Effect of 1α, 25-dihydroxyvitamin D3 on the osteogenic differentiation of human periodontal ligament stem cells and the underlying regulatory mechanism

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Abstract. 1α, 25-dihydroxyvitamin D3 (1,25-D3), an active vitamin D metabolite, is a well-known regulator of osteogenic differentiation. However, how 1,25-D3 regulates osteogenic differentiation in human periodontal ligament stem cells (hPDLSCs) remains to be fully elucidated. The present study aimed to clarify this issue through well-controlled *in vitro* experiments. After hPDLSCs were treated with 1,25-D3, immunofluorescence and western blotting were used to detect the expression of vitamin D receptor; Cell Counting Kit-8 and western blotting were used to assay the cell proliferation ability. Alkaline phosphatase staining, Alizarin Red staining and western blotting were used to detect the osteogenic differentiation. It was found that treating hPDLSCs with 1,25-D3: i) Inhibited cell proliferation; ii) promoted osteogenic differentiation; iii) upregulated the expression of transcriptional coactivator with PDZ-binding motif (TAZ), an important downstream effector of Hippo signaling that has been demonstrated to be involved in the osteogenic differentiation of stem/progenitor cells; and iv) that co-treatment of TAZ-overexpressing hPDLSCs with 1,25-D3 synergistically stimulated the expression of osteogenic markers. These results suggested that the induction of osteogenic differentiation promoted by 1,25-D3 in hPDLSCs involves, at least in part, the action of TAZ.

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Introduction

Periodontal diseases, periapical diseases, jaw osteomyelitis, and oral and maxillofacial tumors can cause alveolar bone tissue loss, which eventually affects chewing function (1-3). Therefore, there is much interest in the use of periodontal tissue engineering to repair bone defects and promote bone regeneration. Human periodontal ligament stem cells (hPDLSCs) are a subtype of mesenchymal stem cells (MSCs) that possess multi-potency to differentiate into cells of chondrogenic, osteogenic, adipogenic and neurogenic lineages *in vitro* (4,5). These cells have been shown to have pluripotent capacity to differentiate into osteoblasts, osteocytes and cementoblasts, and have been used in the regeneration of periodontal tissue (6-8). Therefore, hPDLSCs are a promising cell population for periodontal tissue engineering. It is necessary to have a deep understanding of the underlying regulatory mechanisms that regulate target stem cell proliferation and differentiation prior to practical applications.

1α, 25-dihydroxyvitamin D3 (1,25-D3), an active form of vitamin D, is one of the key factors that regulates bone metabolism. 1,25-D3 functions mainly through binding to its nuclear vitamin D receptor (VDR) (9). 1,25-D3 and VDR are essential for regulation of the osteogenic differentiation of stem/progenitor cells (10,11). Studies have shown that 1,25-D3 can stimulate the mineralization of human osteoblasts and induce the osteogenic differentiation of human MSCs (12-16). Similarly, increasing evidence suggests that 1,25-D3 is essential in promoting osteogenic activity in hPDLSCs (17,18). Further investigations are required to understand the specific regulatory molecular mechanisms of 1,25-D3-induced osteogenic differentiation in hPDLSCs.

The Hippo signaling pathway was initially found to be implicated in organ size control, and subsequent studies have found that the Hippo signaling pathway is highly conserved and may have a similar important role in the context of tissue regeneration (19-21). Transcriptional coactivator with PDZ-binding motif (TAZ) is a key mediator of Hippo signaling that regulates stem cell self-renewal and differentiation in different contexts (22). Similar to other transcriptional coactivators, TAZ shuttles between the cytoplasm and the nucleus

in response to different signaling molecules; through this translocation, TAZ regulates its potential target gene (23). It has been reported that TAZ is a critical mediator for regulating MSC differentiation towards osteoblasts (24,25).

As TAZ has an important function during the osteogenic differentiation of stem cells, it may be possible to capitalize on its osteogenic role in stem cell differentiation to enhance bone regeneration. TAZ-mediated activation is involved in osteoblast differentiation in various cell types upon stimulation with growth factors, cytokines and/or chemical compounds (26-29). Cross-talk between 1,25-D3 and TAZ may also represent an important mechanism to mediate the tissue-specific expression of osteogenic genes. Therefore, the present study aimed to investigate whether the effect of 1,25-D3 on the osteogenic differentiation of hPDLSCs involves the action of TAZ.

Materials and methods

Isolation and culture of hPDLSCs. The present study was approved by the Medical Ethical Committee of the School of Stomatology, Shandong University (Shandong, China; protocol no. 20170303). It has been shown that aging has significantly negative effects on hPDLSC proliferation and differentiation (2). Therefore, a relative narrow age range of subjects (12-16 years old) was selected to avoid the effects of aging on PDLSCs. Prior to commencement of the study, the patients and their parents were informed verbally and in writing. The parents provided written informed consent in accordance with the Declaration of Helsinki. The isolation and culture methods were as reported previously (30,31). Briefly, 10 premolars, extracted for orthodontic reasons from four otherwise healthy patients at the Stomatological Hospital of Shandong University (two girls and two boys, the girls are 13 and 15 years old and underwent teeth extraction in May 2017, the boys are 12 and 16 years old and underwent teeth extraction in October 2017), were used for cell isolation. The harvested teeth were placed in α-MEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with penicillin (400 U/ml, Gibco; Thermo Fisher Scientific, Inc.) and streptomycin (400 mg/ml, Gibco; Thermo Fisher Scientific, Inc.) on ice, and were transported to the laboratory immediately. Human periodontal ligament tissue from the middle third of the tooth root was scraped off and minced into small pieces with an aseptic scalpel. The minced tissues were incubated with collagenase type I (3 mg/ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and dispase (4 mg/ml, Sigma-Aldrich; Merck KGaA) in α-MEM at 37°C for 1 h, followed by filtering through a 70-μm cell strainer to obtain a single cell suspension. The cells were grown in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin in an incubator at 37°C and 5% CO_2. Cells at passages 3-5 were used.

