Connective Tissue Research

ISSN: 0300-8207 (Print) 1607-8438 (Online) Journal homepage: www.tandfonline.com/journals/icts20

# 1,25-Dihydroxyvitamin $D_3$ stimulates odontoblastic differentiation of human dental pulp-stem cells *in vitro*

**Goksen Mucuk, Elif Sepet, Mine Erguven, Ozlem Ekmekcı & Ayhan Bılır**

**To cite this article:** Goksen Mucuk, Elif Sepet, Mine Erguven, Ozlem Ekmekcı & Ayhan Bılır (2017) 1,25-Dihydroxyvitamin $D_3$ stimulates odontoblastic differentiation of human dental pulp-stem cells *in vitro*, Connective Tissue Research, 58:6, 531-541, DOI: 10.1080/03008207.2016.1264395

**To link to this article:** https://doi.org/10.1080/03008207.2016.1264395

Published online: 19 Jan 2017.

Submit your article to this journal

Article views: 417

View related articles

View Crossmark data

Citing articles: 2 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=icts20

CONNECTIVE TISSUE RESEARCH
2017, VOL. 58, NO. 6, 531–541
http://dx.doi.org/10.1080/03008207.2016.1264395

Taylor & Francis
Taylor & Francis Group

# 1,25-Dihydroxyvitamin $D_3$ stimulates odontoblastic differentiation of human dental pulp-stem cells *in vitro*

Goksen Mucuk[a], Elif Sepet[a], Mine Erguven [b], Ozlem Ekmekcı[c] and Ayhan Bılır[d]

[a]Pediatric Dentistry Department, Faculty of Dentistry, Istanbul University, Istanbul, Turkey; [b]Medical Biochemistry Department, Faculty of Medicine, Istanbul Aydın University, Istanbul, Turkey; [c]Biochemistry Department, Cerrahpasa Faculty of Medicine, Istanbul University, Istanbul, Turkey; [d]Histology and Embryology Department, Istanbul Faculty of Medicine, Istanbul Aydın University, Istanbul, Turkey

**ABSTRACT**

***Background***: 1,25-Dihydroxyvitamin $D_3$ (1,25-OH $D_3$) plays an important role in mineralized tissue metabolism, including teeth. However, few studies have addressed its role in odontoblastic differentiation of human dental pulp-stem cells (hDPSCs). ***Aim***: This study aimed to understand the influence of various concentrations of 1,25-OH $D_3$ on the proliferation capacity and early dentinogenesis responses of hDPSCs. ***Materials and Methods:*** hDPSCs were obtained from the impacted third molar teeth. Monolayer cultured cells were incubated with a differentiation medium containing different concentrations of 1,25-OH $D_3$ (0.001, 0.01, and 0.1 µM). All groups were evaluated by S-phase rate [immunohistochemical (IHC) bromodeoxyuridine (BrdU) staining], STRO-1 and dentin sialoprotein (DSP)+ levels (IHC), and alkaline phosphatase (ALP, enzyme-linked immunosorbent assay (ELISA)) levels. ***Results***: The number of cells that entered the S-phase was determined to be the highest and lowest in the control and 0.001 µM 1,25-OH $D_3$ groups, respectively. The 0.1 µM vitamin $D_3$ group had the highest increase in DSP+ levels. The highest Stro-1 levels were detected in the control and 0.1 µM 1,25-OH $D_3$ groups, respectively. The 0.1 µM 1,25-OH $D_3$ induced a mild increase in ALP activity. ***Conclusions***: This study demonstrated that 1,25-OH $D_3$ stimulated odontoblastic differentiation of hDPSCs *in vitro* in a dose-dependent manner. The high DSP + cell number and a mild increase in ALP activity suggest that DPSCs treated with 0.1 µM 1,25-OH $D_3$ are in the later stage of odontoblastic differentiation. The results confirm that 1,25-OH $D_3$-added cocktail medium provides a sufficient microenvironment for the odontoblastic differentiation of hDPSCs *in vitro*.

**ARTICLE HISTORY**
Received 5 March 2015
Revised 13 November 2016
Accepted 21 November 2016
Published online
18 January 2017

**KEYWORDS**
1,25-Dihydroxyvitamin $D_3$; cell culture; dental pulp-stem cells; odontoblastic differentiation; vitamin D

## Introduction

Tissue engineering is a multidisciplinary field that combines scientific knowledge with the aim of developing new tissues and organs (1,2). Tissue engineering has emerged as a novel technique for replacement of lost tissue. Stem cells are well known for their self-renewal and differentiation potential and have been shown to give rise to tissues with structural and functional properties mimicking natural organs (2). Application of these capabilities is useful for tissue engineering to study structure and function, engineering disease models, designing tissues for drug development, and generating replacement tissues that are compatible with the patient's tissues (3). Human dental pulp-stem cells (hDPSCs) are commonly used for not only tooth formation but also the formation of other organs such as the bladder (4).

hDPSCs have the ability to undergo self-renewal and differentiate into neurogenic, osteogenic, dentinogenic, and myogenic cell lineages in different inductive media (5). As a type of adult stem cells, hDPSCs usually perform asymmetric cell division, which gives rise to one daughter cell with stem-cell fate and another that can undergo further cell divisions to generate differentiated progenies. For the lack of specific cell surface markers, the identification of DPSCs mainly relies on their biological features, including small cell volume, high proliferation potency, high clonogenicity, self-renewal, and multiple differentiation potentials (5,6). DPSCs *in vivo* usually remain quiescent within adult dental pulps, but respond during injury to produce progenies with high proliferative potential, which can differentiate into terminally differentiated odontoblasts. Thus, the number of DPSCs in the normal dental pulp remains relatively constant. When one DPSC divides nine times, it generates nine lineage-specific progeny cells plus one unaltered daughter stem cell (7).

STRO-1 is a well-known mesenchymal stem-cell marker for the selection of periodontal ligament stem cells, DPSCs, and adipose-derived SCs (8–12). The sorted stem cells in high purity provide a better cell source for therapeutic

**CONTACT** Elif Sepet elifsepet@hotmail.com Pediatric Dentistry Department, Faculty of Dentistry, Istanbul University, 34093, Capa, Istanbul, Turkey.
© 2017 Taylor & Francis

purposes compared with the heterogeneous unsorted cells, but STRO-1+ cells are usually found in low numbers, and therefore *in vitro* expansion is needed (13,14).

