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ORIGINAL ARTICLE

Odontogenic differentiation of dental pulp-derived stem cells on tricalcium phosphate scaffolds

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Abstract *Background/purpose:* The regeneration of dental-related tissue is a major problem in dentistry. Thus, it is beneficial to develop dental constructs that are fabricated with dental pulp stem cells (DPSCs) and an appropriate scaffold. The present study investigates the level of odontogenic differentiation of human DPSCs on tricalcium phosphate (TCP) scaffolds.
Materials and methods: We isolated pulp stem cells from human third molars and culture-expanded them through several successive subcultures. The cells from passage 3 were then loaded onto TCP scaffolds and treated with odontogenic supplements (OSs) that included vitamin D3 for a period of 21 days. DPSCs cultivated on TCP without OS, a monolayer culture treated with OS, and normal pulp tissue were the controls. We compared the groups in terms of odontogenic differentiation markers.
Results: Alkaline phosphatase (ALP) activity and the amount of culture mineralization, as well as the expression levels of dentin sialophosphoprotein (*DSPP*) and dentin matrix acidic phosphoprotein 1 (*DMP1*) genes tended to be significantly higher in the three-dimensional (3D) cultures treated with OS compared to those 3D cultures without OS and the monolayer culture with OS ($P < 0.05$). The differentiation level in 3D cultures was considerably lower than that in pulp tissue extracted from third molars ($P < 0.05$). 3D culture on TCP without OS showed a level of differentiation indicating an odontogenic property of the TCP biomaterial.
Conclusion: The 3D culture system improves odontogenic differentiation of DPSCs. The differentiation level of the cells in 3D culture is significantly lower than that of odontoblasts present in pulp tissue. TCP biomaterial possesses an odontogenic-inducing property.

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Introduction

During embryonic development, interactions between epithelial and dental papilla cells promote tooth formation by activating mesenchymal cells to generate odontoblasts.[1] During postnatal life, dental repair occurs through odontoblast activity.[2] These cells are believed to be maintained by a population of stem cells in the pulp tissue.[3,4] The odontoblast precursor cells initially isolated by Gronthos et al have been described as colonogenic cells capable of producing densely calcified nodules *in vitro* and a dentin-like structure *in vivo*.[5,6] Subsequent investigations have demonstrated that dental pulp stem cells (DPSCs) are similar to mesenchymal stem cells (MSCs) in that they consist of a fibroblastic morphology with selective adherence to solid surfaces, and have good proliferative potential and the capacity to differentiate *in vitro*.[7] DPSCs possess the potential to give rise to adipocytic, osteocytic, and chondrocytic neuron-like cells as well as hepatocytes *in vitro*.[8,9]

Another characteristic of DPSCs is their capacity for differentiation into odontoblastic cell lineages. Many investigators have attempted to establish and optimize the culture conditions for *in vitro* odontoblast differentiation of DPSCs. According to these studies, for odontoblast differentiation, DPSCs can be cultured in Eagle's basal medium, modified Eagle's medium, alpha-modified Eagle's medium, or Dulbecco's modified Eagle's medium (DMEM) supplemented with various inducers (glycerophosphate, dexamethasone, and vitamin D3), which are the prerequisites of odontoblast differentiation.[10–16] One report has described a model of human pulp cells cultured in RPMI-1640 in which differentiation was observed without supplementation.[17]