Phenotype analysis of isolated hPDLSCs by flow cytometry. After being trypsinized and washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.), the cells were incubated with fluorescent dye-conjugated monoclonal antibodies at 4°C for 20 min in the dark, followed by analysis using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes,

NJ, USA) with a BD Stemflow™ hMSC Analysis kit. The following antibodies (included in the kit) were used: hMSC⁺ cocktail (CD90-FITC, CD105-PerCP-Cy5.5, CD73-APC and CD44-PE), and hMSC⁻ cocktail (CD34-PE, CD11b-PE, CD19-PE, CD45-PE and HLA-DR-PE). Non-specific staining was controlled using isotype-matched antibodies following the manufacturer's protocol (BD Biosciences).

Multipotent differentiation of hPDLSCs. The hPDLSCs were cultured in 6-well dishes at $1x10^5$ cells/well. At 90% confluence, the culture medium was replaced according to the type of differentiation. For osteogenic differentiation, the cells were exposed to osteogenic induction medium (OIM) comprising α-MEM supplemented with 10% FBS), 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 10 nmol/l dexamethasone (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), 10 mmol/l β-glycerophosphate (Beijing Solarbio Science & Technology Co., Ltd.), and 50 mg/l ascorbic acid (Beijing Solarbio Science & Technology Co., Ltd.). The medium was replaced every 3 days. Following 4 weeks of induction, the formation of mineralized nodules was stained with Alizarin Red (AR; Sigma-Aldrich; Merck KGaA) for 5 min at room temperature and detected by an inverted microscope (Olympus Corp., Tokyo, Japan).

For adipogenic differentiation, the cells were exposed to adipogenic induction medium comprising α-MEM containing 10% FBS, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 2 μmol/l dexamethasone (Beijing Solarbio Science & Technology Co., Ltd.), 0.2 mmol/l indomethacin (Sigma-Aldrich; Merck KGaA), 0.01 g/l insulin (Sigma-Aldrich; Merck KGaA) and 0.5 mmol/l isobutyl-methylxanthine (Sigma-Aldrich; Merck KGaA). Following 4 weeks of induction, the lipid droplets in the cells were stained with Oil Red O (Cyagen Biosciences, Suzhou, China) for 30 min at room temperature and detected by an inverted microscope (Olympus Corp.).

For chondrogenic differentiation, the cells were exposed to chondrogenic induction medium containing 2 ng/ml transforming growth factor-β1 (R&D Systems, Inc., Minneapolis, MN, USA), 50 mg/ml L-ascorbic acid, 100 mg/ml sodium pyruvate (Sigma Aldrich; Merck KGaA) and 100 nM dexamethasone (Beijing Solarbio Science & Technology Co., Ltd.). After 4 weeks, chondrogenic differentiation was assessed via Alcian Blue (Beijing Solarbio Science & Technology Co., Ltd.) staining for 5 min at room temperature and detected by an inverted microscope (Olympus Corp.).

Detection of the expression of VDR in hPDLSCs by immunofluorescence. hPDLSCs were plated on 24-well chamber slides at a density of 5,000 cells/well and then treated with or without 10 nM 1,25-D3 (Sigma-Aldrich; Merck KGaA) for 48 h at 37°C. The hPDLSCs were fixed in 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature, permeabilized with 0.1% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min and washed with PBS. The slides were then blocked in goat serum (Cell Signaling Technology, Inc.) for 60 min at room temperature, followed by incubation with primary mouse anti-human VDR monoclonal antibody (1:100; cat. no. sc-13133; Santa Cruz Biotechnology, Inc.,

Dallas, TX, USA) at 4˚C overnight. Following washing with PBS, the cells were incubated with goat anti-mouse secondary antibody (1:200; cat. no. SP-9000; OriGene Technologies, Inc., Beijing, China) for 1 h in the dark at room temperature. The cells were then stained with 4',6-diamidino-2-phenyl-indole (Cell Signaling Technology, Inc.) for 5 min at room temperature and viewed under a fluorescence microscope (Olympus Corp.).

Proliferation assay of hPDLSCs. The cell proliferation ability of the hPDLSCs under different conditions was assayed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cells were seeded in 96-well plates with 3,000 cells/well and were treated with different concentrations of 1,25-D3 (0, 0.1, 1 and 10 nM). The culture media was refreshed every 48 h for each media concentration. Every 24 h, 10 μl CCK-8 reagent was added to the wells. Following a 1.5-h incubation period, the plates were measured for spectrophotometric absorbance at 450 nm. Cell proliferation was also evaluated by western blotting to detect changes in the expression of proliferating cell nuclear antigen (PCNA), a marker essential for cell proliferation (32), following incubation with or without 1,25-D3 for 72 h.

Osteogenic differentiation of hPDLSCs. The hPDLSCs ($1x10^5$ cells/well) were seeded in 6-well plates and cultured in OIM with or without 1,25-D3 (10 nM). For alkaline phosphatase (ALP) staining, the cells were cultured for 7 days. The cells were then washed with PBS and fixed with 4% paraformaldehyde at 4˚C for 30 min. A BCIP/NBT alkaline phosphatase staining kit (Beyotime Institute of Biotechnology, Haimen, China) was used for ALP staining, and the stained samples were then observed with an inverted microscope (Olympus Corp.). For the AR staining, the hPDLSCs were stained with 1% AR for 5 min at room temperature on day 14. The osteogenesis-related proteins, including ALP and osteopontin (OPN), were also detected by western blotting.

Viral transfection and establishment of TAZ-overexpressing stable cell clones of hPDLSCs. Lentiviral vectors overexpressing TAZ were constructed and produced by Shanghai Genechem Company (Shanghai, China). At the third passage, hPDLSCs were seeded in 6-well plates ($0.5x10^5$ cells/well) and cultured to 40% confluence; the cells were then transfected with culture medium containing 8 μg/ml Polybrene (Shanghai Genechem Company) and 100 μl TAZ-overexpression lentivirus particle LV5-homo-TAZ (NM_000116.4) (10^9/ml) as the TAZ-overexpression group. hPDLSCs transfected with 100 μl empty vector lentivirus LV5-Nc (NM_000116.4) (10^9/ml) and 8 μg/ml Polybrene were used as controls. The transfection efficiency of TAZ was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses after 72 h of transfection. All experiments were performed in triplicate and repeated three times.