Dentin is an ectomesenchyme-derived, collagen-containing, calcified tissue that forms the major part of the tooth. Dentinogenesis consists of highly controlled events with the formation of extracellular collagen fibrils and the deposition of plate-like carbonate apatite crystals (15). In dentin, two of the non-collagenous dentin matrix proteins are dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). DSP and DPP are products of a single gene called dentin sialophosphoprotein (DSPP) (16). DSPP processing by tolloid-related protein 1 or by bone morphogenetic protein 1 yields mature DSP and DPP, which are critical for dentin mineralization (17).

Several immunohistochemical (IHC) studies using anti-DSP antibodies showed the presence of DSP in odontoblasts, predentin, and dentin (18–21). DSP is more abundant in the predentin than in the mineralized dentin (20,21). Previous studies have suggested that DSPP also plays a role in enamel biomineralization, being localized to the initial enamel matrix proximal to the dentinoenamel junction (15,21).

Vitamin $D_3$ (cholecalciferol), well accepted as a pleiotropic steroid hormone, performs the principal physiological role of regulating calcium and phosphorus homeostasis and bone mineralization (22). The role of vitamin D in tooth formation is very important, and deficiency of vitamin D leads to hypocalcification of the dentin and enamel, resulting in unmineralized dental structure (23). In humans, vitamin $D_3$ is mainly generated in the skin in response to sun exposure, and then transported to the liver, where it is converted into a circulating form of vitamin D, 25(OH)$D_3$, by 25-hydroxylases. Finally, 25(OH)$D_3$ is transported to the kidney and converted into the most active form, 1,25$(OH)_2D_3$, by the 1-hydroxylase (24,25).

1,25-OH $D_3$, the physiologically active form of vitamin D, and its synthetic analogs are being increasingly recognized for their potent antiproliferative, cell-differentiation-promoting, immunomodulator, and anti-inflammatory activities (26–28).

It was hypothesized that 1,25-OH $D_3$ might induce proliferation capacity and odontoblastic differentiation of hDPSCs. The aim of this study was to understand the role of various vitamin D concentrations on the proliferation capacity and early dentinogenesis responses of hDPSCs.

## Materials and methods

### Cell culture

The study was approved by the ethics committee of Istanbul University, Istanbul Faculty of Medicine (2010/1106-375). Human-impacted third molars were obtained with informed consent. Dental pulp tissue explants were obtained from completely impacted third molar teeth of 10 patients aged 19–25 years.

The tooth samples were stored in a sterile container containing the culture medium (Techno Plastic Products, Trasadingen, Switzerland) and transported to the lab for sample processing. The dental pulp tissues were extracted from teeth in the laboratory under the laminar hood for primary cell cultures. The tissue fragments were minced using sterilized scalpels. The pulp tissue was digested in a solution of 3 mg/mL collagenase type I (Invitrogen, Carlsbad, CA) and 4 mg/mL dispase (Invitrogen) in Dulbecco's modified Eagle's medium (DMEM)-F12 nutrient mixture (Biological Industries, Israel) supplemented with 10% fetal bovine serum (FBS) and 100 µM L-ascorbic acid for 1 h at 37 °C in 5% $CO_2$. After centrifugation, the supernatant was removed and the cells were washed three times with DMEM-F12 supplemented with 10% FBS, and then the single-cell suspensions ($5 \times 10^5$/well) of the dental pulp were incubated in six-well culture plates (TPP) with DMEM-F12, 15% FBS (Scientific Labs, Yorkshire, UK), 1% antibiotics/antimycotic (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Biological Industries) at 37 °C under 5% $CO_2$ humidified air. Cell culture plates and flasks were monitored daily for cell growth with medium changes three times every week. Cells from all 10 donors were used in each assay.

### Experimental design

Osteo-odontoblastic culture medium (OCM) was prepared to provide the conditions for cellular differentiation and the induction of calcified matrix deposition by following the extensively reported method in previous (21–25) studies. OCM was prepared using DMEM-F12, dexamethasone (DEX; Sigma–Aldrich, Germany) ($10^{-8}$ M), 0.1% ethanol (as vehicle), bone morphogenic protein-2 (50 ng/mL), transforming growth factor beta-1 (20 ng/mL), and fibroblast growth factor-2 (5 ng/mL). Different concentrations of 1,25-OH $D_3$ (0.001, 0.01, and 0.1 µM) were added to the experimental cultures. The groups were determined as follows:

- Group 1 represented 0.1 µM 1,25-OH $D_3$ with OCM.
- Group 2 represented 0.01 µM 1,25-OH $D_3$ with OCM.
- Group 3 represented 0.001 µM 1,25-OH $D_3$ with OCM.
- Positive control group with OCM.

- Negative control group with basic cell culture (BCC).

All groups were evaluated by cell number (counted using a hemocytometer), S-phase rate (IHC BrdU staining), STRO-1 and DSP levels (IHC), and alkaline phosphatase activity (ALP, ELISA) levels for 7 days.

### Cell proliferation

DPSCs were seeded at a concentration of $1 \times 10^4$ per well into 24-well culture plates containing 1 mL of the medium. The medium was changed every day. After attachment, test solutions were applied for differentiation for 7 days. The cells were harvested after 7 days, and the total cell number was determined using an automated cell counter (Nucleocounter, Allerod, Denmark).

### S-phase evaluation by BrdU staining

The number of cells in the S-phase of the cell cycle was determined by a BrdU staining technique. DPSCs were seeded at a concentration of $1 \times 10^4$ on circular microscope cover glasses (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) in 24-well culture plates containing 1 mL of the medium. The medium was changed every day. After attachment, test solutions were applied for differentiation for about 7 days. After 7 days, BrdU (Invitrogen, Frederick, MD) was added as described previously (29,30) to all wells (final concentration of 20 μM of BrdU) for 2 h at 37 °C. The cells were then fixed in 70% cold ethanol at –20 °C for 30 min. Endogenous peroxidase activity was quenched with 0.5% $H_2O_2$ in methanol. Double-stranded DNA was denatured with 4 N HCl at 37 °C for 30 min. After washing, a nonspecific blocking reagent (Invitrogen) was used to prevent nonspecific binding. The primary and secondary antibodies used were the monoclonal mouse (Invitrogen; 1:50 dilution, overnight at 4 °C) and biotinylated goat anti-mouse antibodies (1:200 dilution, 1 h at room temperature; Invitrogen), respectively. After washing, peroxidase-conjugated streptavidin (Lab Vision Co., Fremont, CA) was applied, and the aminoethylcarbazole chromogen was used. Sections were counterstained with Mayer's hematoxylin to enhance nuclear staining. For negative controls, adjacent sections were processed through the same steps without primary and secondary antibodies and labels. Cells with red nuclei were accepted as the BrdU-labeling index (LI)-positive cells.