In addition to two-dimensional cultures, some authors have attempted to establish three-dimensional (3D) culture conditions for DPSC differentiation. In these studies, different types of biomaterials have been evaluated. Zhang et al have observed that the cultivation of DPSCs on 3D titanium scaffolds did not alter the odontogenic property of DPSCs.[16] Wang et al investigated the odontogenic differentiation of human DPSCs on nanofibrous poly (L-lactic acid) (NF-PLLA) scaffolds. They have reported that the odontogenic differentiation of DPSCs can be achieved on NF-PLLA scaffolds and is enhanced by the presence of bone morphogenetic protein (BMP)-7.[18] These authors have presented NF-PLLA as a promising scaffold for dentin regeneration.[19] Zheng et al have tested the biocompatibility of four different types of 3D scaffolds, namely pure poly(lactide-co-glycolic acid) (PLGA), PLGA/hydroxyapatite (HA), PLGA/tricalcium phosphate (TCP), and PLGA/calcium carbonate hydroxyapatite for tooth-tissue regeneration. They found that the PLGA/TCP scaffold performed better than the other three scaffolds in terms of dentin formation.[20]

In the above-mentioned studies, DPSCs were seeded onto scaffolds, after which they differentiated into odontoblastic lineages. The differentiation levels were then measured and compared to those of the other groups. There was no positive control (i.e., pulp tissue) to determine the differentiation level of the cultured cells in comparison to the tissue odontoblasts. We have taken this into consideration in the present study, which is an attempt to establish a 3D differentiation culture using human DPSCs and pure TCP, a totally biocompatible scaffold evidenced by many animal and human studies.

Materials and methods

Scaffolds

We used Kasios TCP (Kasios, Lanauguet, France), which is commercially available in the form of granules, sticks, blocks, and wedges. It has been described as a synthetic bone substitute made of a pure beta-TCP. Granules of 3 mm dimension were used in this study. TCP possesses completely interconnected pores with average diameters of 200–500 μm and a mean porosity of 60–80%.

Cell culture

The use of human extracted teeth for this study was approved by the local research Ethics Committee at the Royan Institute. Human third molars were collected from young male patients (age 20–25 years) at private dental clinics. The teeth were transferred to the cell culture laboratory where stem cells from the pulp tissue were isolated according to a previously published method, with some modifications.[18] The tooth was cut around the root–enamel boundary using dental fissure burs, and then pulp tissue was gently removed from the chambers and digested with an enzyme solution that consisted of 3 mg/mL collagenase type I and 4 mg/mL dispase (both from Sigma, St Louis, MO, USA) for 30 minutes at 37°C. The digest was added to 3 mL DMEM (Gibco, Paisley, UK) supplemented with 15% fetal bovine serum (FBS; Gibco) and centrifuged at 400*g* for 5 minutes. The pellet was then suspended in fresh medium, plated in a six-well culture plate at 10^4 cells/well and incubated in an atmosphere of 5% CO_2 at 37°C. The culture medium was changed twice weekly until confluency.

At this time, the cultures were trypsinized and subcultured at 1:3 ratios (Passage 1). Several additional subcultures provided adequate cells (Passages 3, 4, and 5) for the following experiments. Since similar results were obtained when the cells from different passages were used, representative results are shown in this paper for the Passage 3 cells.

Cell characterization

Multilineage differentiation

For chondrogenic differentiation, 2.5×10^4 cells from passage 3 DPSCs were pelleted at 400*g* for 5 minutes. DMEM supplemented with 10 ng/mL transforming growth factor-β3

(Sigma, Germany), 10 ng/mL BMP-6 (Sigma, Germany), 50 mg/mL insulin—transferrin—selenium premix (Sigma, Germany), 1.25 mg bovine serum albumin (Sigma, Germany), and 1% FBS were added to the pellets. The cultures were maintained for 3 weeks, during which the medium was exchanged twice weekly. Next, a number of differentiated pellets were histologically prepared, cut into 5-μm-thick sections, and stained with toluidine blue for metachromatic matrix detection.

For osteogenic differentiation, confluent cultures of passaged-3 DPSCs were treated with DMEM supplemented with 50 mg/mL ascorbic 2-phosphate (Sigma, St Louis, MO, USA), 10 nM dexamethasone (Sigma, USA), and 10 mM β-glycerol phosphate (Sigma, USA) for 3 weeks. During this period, the culture medium was exchanged twice a week. The cultures were then stained by Alizarin Red for mineralized matrix.