RT-qPCR analysis. TRIzol (1 ml) was added to the wells and total RNA was extracted from the cells according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.).

The total RNA (1 μg) was reverse transcribed to cDNA using a SuperScript™ II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.). The temperature protocol was as follows: 42˚C for 2 min, and 4˚C for 30 min; followed by 37˚C for 15 min at 85˚C for 5 sec, and 4˚C for 30 min. These generated cDNA samples were then amplified with RT-qPCR in 20 μl of the reaction system, which contained the 10 μl SYBR® Primix Ex Taq™ (Takara Bio, Inc., Otsu, Japan), 2 μl cDNA, 0.4 μl each primer and 7.2 μl RNase-free H_2O, following the manufacturer's protocol. RT-qPCR analysis was performed on a Roche Light Cycler® 480 (Hoffmann-La Roche Ltd., Basel, Switzerland). The thermal cycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (33), normalizing with GADPH levels. The primers used were as follows: GAPDH, forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACGCCAGTGGA-3'; TAZ, forward 5'-CCTCTTCAATGATGTAGAGTCTGC-3' and reverse 5'-AGTGATTACAGCCAGGTTAGAAAG-3'.

Total protein isolation and western blotting. The primary monoclonal antibodies used included rabbit anti-human TAZ (cat. no. cst70148; Cell Signaling Technology, Inc.; 1:1,000), rabbit anti-human ALP (cat. no. ab108337; Abcam, Cambridge, UK; 1:20,000), mouse anti-human OPN (cat. no. sc-21742; Santa Cruz Biotechnology, Inc; 1:500), rabbit anti-human PCNA (cat. no. WL0341c; Wanlei Biotechnology Co., Ltd., Shanghai, China; 1:1,000), and anti-human GAPDH monoclonal (cat. no. HRP-60004, ProteinTech Group, Inc., Chicago, IL, USA; 1:20,000). The cells were lysed in radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 1% phenylmethylsulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was quantified using the bicinchoninic acid (Beijing Solarbio Science & Technology Co., Ltd.) method. Subsequently, 20 μg of protein were separated electrophoretically with 10% SDS-PAGE and was then electroblotted onto a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with nonfat milk (5%; Beijing Solarbio Science & Technology Co., Ltd.), the membranes were incubated with the primary monoclonal antibodies against TAZ, ALP, OPN or GAPDH (loading control) at 4˚C overnight. The cells were then labeled with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (cat. no. 7076S) or goat anti-rabbit IgG (cat. no. 7074S) from Santa Cruz Biotechnology, Inc. The protein bands were detected using the chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA) and protein levels were analyzed using ImageJ 1.47V software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were repeated at least three times and data are presented as the mean ± standard error of mean. Data were statistically analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) with Student's two-tailed t-test for two groups of data or one-way analysis of variance with Tukey's post hoc test for multiple groups of data. P<0.05 was considered to indicate a statistically significant difference.
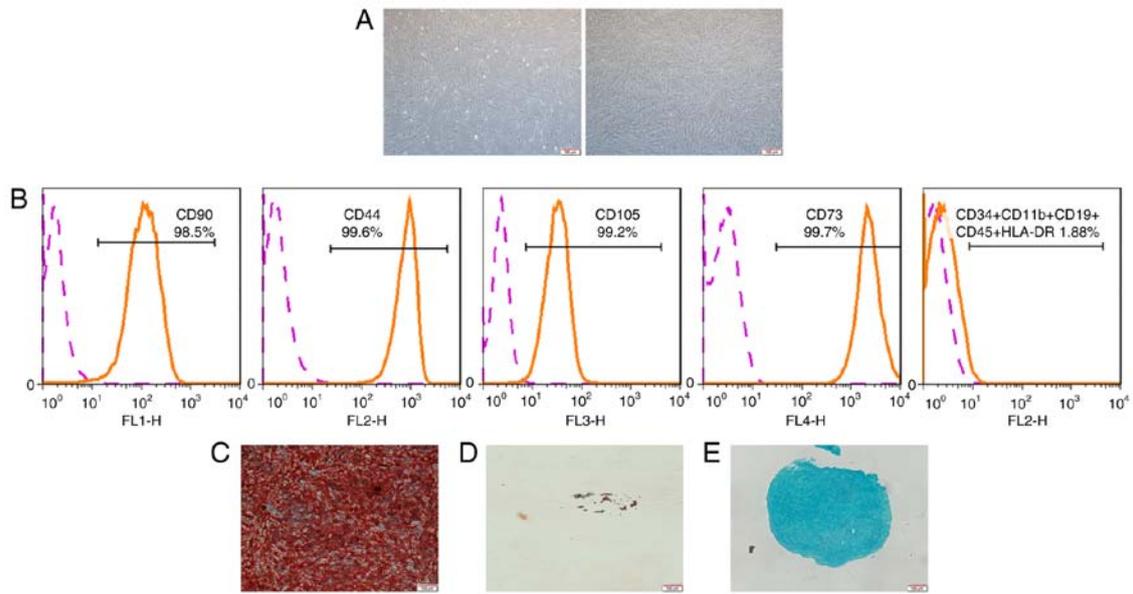
Figure 1. Characterization of hPDLSCs. (A) Morphological characterization of hPDLSCs. Primary culture (left) and culture at passage 4 (right) are shown. (B) Flow cytometric analysis showed that PDLSCs were positive for CD90, CD44, CD105, CD73, and negative for CD34, CD11b, CD19, CD45 and HLA-DR (as demonstrated by the orange line). Pink line represents non-specific staining with isotype-matched antibodies. (C) Following 4 weeks of culture in osteogenic induction medium, the cells were stained with Alizarin Red. (D) Following 4 weeks of culture in adipogenic induction medium, the cells were stained with Oil Red O. (E) Following 4 weeks of culture in chondrogenic induction medium, the cells were stained with Alcian Blue (scale bar, 100 μm). hPDLSCs, human periodontal ligament stem cells.
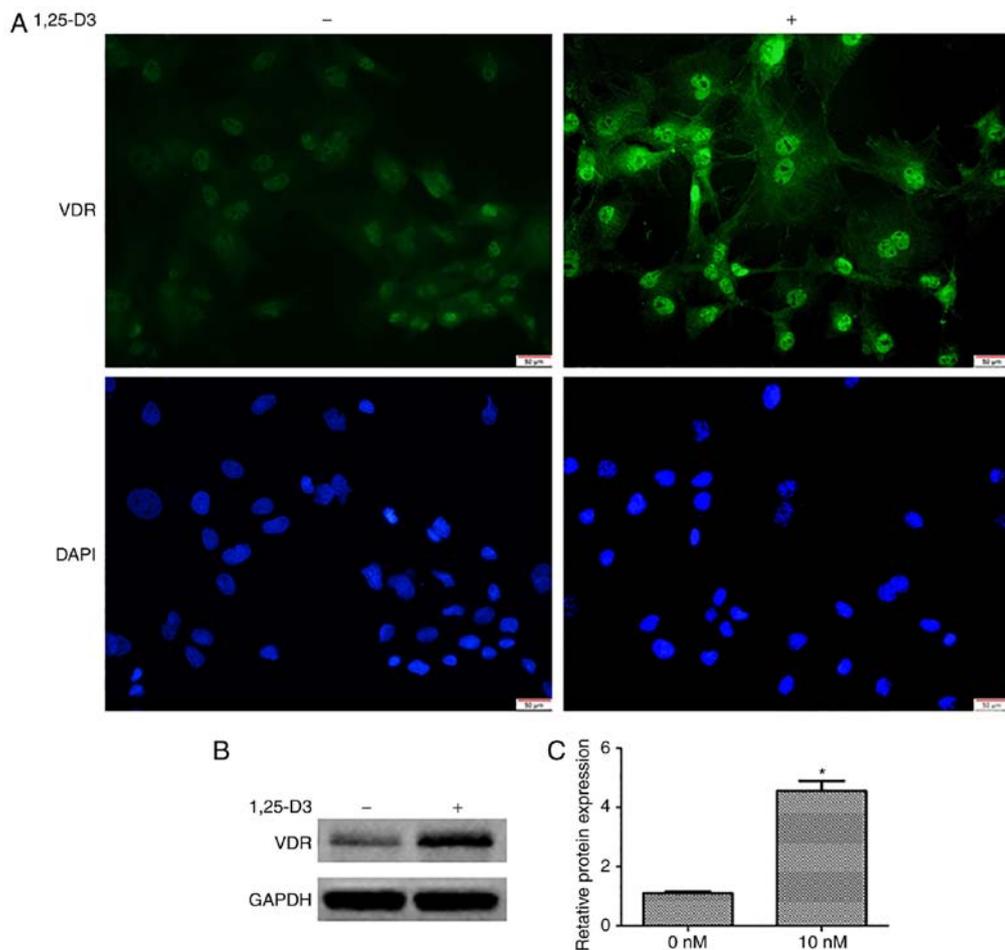