Photographs were taken using a camera attached to a light microscope. All BrdU-labeled cells were counted by two investigators. Preparations without artifact and bubbles were selected and 30 areas were counted. The BrdU LI was calculated by the following formula: number of positively stained cells/total number of cells counted × 100.

### Determination of Stro-1 and DSP+ levels

Immunocytochemical staining of cells was performed as described previously (31), before and after incubation with OCM. DPSCs were seeded at a concentration of $1 \times 10^5$/mL on circular microscope cover glasses in 24-well culture plates. After attachment, test materials were applied for differentiation for 7 days. IHC was performed following the instructions of a commercial kit (Histostain-Bulk-SP Detection Kit, Invitrogen Life Technologies, Cat no. 85-8943). The cells were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS containing 0.1% Triton X-100 (Sigma–Aldrich) at 4 °C for 30 min. After rinsing in PBS, the samples were blocked with 2.5% bovine serum albumin in PBS for 30 min at room temperature and subsequently incubated at 37 °C for 2 h with the following primary antibodies: STRO-1 (1:50 dilution; mouse IgM, dilution range 1:100–1:400; Santa Cruz Biotechnology, Heidelberg, Germany) and DSP (1:50 dilution; goat IgG, dilution range 1:100–1:400; Santa Cruz Biotechnology). After several washes in PBS, the samples were incubated with secondary antibodies (1:1500 dilution, biotinylated goat anti-mouse, Lab Vision Co.) at 37 °C for 1 h. After rinsing in PBS, the samples were counterstained in diaminobenzidine. Staining omitting primary and secondary antibodies or chromogen served as controls. Sections were counterstained with Mayer's hematoxylin. STRO-1+ (brown-stained cell surface) and DSP+ (brown-stained) cells were determined and counted under the light microscope with several magnifications by two investigators. Preparations without artifact and bubbles were selected and 25 areas were counted. The staining was calculated by the following formula: number of positively stained cells/total number of cells counted × 100.

### Determination of ALP activity by ELISA

ALP activity was measured by ELISA using the commercial kit (The Metra BAP EIA kit; Quidel, CA). The procedure was performed according to the manufacturer's instructions. ALP was expressed in IU/L, where 1 unit represented 1 mmol of *p*-nitrophenyl phosphate (pNPP) hydrolyzed per minute at 25 °C in 2-amino-2-methyl-1-propanol buffer. ALP activity was determined from the absorbance of p-NP at a wavelength of 410 nm using a microplate reader (Elx800 Bio-Tek Instruments Inc., Winooski, VT). The protein content of each well was determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA). ALP activity was expressed as the production of

nanomoles of p-nitrophenylphosphate per microgram of cell protein per minute and expressed as nmol/mg protein/min.

### Statistical analyses

The data were expressed as the means ± standard deviation and analyzed using the NCSS software system (Number Cruncher Statistical System, 2007, Kaysville, UT) applying analysis of variance, Tukey's and Pearson correlation tests; $p$ values less than 0.05 were considered statistically significant.

## Results

### Cell proliferation

We first evaluated the effect of Vitamin $D_3$ on dental pulp cell (DPC) proliferation using direct cell counting. We found that the 0.001, 0.01, and 0.1 μM vitamin $D_3$-added OCM groups demonstrated decreased cell numbers of 6.02 ± 1.8, $p = 0.0001$; 6.73 ± 2.2, $p = 0.001$; and 11.49 ± 6.43, $p = 0.001$, respectively, compared with the OCM group (14.70 ± 5.55).

### S-phase evaluation

S-phase cells were identified by BrdU labeling. Light microscopic photographs of BrdU-positive cells are represented in Figure 1. Cells with red nuclei showed BrdU positivity, indicating that they were in the S-phase. We found that the 0.001, 0.01, and 0.1 μM vitamin $D_3$-added OCM groups demonstrated decreased DNA synthesis of 5.52 ± 2.43, 6.62 ± 2.28, and 12.1 ± 3.09, respectively, compared with the OCM (15.83 ± 3.61) and BCC (13.22 ± 2.23) groups. The number of cells that entered the S-phase was determined as the highest in the control and the lowest in the 0.01 and 0.001 μM 1,25-OH $D_3$ groups. Low concentration decreased the DNA synthesis ($p = 0.0001$), (Figure 2).

### Alterations of Stro-1 and DSP+ levels

STRO-1 and DSP+ cells were identified by IHC staining.

### DSP+ levels

IHC staining of DSP at cells cultured with induction media for 7 days is represented in Figure 3.

The 0.001, 0.01, and 0.1 μM vitamin $D_3$-added OCM groups demonstrated increased DSP levels of 14.41 ± 3.5, 36.16 ± 5.98, and 45.77 ± 3.18, respectively, in a dose-dependent manner, compared with the OCM (26.61 ± 3.04) and BCC (27.53 ± 11.23) groups (Figure 4).

The 0.1 μM 1,25-OH $D_3$ group induced the highest increase in DSP+ levels compared with other groups ($p = 0.0001$).

### Stro-1+ levels

IHC staining of Stro-1 at cells cultured with induction media for 7 days is represented in Figure 5.

The 0.001, 0.01, and 0.1 μM vitamin $D_3$-added OCM groups demonstrated increased Stro-1 levels of 4.86 ± 3.92, 13.6 ± 2.65, and 22.86 ± 6.66, respectively, in a dose-dependent manner.

The highest Stro-1 levels were detected in the control (42.71 ± 3.67) and 0.1 μM 1,25-OH $D_3$ groups, respectively ($p = 0.0001$). The lowest levels were determined in the 0.001 μM 1,25-OH $D_3$ and BCC (11.78 ± 8.47) groups ($p = 0.0001$) (Figure 6).

### ALP activity

ALP activity was measured by ELISA. We found that the 0.001, 0.01, and 0.1 μM vitamin $D_3$-added OCM groups demonstrated increased ALP levels of 217.33 ± 11.17, 201.30 ± 17.83, and 169.42 ± 8.35, respectively, compared with the OCM (164.45 ± 10.81) and BCC (155.30 ± 6.61) groups.

The vitamin $D_3$ groups had increased ALP levels in comparison to the control groups ($p < 0.05$). The 0.1 μM 1,25-OH D3 group demonstrated decreased ALP levels in comparison to other vitamin $D_3$ groups ($p < 0.05$). Moreover, 0.1 μM 1,25-OH $D_3$ induced a mild increase in ALP activity ($p > 0.05$) (Figure 7).