For adipogenic differentiation, the confluent cultures were treated with differentiation-inducing medium that consisted of DMEM supplemented with 50 μg/mL ascorbic acid 3-phosphate, 100 nM dexamethasone, and 50 μg/mL indomethacin. After 3 weeks, the cultures were examined by Oil Red O staining for lipid droplets. During the differentiation period, the culture medium was exchanged twice weekly.

Flow cytometry

In order to characterize the cells regarding their surface epitopes, flow cytometric analysis was performed. The following antibodies were applied to stain the cells: fluorescein isothiocyanate (FITC)-conjugated CD31, CD33, CD45, and CD90; and phycoerythrin (PE)-conjugated CD105, CD11b, CD34, CD44, and CD73 (all purchased from Becton Dickinson, Franklin Lakes, NJ, USA). About 10^6 passaged-3 cells were placed in 5 mL tubes. We then added 5 μL of either PE- or FITC-conjugated mouse antihuman antibody and 5 μL of blocking buffer, and then incubated the cells at 4°C for 20—25 minutes in the dark; after this, they were washed with 1 mL washing buffer [phosphate-buffered saline (PBS) supplemented with 1% FBS] and centrifuged at 400*g*. The cell pellet was then suspended in 300—500 μL washing buffer and analyzed by flow cytometry (FACSCalibur cytometer equipped with 488 nm argon lasers; Becton Dickinson, Franklin Lakes, NJ, USA). In this study, Immunoglobulin G (IGG)2 and IGG1 were used as isotope controls. WinMDI software (WinMDI 2.8 is free software designed by J. Trotter) was used to analyze the flow cytometric results.

Cell seeding

TCP scaffolds were autoclaved and then soaked in DMEM medium prior to cell seeding. A total of 2.5×10^5 passaged-3 cells were suspended in 0.2 mL DMEM and placed on the top surface of each 3 mm scaffold granule. Cells were allowed to attach to the ceramics for 2 hours at 37°C before being provided with either an odontogenic medium that consisted of DMEM supplemented with 500 nM vitamin D3, 50 mg/mL ascorbic 2-phosphate, 10 nM dexamethasone, and 10 mM β-glycerol phosphate or DMEM alone. Monolayer cultures treated with odontogenic medium were used as the controls. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 for 21 days with twice-weekly medium exchanges. To determine the loading efficiency, we quantified the cells present within the culture medium a few hours after cell loading. The loading efficiency was 80%. The scaffolds were then prepared for light microscopy (LM) and scanning electron microscopy (SEM) to observe the cells within the scaffold.

Light microscopy

The scaffold/cell constructs (Day 7) were fixed in 10% formalin and decalcified in 15% nitric acid for 24 hours. The samples were dehydrated in isopropanol (Merck, Darmstadt, Germany), cleared in xylene (Merck), and finally embedded in paraffin wax (Leica, Bensheim, Germany). Three representative sequential sections with 5 mm thicknesses at a defined depth of 0.75 mm were selected for LM evaluation.

Scanning electron microscopy

MSCs-loaded scaffolds (Day 7) were fixed in 2.5% glutaraldehyde at 4°C for 24 hours and then washed with PBS. The samples were sequentially dehydrated with increasing concentrations of ethanol (30%, 50%, 80%, and 100%), coated with gold, and visualized at an accelerating voltage using a Zeiss scanning electron microscope (Zeiss, München, Germany).

Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity of differentiated cells on TCP scaffolds and monolayer cultures was measured using a para-nitrophenyl phosphate (PNPP) kit (Cayman Chemical Company, Ann Arbor, Michigan 48108, USA) according to the manufacturer's instructions. Scaffolds were briefly washed with PBS and sonicated in PBS solution. Next, we mixed 200 μL of the cell lysis solution with 200 μL of the PNPP substrate, which was then incubated for 30 minutes. The reaction was stopped by the addition of 300 μL NaOH, and enzyme activity was determined by measuring absorbance at 405 nm.