Figure 2. 1,25-D3 induces the expression and nuclear localization of VDR in hPDLSCs. (A) Immunofluorescence staining of cultured hPDLSCs for VDR (green). Cells were counterstained with DAPI (blue) to visualize nuclei (scale bar, 50 μm). (B) Western blot analysis and (C) protein band density quantitative analysis of protein expression of VDR at 48 h of culture. *P<0.05, vs. control (0 nM group). hPDLSCs, human periodontal ligament stem cells; VDR, vitamin D receptor; DAPI, 4',6-diamidino-2-phenylindole.
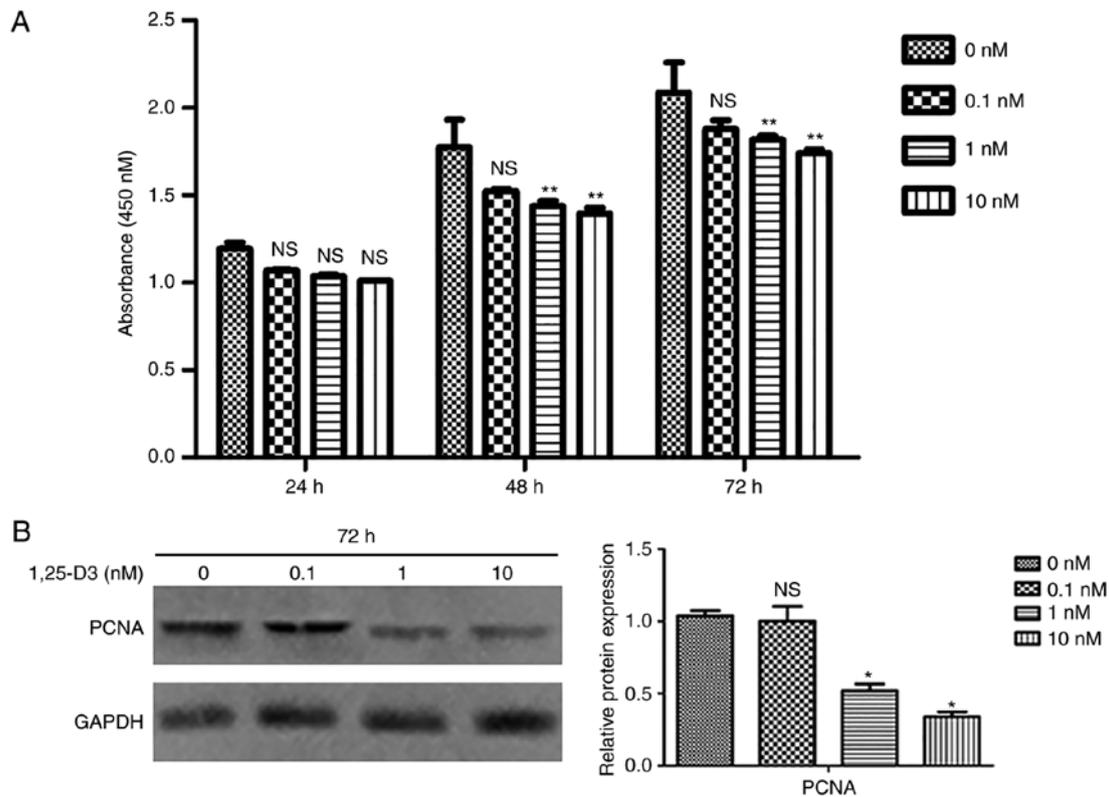
Figure 3. 1,25-D3 inhibits the proliferation of hPDLSCs. (A) Effects of different concentrations of 1,25-D3 (0, 0.1, 1 and 10 nM) on the proliferation of hPDLSCs were analyzed by CCK-8 at 24, 48 and 72 h following treatment. Cell proliferation was significantly inhibited by 1,25-D3 (1 and 10 nM) in hPDLSCs at 48 and 72 h. (B) Western blotting showed that 1,25-D3 downregulated the protein expression of PCNA in hPDLSCs following treatment. $^*P<0.05$ and $^{**}P<0.01$, vs. control (0 nM group). hPDLSCs, human periodontal ligament stem cells; PCNA, proliferating cell nuclear antigen; NS, not significant.