## Discussion

Dentin is the mineralized tissue comprising the body of a tooth. Dentin consists of 70% mineral, 20% organic matrix, and 10% water. Type I collagen and DSPP-derived proteins are the most abundant proteins in dentin (32). Sibling proteins, such as bone sialoprotein (BSP), DMP-1, and DSPP, all play positive roles in the process of odontoblast differentiation and maturation (33). Expression of a DSP transgene in the DSPP null background indicated distinct roles of DSP and DPP in dentin mineralization, with DSP regulating the initiation of dentin mineralization, and DPP being involved in the maturation of mineralized dentin (34). Human pulp cells can be induced *in vitro* to differentiate into cells of odontoblastic phenotype, characterized by polarized cell bodies and the accumulation of mineralized nodules (35).

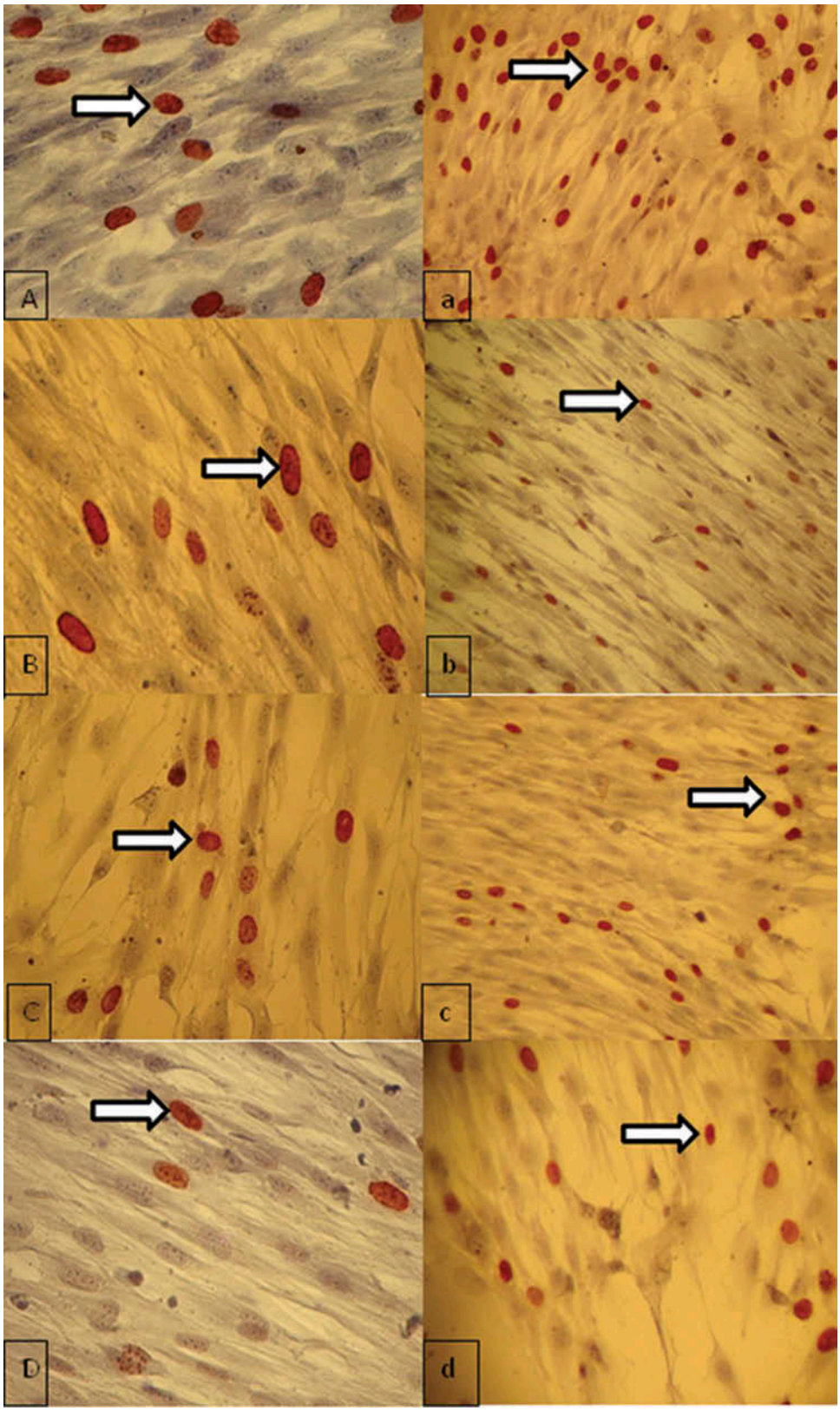


**Figure 1.** Representative immunohistochemistry images of hDPSC BrdU-positive cells. Arrowheads indicate BrdU-positive nucleus (red stain). Control (OCM) Group: A (×40), a (×10); 0.1 µM 1,25-OH $D_3$: B (×40), b (×10); 0.01 µM 1,25-OH $D_3$: C (×40), c (×10); 0.001 µM 1,25-OH $D_3$: D (×40), d (×10).

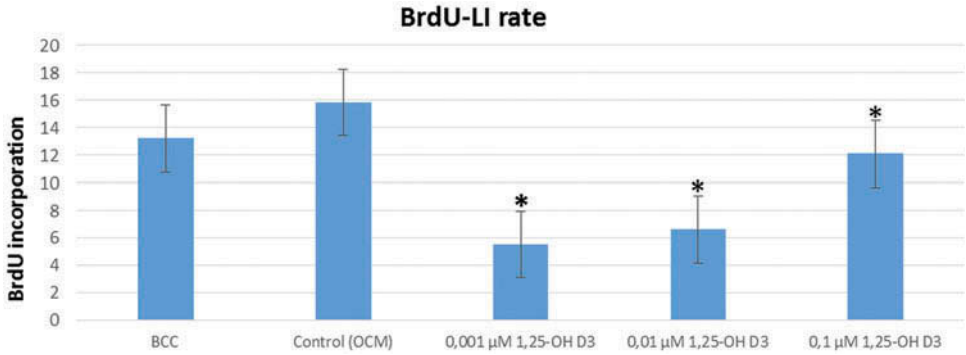


**Figure 2.** Effect of induction media (1,25-OH $D_3$-supplemented OCM) on hDPSC BrdU-LI rate. The bars represent means ± SD (n = 3). * represents a significant difference compared with the control ($p < 0.05$).

