in hPDLs as determined by the upregulation of

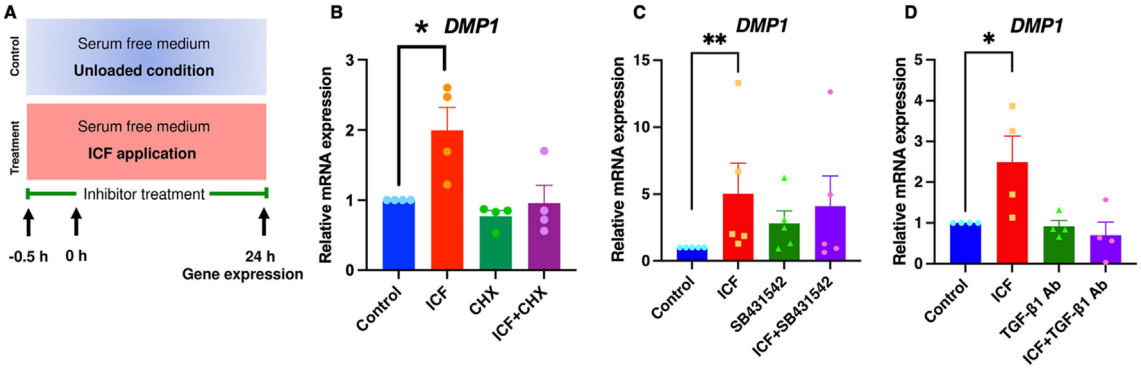

Figure 3 TGF-β pathway regulated ICF-induced *DMP1* expression in hPDLSCs. The experimental scheme was illustrated (A). hPDLSCs were treated with the cycloheximide (CHX) (B), or SB431542 (a TGF-β inhibitor) (C), or neutralizing TGF-β1 antibody (D) 30 min prior to ICF application. After cells were exposed to ICF for 24 h, the *DMP1* mRNA expression was examined using qPCR. Bars indicated a significant difference between conditions (*$P < 0.05$, **$P < 0.01$).

mineral deposition and the osteogenic marker gene expression.[8] The present study described the inductive effect of ICF on DMP1 expression in hPDLSCs, indicating the role of ICF in osteogenic differentiation in this cell type.

We also demonstrated in the present study that ICF-induced *DMP1* expression can be inhibited by cycloheximide pretreatment, suggesting the involvement of the intermediate molecule. We found that this regulation could occur through TGF-β signaling as confirmed by inhibition experiments and exogenous treatment. For the inhibition experiment, SB431542, the ALK5 inhibitor, was employed at the concentration of 4 μM according to our previous study, The effect of intermittent compressive force (ICF) on cells demonstrated that 4 μM of SB431542 inhibits ICF upregulated sclerostin,[12] periostin,[12] *HES1*,[26] *HEY1*,[26] *NOTCH2*,[26] *NOTCH3*[26] and osterix[8] expression in hPDLSCs. In addition, the inhibitor at this concentration attenuates the ICF-induced *in vitro* mineralization in hPDLSCs.[8] Moreover, in other cell types, 4 μM of SB431542 abolishes the effect of ICF suppressed *Sost* expression in murine pre-osteoblast cell line,[27] and the effect of ICF increased Ccnd1 and *Cdk 6* in mouse-induced pluripotent stem cells.[22]

TGF-β signaling is crucially involved in periodontal ligament homeostasis. Tgfbr2 knockdown in mesenchymal progenitor cells results in the disorganized collagen bundles and the reduction of cell number corresponds with the reduction of β-catenin and periostin expression in the periodontal ligament.[28] TGF-β signaling pathway differentially regulates osteo/odontogenic differentiation depending on protein subtypes and cell types.[29] In this respect, TGF-β1 promotes but TGF-β2 inhibits osteogenic differentiation in bone marrow-derived mesenchymal stem cells.[29] Our previous report showed that ICF upregulated *TGFB1* but not *TGFB2* and *TGFB3* mRNA expression in hPDLs.[8] Corresponding with the present study, ICF-induced *DMP1* mRNA expression was attenuated by the inhibitor of TGF-β receptor and TGF-β1 neutralized antibody. Exogeneous TGF-β1 treatment led to the upregulation of *DMP1*. Taken all evidence together, TGF-β1 could participate in the osteogenic inductive effects of ICF in hPDLSCs. However, the role of DMP1 in ICF-induced osteogenic differentiation in hPDLSCs is required for further investigation.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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