Results

Characterization of hPDLSCs. The hPDLSCs exhibited a typical spindle-shaped morphology (Fig. 1A). In flow cyto-metric analysis, the hPDLSCs negatively expressed CD34, CD11b, CD19, CD45, HLA-DR, but positively expressed CD90, CD44, CD105, CD73 (Fig. 1B). This indicated that hPDLSCs exhibit a similar phenotypic characterization as MSCs. Furthermore, the hPDLSCs demonstrated multi-potency, as evidenced by the formation of mineralized nodules, lipid droplets and cartilage following induc-tion (Fig. 1C-E).

1,25-D3 upregulates the expression of VDR in hPDLSCs. To determine the expression profile of the VDR in hPDLSCs and further assess whether its expression is induced by 1,25-D3, the expression of VDR was measured by immunofluores-cence and western blotting. The cells were incubated in the presence or absence of 10 nM 1,25-D3 for 48 h. Under basal conditions (0 nM), the specific immunofluorescence signal for VDR (green) was weak. However, the expression of VDR was upregulated following 48 h incubation with 10 nM 1,25-D3 (Fig. 2A).

The upregulation of VDR in response to 1,25-D3 was further validated by western blotting. Following culture with 10 nM 1,25-D3 for 48 h, the quantitative results from the western blotting revealed an increase in the expression of VDR relative to the controls (0 nM) (Fig. 2B and C).

1,25-D3 inhibits the proliferation of hPDLSCs. To evaluate the effect of 1,25-D3 on hPDLSC proliferation, the CCK-8 proliferation assay was performed. The hPDLSCs were treated with different concentrations of 1,25-D3 (0, 0.1, 1 and 10 nM) for 24, 48, and 72 h. Starting at 48 h, 1 and 10 nM 1,25-D3 induced a significant reduction in cell proliferation compared with the control cells (Fig. 3A). To further corroborate these findings, the expression of PCNA was examined by western blotting, and decreased expression of PCNA was confirmed at the protein level (Fig. 3B).