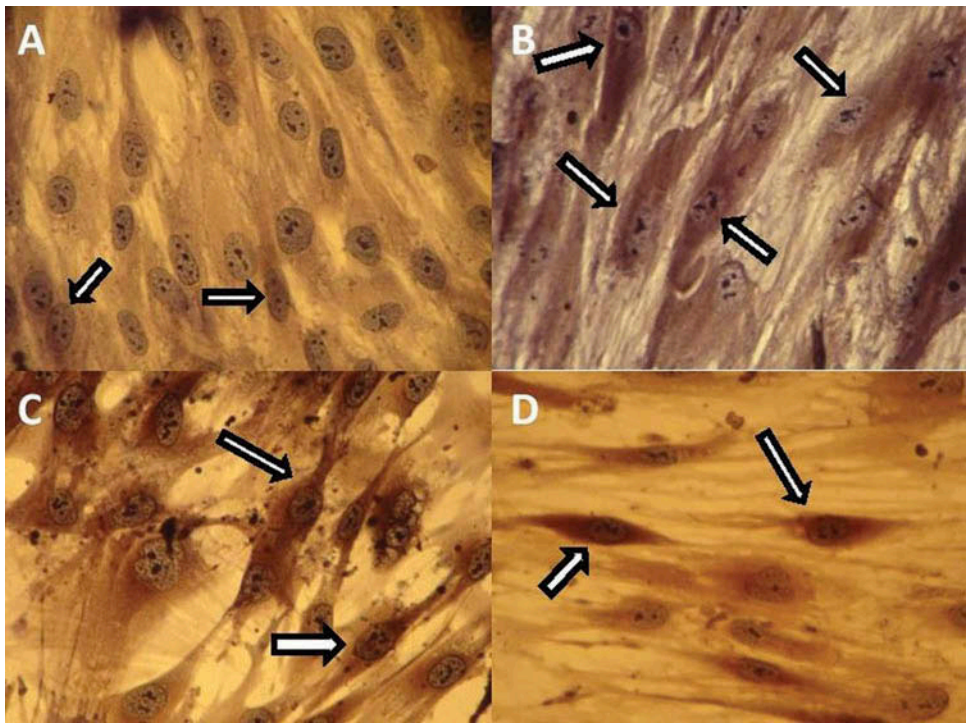


**Figure 3.** Representative immunohistochemistry images of DSP+ hDPSCs cultured in induction media for 7 days. A, Control (OCM) (×40); B, 0.1 µM 1,25-OH $D_3$ (×40); C, 0.01 µM 1,25-OH $D_3$ (×40); D, 0.001 µM 1,25-OH $D_3$ (×40). Arrowheads indicate DSP+ cells.

DPSCs obtained from impacted third molar teeth were studied extensively for their excellent proliferation and multipotential differentiation capacity (36).

DEX, together with ascorbic acid and β-glycerophosphate, is the most commonly used osteogenic inducer for human mesenchymal stem cells (37).

Vitamin $D_3$ in combination with β-glycerophosphate and ascorbic acid has been reported as more potent than DEX as an agent that induces osteogenic differentiation in human dental pulp cells (hDPCs) and human dental follicle cells (hDFCs) *in vitro* (38). There are reports suggesting that vitamin $D_3$ induced ALP activity and mineralization of hDPCs and hDFCs in basal cell culture medium without osteogenic factors, although it showed high effects in osteogenic medium (38,39). It was demonstrated that vitamin D metabolites, in combination with other commonly used osteogenic factors, increased the matrix maturation and mineralization in human osteoblast precursor cell line (40).

Cultures of ameloblasts and odontoblasts often use osteogenic media (41). It was reported that the combination of Dex, β-glycerophosphate, and vitamin $D_3$ is critical for the differentiation of human dental pulp-derived cells into odontoblasts (42).

In this study, cells were cultured in OCM containing DMEM-F12, DEX, bone morphogenic protein-2, transforming growth factor beta-1, and fibroblast growth factor-2 and different concentrations of

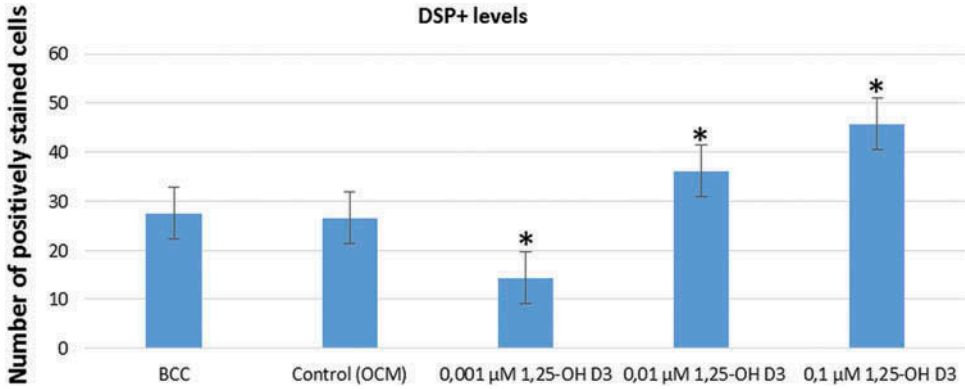


**Figure 4.** Effect of induction media (1,25-OH $D_3$-supplemented OCM) on hDPSC DSP expression. The bars represent means ± SD (n = 3). * represents a significant difference compared with the control ($p < 0.05$).