Evaluation of culture mineralization

At the end of the differentiation period, the amount of Alizarin Red staining for the scaffolds with and without vitamin D3 and the monolayer cultures were quantified using an Osteogenesis Quantification Kit (Chemicon® International, Hofheim, Germany). This analysis was performed by determining the optical density 405 values of a set of known Alizarin Red concentrations and comparing these values to those obtained from the cultures. Based on the manufacturer's instructions, the cells/scaffold constructs were fixed in 10% formaldehyde for 15 minutes, stained with Alizarin Red for 10 minutes, and washed with distilled water. Precipitated red matrix was then solubilized in 10% acetic acid (Sigma, USA). The optical density of the solution was recorded at 405 nm with a microplate reader and compared to that of known Alizarin Red concentrations as provided by the manufacturer. Since TCP as the structural component of scaffold may stain with Alizarin Red, we used the scaffold without cells as the control in order to exclude the amount of calcium in the scaffold itself.

Quantitative real-time RT-PCR

To quantify relative gene expression levels, total RNA was extracted from approximately 10^6 cells from each samples (the cultures and pulp tissue) using Trizol (Invitrogen, Paisley, UK). cDNA was synthesized from 2 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. mRNA levels for dentin matrix acidic phosphoprotein (*DMP1*) and dentin sialophosphoprotein (*DSPP*) (as odontogenic differentiation marker genes) were measured by real-time reverse transcriptase polymerase chain reaction (RT-PCR; StepOne Real-Time PCR; Applied Biosystems, San Francisco, CA, USA). The 20 μL reaction contained 2 μL cDNA from each sample mixed with 10 μL SYBR Green PCR Mastermix (ABI), 1 μL each of sense and antisense primers, and 6 μL RNase/DNase-free water. The PCR conditions were incubation at 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds and at 60°C for 60 seconds.

The gene expression levels of the target genes *DMP1* and DSPP were determined based on threshold PCR cycle-values [C_t (target)] following the instructions of Applied Biosystems. Relative quantification was derived using the Comparative CT Method using $2^{-\Delta\Delta Ct}$, where the amount of target was