1,25-D3 promotes the osteogenic differentiation of hPDLSCs. In order to elucidate the role of 1,25-D3 in osteogenic differentiation and mineralization, ALP and AR staining were performed on hPDLSCs cultured with OIM with or without 10 nM 1,25-D3 for 7 and 14 days, respectively. The ALP staining of hPDLSCs treated with 10 nM 1,25-D3 for 7 days was more extensive than that in the corresponding group cultured without the drug (Fig. 4A). In the normal basic medium group, no calcium nodules were observed following incubation for 14 days; in the osteogenic induction group, there was notable calcium nodule formation. The hPDLSCs treated with 10 nM 1,25-D3 had a high number of calcium nodules than those cultured without the drug for 14 days (Fig. 4B). Furthermore, the protein expression of osteogenic markers were examined in hPDLSCs cultured in OIM with or without 10 nM 1,25-D3 for 7 and 14 days. Consistently, the cells cultured in OIM with 10 nM 1,25-D3 expressed significantly
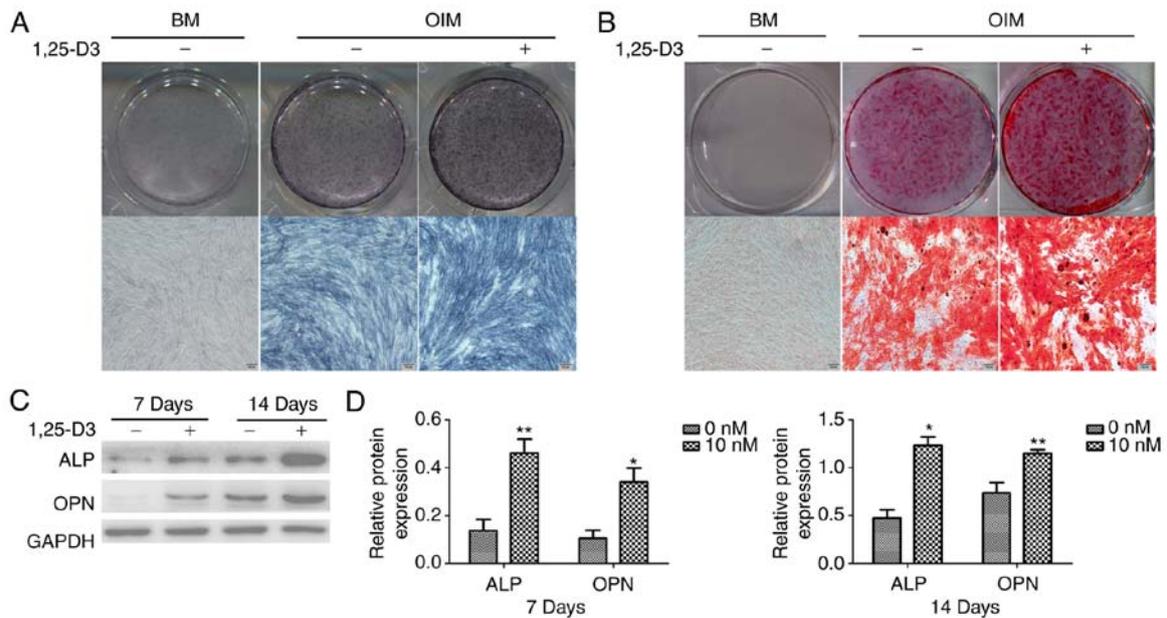
Figure 4. 1,25-D3 promotes the osteogenic differentiation of hPDLSCs. (A) Osteogenic differentiation following 7 days incubation was examined by ALP staining. In the BM group, the expression was ALP was undetectable. The expression of ALP in hPDLSCs cultured in OIM with 10 nM 1,25-D3 was more extensive than in those cultured in OIM alone. (B) Mineralization following 14 days incubation was examined by AR staining. No calcium nodules were observed in the BM group. In the OIM group, the presence of 10 nM 1,25-D3 significantly enhanced AR staining (scale bar, 100 μm). (C) Expression levels of osteogenic markers were detected by western blotting. The addition of 10 nM 1,25-D3 significantly upregulated the expression levels of ALP and OPN in hPDLSCs. (D) Quantitative analysis of protein band density. *P<0.05 and **P<0.01, vs. 0 nM. hPDLSCs, human periodontal ligament stem cells; BM, basic medium; osteogenic induction medium; ALP, alkaline phosphatase; OPN, osteopontin.
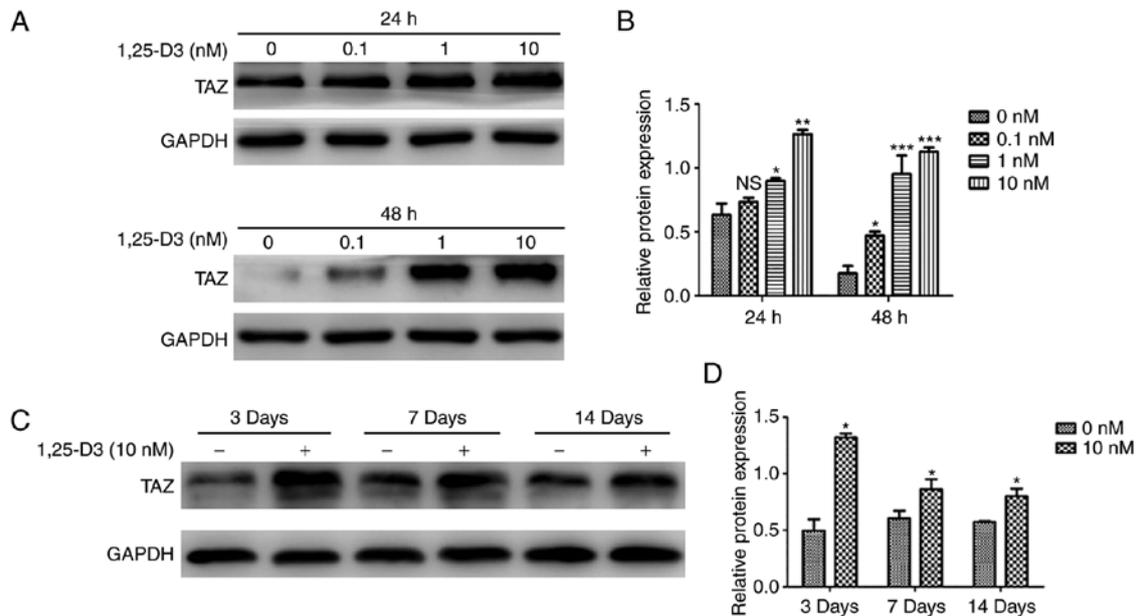

Figure 5. 1,25-D3 treatment upregulates TAZ in hPDLSCs. (A) Western blot analysis showed that 1,25-D3 upregulated the protein expression of TAZ in PDLSCs cultured with BM for 24 and 48 h in a dose-dependent manner. (B) Quantification of the western blot data from A. (C) 1,25-D3 (10 nM) upregulated the protein expression of TAZ in PDLSCs in the context of osteogenic induction for 3, 7 and 14 days. (D) Quantification of the western blot data from C. *P<0.05, **P<0.01 and ***P<0.001, vs. 0 nM. hPDLSCs, human periodontal ligament stem cells; TAZ, transcriptional coactivator with the PDZ-binding motif; NS, not significant.

higher levels of ALP and OPN than those cultured in OIM without the drug (Fig. 4C and D).