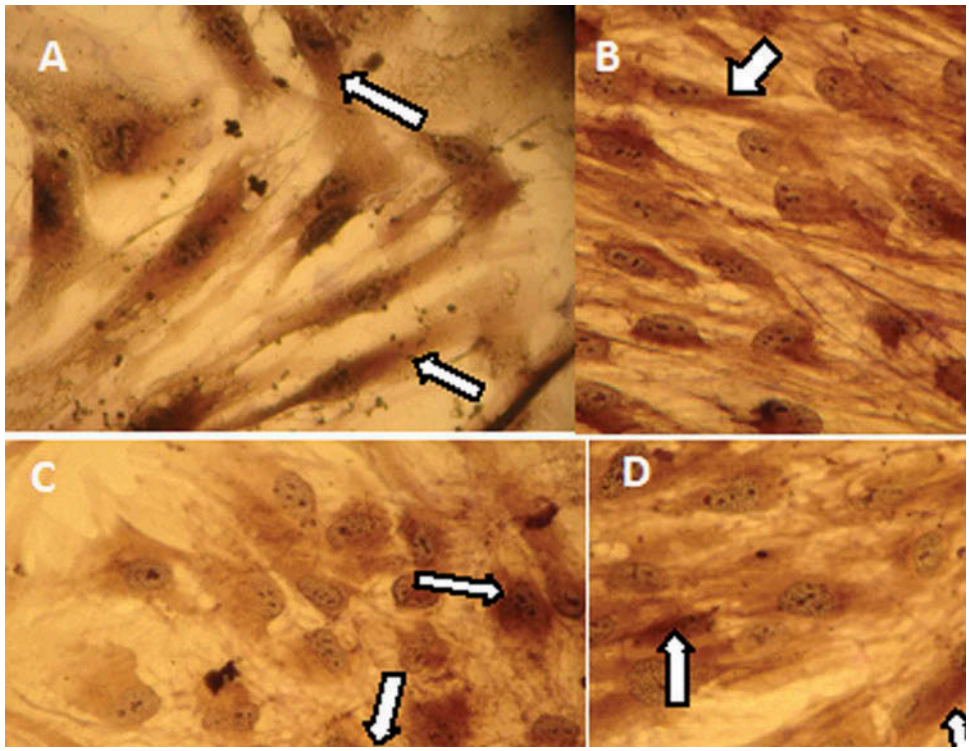


**Figure 5.** Representative immunohistochemistry images of Stro-$1^+$ hDPSCs cultured in induction media for 7 days. A, Control (OCM) (×40); B, 0.1 µM 1,25-OH $D_3$ (×40); C, 0.01 µM 1,25-OH $D_3$ (×40); D, 0.001 µM 1,25-OH $D_3$ (×40). Arrowheads indicate Stro-$1^+$ cells.

1,25-OH $D_3$. The OCM was prepared to provide the conditions for the differentiation of human dental pulp-derived cells into odontoblasts according to previous (21–25) studies.

One of the advantages of studying mineralizing in culture is the ability to modify the environment in which mineralization is occurring to test the hypotheses about the factors affecting mineral deposition (41). Culture media are usually supplemented with serum (to provide the required growth factors) or cocktails containing specific growth factors (43,44).

While these generally do not alter the supersaturation of the solution, they can and do alter the rate of mineral deposition and the physicochemical properties of the matrix (41).

We used a cocktail medium consisting of osteogenic inducers and growth factors for the odontoblastic differentiation of hDPSCs in order to alter the rate of mineral deposition and the physicochemical properties of the matrix. Additionally, we preferred to use the cocktail medium to increase the differentiative potential of pulp cells by mediating intracellular communication.

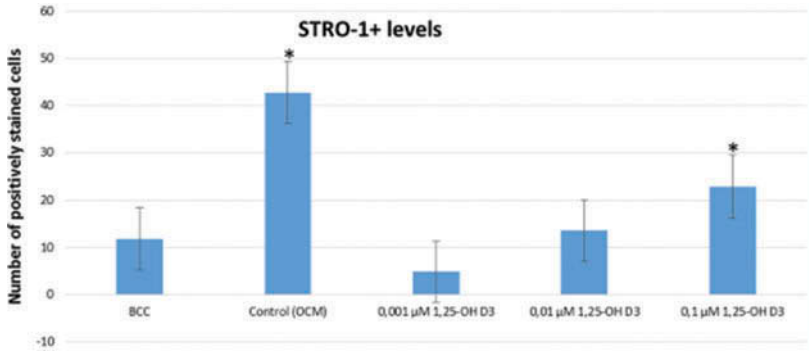


**Figure 6.** Effect of induction media (1,25-OH $D_3$-supplemented OCM) on hDPSC Stro-1 expression. The bars represent means ± SD (n = 3). * represents a significant difference compared with the control ($p < 0.05$).

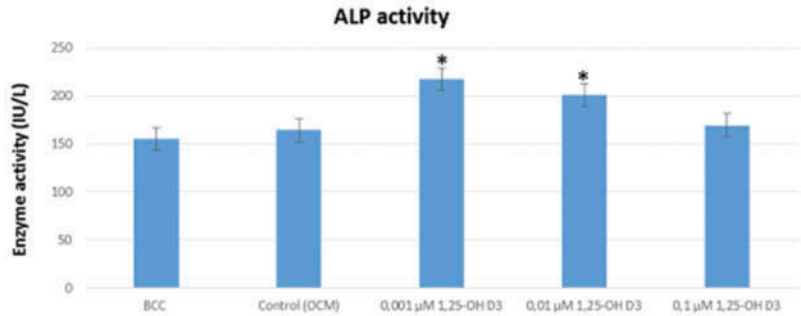


**Figure 7.** Effect of induction media (1,25-OH $D_3$-supplemented OCM) on hDPSC ALP activity. The bars represent means ± SD (n = 3). * represents a significant difference compared with the control ($p < 0.05$).

In the current study, OCM with 1,25-OH $D_3$ supplements increased the odontoblastic differentiation of hDPSCs, whereas OCM treatment alone was ineffective on the proliferation of cells undergoing odontoblast-like differentiation. OCM and BCC media resulted in a similar number of DSP+ cells. It can be explained that the heterogeneous nature of dental pulp-stem cells at different differentiation stages along odontoblastic lineage had a strong contribution in cell behavior and activity.

Additions of 0.01 and 0.1 µM 1,25-OH $D_3$ in the culture medium induced a 1.3- and 1.7-fold increase in the number of DSP+ cells, respectively.

This study revealed that the addition of 1,25-OH $D_3$ to hDPSCs failed to increase Stro-1+ cell numbers compared with OCM-treated cells. Moreover, 0.01 and 0.001 µM 1,25-OH $D_3$ inhibited the cell numbers in hDPSCs. The results were consistent with previous studies conducted on hMSCs, hDPCs, and hDFCs, wherein 1,25-OH $D_3$ inhibited the proliferation of Stro-1+ hDPSCs (38,45). It can be explained that 1,25-OH $D_3$ suppressed the cells within the S-phase of the cell cycle.

The incorporation of BrdU into DNA has been very useful in different biological systems to determine the fraction of S-phase cells (46). The cell cycle is tightly controlled by many regulatory mechanisms that either permit or restrain its progression (47). The signaling pathways that control these processes are central to the functioning of all multicellular life, and any drawbacks in the cell cycle control may lead to chromosome alterations, loss of cellular growth control, and the induction of cell transformation (48). Besides the effects of many regulatory mechanisms, the proliferation rate between donor cell lines might play a vital role in BrdU assay.

The length of S-phase varies from a few minutes to several hours in different organisms and cell types (49). Both exogenous and endogenous factors constantly stress and damage cells, including stem cells (50). Under unfavorable conditions such as nutrient and growth factor deprivation or high cell density, cells can reversibly withdraw from the cell cycle and enter into a resting non-proliferative gap (47). Akpinar et al. showed that SHED cells and DPSCs had a similar cell-cycle distribution, with most of the cells (78%) in the G1 phase of the cell cycle, whereas cells in the S- and G2-phases were present in lower proportions (12% and 10%) (51). In this study, the decrease in the number of cells that entered the S-phase can also be explained by the cessation of proliferation.