1,25-D3 treatment upregulates TAZ in hPDLSCs. To determine whether the 1,25-D3-induced osteogenic differentiation of hPDLSCs involves the modulation of TAZ, the effects of 1,25-D3 on the expression of TAZ were examined in hPDLSCs cultured with basic medium for 24, 48 h or OIM for 3, 7, and 14 days. It was found that 1,25-D3 upregulated the expression of TAZ in the hPDLSCs incubated with basic medium (Fig. 5A and B). Similarly, the protein levels of TAZ were significantly upregulated by treatment with
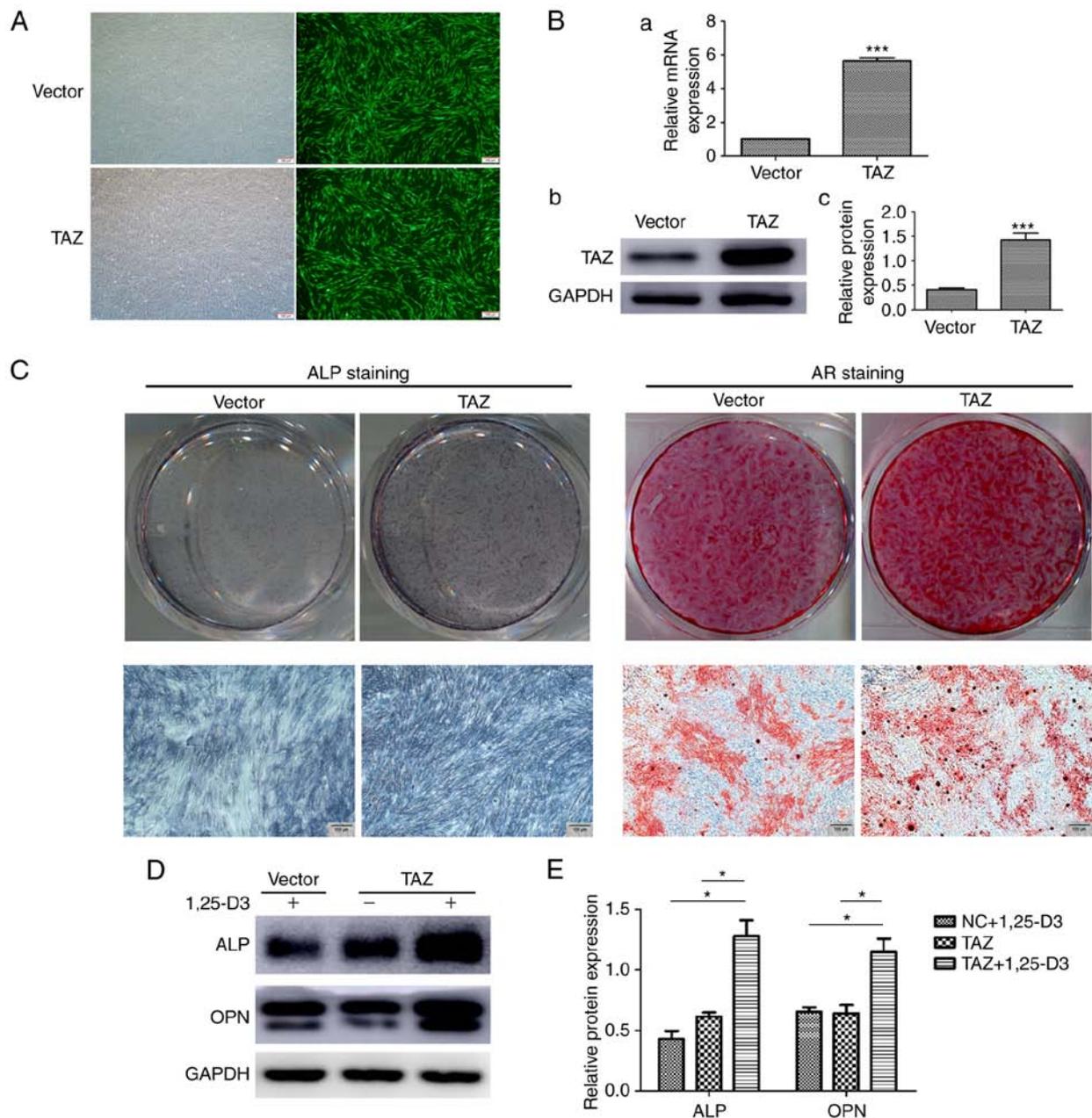
Figure 6. Potential interaction of 1,25-D3 and TAZ on the osteogenic differentiation of hPDLSCs. (A) Fluorescence detection of hPDLSCs transduced with lentiviral expression vectors. Upper panels show cells transduced with normal lentivirus vectors (vector), lower panels show cells transduced with TAZ overexpressing lentiviral vectors (TAZ). Scale bar, 100 mM (B) (a) mRNA and (b) protein levels of TAZ, (c) quantification of the protein expression levels were significantly increased following transfection with lentiviral vectors expressing TAZ, compared with those in the control. (C) ALP staining of TAZ-overexpressing hPDLSCs showed enhanced osteogenic differentiation following osteogenic induction *in vitro* for 7 days. AR staining of the TAZ-overexpressing hPDLSCs showed increased mineralization following osteogenic induction for 14 days. (D) Following culture with OIM for 7 days, co-treatment of 1,25-D3 with TAZ overexpression significantly enhanced protein expression levels of ALP and OPN compared with levels when treated with TAZ overexpression or 1,25-D3 alone. (E) Quantification of the data from D. $^*P<0.05$, $^{***}P<0.001$ (scale bar, 100 μm). hPDLSCs, human periodontal ligament stem cells; TAZ, transcriptional coactivator with the PDZ-binding motif; ALP, alkaline phosphatase; AR, Alizarin Red.

10 nM 1,25-D3 in the hPDLSCs incubated with OIM for 3, 7 and 14 days (Fig. 5C and D).

Potential interaction of 1,25-D3 and TAZ in the osteogenic differentiation of hPDLSCs. To further elucidate the mechanism of 1,25-D3 on the osteogenic differentiation of PDLSCs, the hPDLSCs were transfected with lentiviral vectors containing the human TAZ cDNA sequence to obtain stable cell clones. The overexpression of TAZ in hPDLSCs was verified by RT-qPCR and western blot analyses (Fig. 6A and B).

Subsequently, the hPDLSCs overexpressing with TAZ were examined for their osteogenic differentiation capacity *in vitro*. Following osteogenic induction, the TAZ-overexpressing hPDLSCs exhibited marked ALP staining on day 7 and enhanced mineralization, revealed by AR staining, on day 14 (Fig. 6C).

Taken together, the results obtained revealed that TAZ was critical for driving the osteogenic differentiation of hPDLSCs and was upregulated by 1,25-D3 treatment. Subsequently, whether there is an interaction between 1,25-D3

and TAZ in the osteogenic differentiation of hPDLSCs was investigated. Osteogenic markers in the TAZ-overexpressing hPDLSCs treated with or without 10 nM 1,25-D3 were examined. Following culture with OIM for 7 days, the cells overexpressing TAZ treated with 1,25-D3 expressed markedly enhanced protein levels of ALP and OPN, compared with TAZ overexpression or 1,25-D3 treatment alone (Fig. 6D). This data suggest that overexpression of TAZ and treatment with 1,25-D3 synergistically stimulate the expression of osteogenic markers in hPDLSCs.

Discussion

Alveolar bone defects due to periodontal diseases, periapical diseases, jaw osteomyelitis and maxillofacial tumors are associated with, not only functional, but also aesthetic and psychological problems. A stem cell-based tissue-engineering approach is perceived as a promising therapeutic alternative for bone defects, which has revolutionized current treatment paradigms (34). The periodontal ligament itself contains PDLSCs, which allow for the self-renewal and regeneration of other tissues, including cementum and alveolar bone. It has been reported that PDLSCs are able to undergo multilineage differentiation, for example to chondrogenic, osteogenic and adipogenic lineages, when exposed to suitable inductive conditions (35). Additionally, the data obtained in the present study showed the potential of PDLSCs to form calcified deposits *in vitro*. However, it remains challenging to fully exploit the therapeutic potential of hPDLSCs for bone repair and regeneration.

Skeletal development is controlled by multiple osteogenic signals, and 1,25-D3 is one physiological regulator of osteogenic differentiation. 1,25-D3 exerts its biological action through binding to VDR, a member of the nuclear hormone receptor superfamily (36). In the present study, the addition of 1,25-D3 upregulated the expression of VDR in hPDLSCs. This result is consistent with a study on H9c2 cardiac cells, which showed that the expression of VDR increased upon incubation with 1,25-D3 (37). Through binding to VDR, 1,25-D3 has been found to inhibit the proliferation of certain cells, including osteoblasts, osteoclasts (38) and MSCs (36,39). Similarly, the present study study revealed that treatment with 1,25-D3 inhibited the proliferation of hPDLSCs.

As a systemic factor, 1,25-D3 is a key signaling moiety that is well known for its roles in the regulation of bone metabolism. For example, the expression of OPN is upregulated by 1,25-D3 in osteoblast cells (40). 1,25-D3 is also essential in osteocalcin synthesis in bone cells *in vitro* (41). In addition, treatment with 1,25-D3 significantly increases the activity of ALP in hMSCs (36).

In the present study, it was hypothesized that 1,25-D3 may enhance the osteogenic differentiation of hPDLSCs *in vitro*. The results revealed enhanced ALP staining of the hPDLSCs treated with 10 nM 1,25-D3 in OIM relative to the control groups. In addition, hPDLSCs treated with 10 nM 1,25-D3 exhibited significantly more calcium nodules than those cultured without the drug for 14 days. ALP is a key osteogenic maker expressed at the early stage of osteogenic differentiation (42), whereas OPN is a marker reflecting osteogenic maturation and bone formation (43). The results of the present study confirmed that

the expression of ALP and OPN were upregulated by 1,25-D3 in hPDLSCs. Although there is sufficient evidence supporting the enhanced effects of 1,25-D3 on the osteogenic differentiation of hPDLSCs, the mechanism of 1,25-D3 in hPDLSC osteogenic differentiation remains to be fully elucidated.

The Hippo pathway is a key pathway in controlling tissue regeneration. Inhibition of the Hippo pathway causes an accumulation of TAZ and its eventual translocation into the nucleus where it activates the transcription of its target genes, which are required for osteogenic differentiation (44). Of note, canonical Wnt signaling promotes the differentiation of MSCs into osteoblasts through TAZ stabilization and upregulation (45). Zinc-finger transcriptional factors Snail/Slug interact with TAZ to form complexes that induce the osteogenesis of skeletal stem cells (46). Furthermore, the overexpression of TAZ in osteoblasts significantly enhances bone formation in transgenic mice (47). TAZ has been found to drive osteogenic differentiation in stem cells from a diverse source, and it has been reported that TAZ can regulate the sensitivity of intra-hepatic cholangiocarcinoma cells to vitamin D *in vivo* (48). Taking all available data into consideration, it was hypothesized that the osteogenic effect of 1,25-D3 in hPDLSCs involve the actions of TAZ. The results of the present study indicated that 1,25-D3 enhanced the protein levels of TAZ in hPDLSCs in the context of either basic medium or OIM.

To further examine the underlying mechanism, the present study generated stable TAZ-overexpressing cell clones of hPDLSCs. The results revealed that the overexpression of TAZ promoted the osteogenic differentiation of hPDLSCs *in vitro*. As it was found that the protein expression of TAZ was increased in hPDLSCs following treatment with 1,25-D3, and that TAZ was found to be associated with osteogenic differentiation in several previous studies and the present study, the subsequent aim was to understand the potential interaction between 1,25-D3 and TAZ on the osteogenic differentiation of hPDLSCs. The osteogenic marker expression levels of hPDLSCs were examined under the induction of osteogenic differentiation following 1,25-D3 treatment and TAZ overexpression, alone or in combination. The results showed that the combination of 1,25-D3 treatment and TAZ overexpression potently enhanced the protein expression levels of ALP and OPN in hPDLSCs cultured in OIM. These data showed that the treatment of hPDLSCs overexpressing TAZ with 1,25-D3 synergistically stimulated osteogenic marker expression. This synergistic stimulation may be important in the osteogenic differentiation of hPDLSCs, however, the precise underlying mechanism for this synergistic interaction remains to be elucidated.

In conclusion, the present study confirmed that 1,25-D3 treatment enhanced the osteogenic differentiation of hPDLSCs, and indicated that this pro-osteogenic effect involves, at least in part, the actions of TAZ, a key Hippo signaling pathway effector. These insights may be valuable for therapeutic strategies, including preprogramming stem cells prior to clinical use. However, the present study focused only on whether TAZ was involved in the effect of 1,25-D3 on the osteogenic differentiation of hPDLSCs. Future detailed investigations with an inhibitor of TAZ or TAZ-knockdown are warranted to determine the underlying mechanisms of TAZ in the osteogenic effect of 1,25-D3.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XX designed the experiments. YJ and PZ performed the experiments. YX, LJ, YZ, XW and BZ analyzed the data and YJ wrote the manuscript.

Ethics approval and consent to participate

This study was approved by the Medical Ethical Committee of School of Stomatology, Shandong University (protocol no. 201 70303). Each participant provided written informed consent in accordance with the Declaration of Helsinki.

Patient consent for publication

Each participant provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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