The differentiation and mineralization of osteoblasts and odontoblasts involve an initial period of extracellular matrix synthesis and proliferation, which is followed by

cell differentiation (52). ALP is also one of the markers of differentiation of DPSCs and plays an important role in the formation of calcified tissue and extracellular matrix (53).

Morsczeck (54) reported that DEX+ osteogenic supplements stimulated ALP activity and mineralization when the cells were cultured for 4 weeks (54). It was reported that the addition of 1,25-OH $D_3$ metabolite to osteogenic DEX+ supplements significantly increased the ALP activity of hDPSCs (42). Lee et al. reported that DEX treatment stimulated the ALP activity of human dental pulp cells from passages 1–4 in a time course after 8 days (55).

The results of this study showed that 0.01 and 0.001 μM 1,25-OH $D_3$ and DEX in addition to osteogenic supplements increased the ALP activity in hDPSCs after 7 days. A weak response to ALP activity was observed in the 0.1 μM 1,25-OH $D_3$ group. This response may be explained by the stages of differentiation. DSP protein has been identified as a late-stage marker of odontoblasts, the expression of which indicates the mature differentiation of odontoblasts (32). ALP is expressed early in the developing osteoblast, during the phase of matrix deposition, and is down-regulated in calcifying osteoblasts (56). The high DSP+ cell number and a mild increase in ALP activity suggest that DPSCs treated with 0.1 μM 1,25-OH $D_3$ are in the later stage of odontoblastic differentiation.

## Conclusions

In conclusion, this study demonstrated that 1,25-OH $D_3$ stimulated odontoblastic differentiation of hDPSCs *in vitro* in a dose-dependent manner. The results confirm that 1,25-OH $D_3$-added cocktail medium provides a sufficient microenvironment for the odontoblastic differentiation of hDPSCs *in vitro*.

## Funding

This study was supported by the Research Fund of Istanbul University, Project No. 15176.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

## ORCID

Mine Erguven http://orcid.org/0000-0002-6583-0684

## References

1. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell 2008;2:313–319.
2. Lymperi S, Ligoudistianou C, Taraslia V, Kontakiotis E, Anastasiadou E. Dental stem cells and their applications in dental tissue engineering. Open Dent J 2013;7:76–81.
3. Knowlton S, Cho Y, Li XJ, Khademhosseini A, Tasoglu S. Utilizing stem cells for three-dimensional neural tissue engineering. Biomater Sci 2016 Feb 18. [Epub ahead of print]
4. Song B, Jiang W, Alraies A, Liu Q, Gudla V, Oni J, Wei X, Sloan A, Ni L, Agarwal M. Bladder smooth muscle cells differentiation from dental pulp stem cells: future potential for bladder tissue engineering. Stem Cells Int 2016;2016:6979368.
5. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. J Dent Res 2002;81(8):531–535.
6. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 2009;88(9):792–806.
7. Yu J, He H, Tang C, Zhang G, Li Y, Wang R, Shi J, Jin Y. Differentiation potential of STRO-$1^+$ dental pulp stem cells changes during cell passaging. BMC Cell Biol 2010;11:32–38.
8. Pisciotta A, Carnevale G, Meloni S, Riccio M, De Biasi S, Gibellini L, Ferrari A, Bruzzesi G, De Pol A. Human Dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. BMC Dev Biol 2015;15:14. doi:10.1186/s12861-015-0065-x.
9. Liu M, Sun Y, Liu Y, Yuan M, Zhang Z, Hu W. Modulation of the differentiation of dental pulp stem cells by different concentrations of β-glycerophosphate. Molecules 2012;17:1219–1232.
10. Nakahara T, Nakamura T, Kobayashi E, Kuremoto K, Matsuno T, Tabata Y, Eto K, Shimizu Y. In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells. Tissue Eng 2004;10(3–4):537–544.
11. Tatullo M, Marrelli M, Shakesheff KM, White LJ. Dental pulp stem cells: function, isolation, and applications in regenerative medicine. J Tissue Eng Regen Med 2015;9:1205–1216.
12. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng 2006;12:2813–2823.
13. Itaya T, Kagami H, Okada K, Yamawaki A, Narita Y, Inoue M, Sumita Y, Ueda M. Characteristic changes of periodontal ligament-derived cells during passage. J Periodontal Res 2009;44:425–433.
14. Iwata T, Yamato M, Zhang Z, Mukobata S, Washio K, Ando T, Feijen J, Okano T, Ishikawa I. Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. J Clin Periodontol 2010;37:1088–1099.

15. Paine ML, Luo W, Wang HJ, Bringas P, Ngan AYW, Miklus VG, Zhu DH, MacDougall M, White SN, Snead ML. Dentin sialoprotein and dentin phosphoprotein overexpression during amelogenesis. J Biol Chem 2005;280:31991–31998.
16. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. J Biol Chem 1997;272:835–842.
17. Ritchie HH, Yee CT, Tang XN, Dong ZH, Fuller RS. DSP-PP precursor protein cleavage by tolloid-related-1 protein and by bone morphogenetic protein-1. PLoS One 2012;7:e41110.
18. Baba O, Qin C, Brunn JC, Jones JE, Wygant JN, McIntyre BW, Butler WT. Detection of dentin sialoprotein in rat periodontium. Eur J Oral Sci 2004;112:163–170.
19. Butler WT, Bhown M, Brunn JC, D'Souza RN, Farach-Carson MC, Happonen RP, Schrohenloher RE, Seyer JM, Somerman MJ, Foster RA, Tomana M, van Dijk S. Isolation, characterization, and immunolocalization of a 53-kDal dentin sialoprotein (DSP). Matrix 1992;12:343–351.
20. Bronckers AL, D'Souza RN, Butler WT, Lyaruu DM, van Dijk S, Gay S, Woltgens JH. Dentin sialoprotein: biosynthesis and developmental appearance in rat tooth germs in comparison with amelogenins, osteocalcin and collagen type-I. Cell Tissue Res 1993;272:237–247.
21. Baba O, Qin C, Brunn JC, Wygant JN, McIntyre BW, Butler WT. Colocalization of dentin matrix protein 1 and dentin sialoprotein at late stages of rat molar development. Matrix Biol 2004;23:371–379.
22. Holick MF. Vitamin D and bone health. J Nutr 1996;126:1159–1164.
23. van Driel M, Koedam M, Buurman CJ, Roelse M, Weyts F, Chiba H, Uitterlinden AG, Pols HA, van Leeuwen JP. Evidence that both 1alpha,25-dihydroxyvitamin D3 and 24-hydroxylated D3 enhance human osteoblast differentiation and mineralization. J Cell Biochem 2006;15:99(3):922–935.
24. MacLaughlin JA, Anderson RR, Holick MF. Spectral character of sunlight modulates photosynthesis of previtamin D3 and its photoisomers in human skin. Science 1982;216:1001–1003.
25. Haddad JG, Matsuoka LY, Hollis BW, Hu YZ, Wortsman J. Human plasma transport of vitamin D after its endogenous synthesis. J Clin Invest 1993;91:2552–2555.
26. Bikle D. Nonclassic actions of vitamin D. J Clin Endocrinol Metab 2009;94:26–34.
27. Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. Brain 2009;132:1146–1160.
28. Krishnan AV, Feldman D. Mechanisms of the anticancer and anti-inflammatory actions of vitamin D. Ann Rev Pharmacol Toxicol 2011;51:311–336.
29. Craig CG, Tropepe V, Morshead CM, Reynolds BA, Weiss S, van der Kooy D. In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci 1996;16:2649–2658.
30. Magavi SS, Macklis JD. Identification of newborn cells by BrdU labeling and immunocytochemistry in vivo. Methods Mol Biol 2008;438:335–343.
31. Burry RW. Controls for immunocytochemistry: an update. J Histochem Cytochem 2011;59:6–12.
32. Yamakoshi Y. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). J Oral Biosci /JAOB, Japanese Assoc Oral Biol 2009;51(3):134. doi:10.2330/joralbiosci.51.134.
33. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. Crit Rev Oral Biol Med 2004;15:126–136.
34. Suzuki S, Sreenath T, Haruyama N, Honeycutt C, Terse A, Cho A, Kohler T, Muller R, Goldberg M, Kulkarni AB. Dentin sialoprotein and dentin phosphoprotein have distinct roles in dentin mineralization. Matrix Biol 2009;28(4):221–229.
35. Lee JH, Lee DS, Choung HW, Shon WJ, Seo BM, Lee EH, Cho JY, Park JC. Odontogenic differentiation of human dental pulp stem cells induced by preameloblast-derived factors. Biomaterials 2011;32:9696–9706.
36. Papaccio G, Graziano A, d'Aquino R, Graziano MF, Pirozzi G, Menditti D, De Rosa A, Carinci F, Laino G. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 2006;208:319–325.
37. Kadar K, Kiraly M, Porcsalmy B, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, Kallo K, Szabo EL, Gera I, Gerber G, Varga G. Differentiation potential of stem cells from human dental origin—promise for tissue engineering. J Physiol Pharmacol 2009;60:167–175.
38. Khanna-Jain R, Vuorinen A, Sandor GK, Suuronen R, Miettinen S. Vitamin D3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells. J Steroid Biochem Mol Biol 2010;122:133–141.
39. Woo SM, Lim HS, Jeong KY, Kim SM, Kim WJ, Jung JY. Vitamin D promotes odontogenic differentiation of human dental pulp cells via ERK activation. Mol Cells 2015;38:604–609.
40. Mason SS, Kohles SS, Winn SR, Zelick RD. The influence of vitamin D metabolism on gene expression, matrix production and mineralization during osteoprecursor cell-based bone development. J Endocrinol Metab 2014;4:1–12.
41. Boskey AL, Roy R. Cell culture systems for studies of bone and tooth mineralization. Chem Rev 2008;108:4716–4733.
42. Tonomura A, Sumita Y, Ando Y, Iejima D, Kagami H, Honda MJ, Ueda M. Differential inducibility of human and porcine dental pulp-derived cells into Odontoblasts. Connect Tissue Res 2007;48:229–238.
43. Tsuboi T, Mizutani S, Nakano M, Hirukawa K, Togari A. FGF-2 regulates enamel and dentine formation in mouse tooth germ. Calcif Tissue Int 2003;73:496–501.
44. Fu H, Doll B, McNelis T, Hollinger JO. Osteoblast differentiation in vitro and in vivo promoted by Osterix. J Biomed Mater Res A 2007;83:770–778.
45. Feng Y, Sun Y, Jia W, Zhang C. Platelet-rich plasma and 1,25(OH)(2) vitamin D(3) synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells. Biotechnol Lett 2010;32:635–642.
46. Drouin R, Lemieux N, Richer CL. Analysis of DNA replication during S-phase by means of dynamic chromosome banding at high resolution. Chromosoma 1990:99:273–280.

47. de Carvalho LKH, de Araujo AVP, da Silva MGL, Laiso RAN, Maria DA. Response proliferative capacity of undifferentiated stem cells of obtained human adult dental follicle. Stem Cell Discovery 2014;4:125–137.
48. Agami, R., Bernards, R. Distinct ınitiation and maintenance mechanisms cooperate to ınduce G1 cell cycle arrest in response to DNA damage. Cell 2000;102:55–66.
49. Blumenthal AB, Kriegstein HJ, Hogness DS. The units of DNA replication in Drosophila melanogaster chromosomes. Cold Spring Harbor Symp Quant Biol 1974;38:205–223.
50. Turinetto V, Vitale E, Giachino C. Senescence in human mesenchymal stem cells: functional changes and ımplications in stem cell-based therapy. Int J Mol Sci 2016;17:1164. doi:10.3390/ijms17071164.
51. Akpinar G, Kasap M, Aksoy A, Duruksu G, Gacar G, Karaoz E. Phenotypic and proteomic characteristics of human dental pulp derived mesenchymal stem cells from a natal, an exfoliated deciduous, and an impacted third molar tooth. Stem Cells Int. 2014;2014: Article ID 457059.
52. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 2000;97:13625–13630.
53. Liu Q, Cen L, Yin S, Chen L, Liu G, Chang J, Cui L. A comparative study of proliferation and osteogenic differentiation of adipose-derived stem cells on akermanite and beta-TCP ceramics. Biomaterials 2008;29:4792–4799.
54. Morsczeck C. Gene expression of runx2, osterix, c-fos, DLX-3, DLX-5, and MSX-2 in dental follicle cells during osteogenic differentiation in vitro. Calcif Tissue Int 2006;78(2):98–102.
55. Lee DH, Kim NR, Ahn SJ, Yang HC. Effect of passage number on human dental pulp cell proliferation and differentiation. Biomat Res 2006;10(2):74–77.
56. Taskiran D, Evren V. Stimulatory effect of 17β-estradiol on osteogenic differentiation potential of rat adipose tissue-derived stem cells. Gen Physiol Biophys 2011;30:167–174.