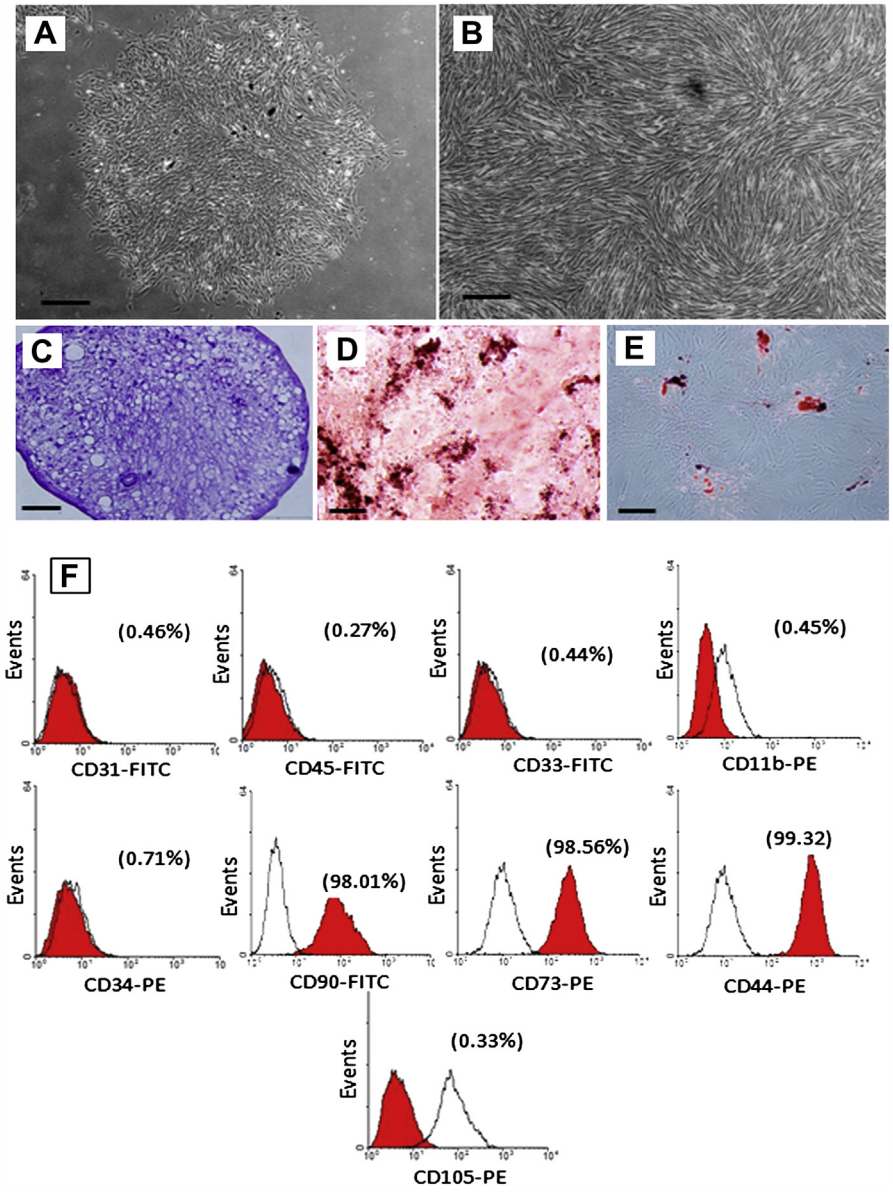

Figure 1 Dental pulp stem cells (DPSCs). (A) In primary cultures, the cells formed large colonies that consisted of fibroblastic cells (bar = 200 μm). (B) The colonies increased in size, becoming confluent (bar = 100 μm). (C) Cartilage differentiation of DPSCs stained by toluidine blue (bar = 200 μm). (D) Osteogenic differentiation of DPSCs stained by Alizarin Red (bar = 200 μm). (E) Adipose differentiation of DPSCs stained by Oil Red O (bar = 200 μm). (F) The cells had a surface antigenic profile similar to those of mesenchymal stem cells. While endothelial and hematopoietic markers were present in a very low percentage of the cells, the mesenchymal markers were expressed by the majority of the cell population. FITC = fluorescein isothiocyanate; PE = phycoerythrin.

normalized to an endogenous control (GAPDH) relative to a calibrator (untreated passaged-3 hDPSCs). The following primers were utilized: *DMP1* (forward: 5′-GCAGAGTGAT GACCCAGAG-3′, reverse: 5′-GCTCGCTTCTGTCATCTTCC-3′); *DSPP* (forward: 5′-CCATTCCAGTTCCTCAAAGC-3′, reverse: 5′-CTGCCCACTTAGAGCCATTC-3′); and GAPDH (forward: 5′-CT CATTTCCTGGTATGACACC-3′, reverse: 5′-CTTCCTCCTGTGCT CTTGCT-3′).

Statistical analysis

In this study, five independent experiments were performed for confirmation of the results. All values were stated as means ± SD. We used analysis of variance to analyze the results of ALP activity and culture mineralization. Real-time PCR data were analyzed with the Tukey and least significant difference tests. A value $P < 0.05$ was considered statistically significant.

Results

Cell culture

A few days after the initiation of the primary culture, we observed several large colonies that consisted of fibroblastic cells (Fig. 1A) that increased in size and then became confluent. On Day 10, the culture consisted of a uniform monolayer of fibroblast-like cells (Fig. 1B). The cells maintained their spindle-shaped morphology during the passages.

Multilineage differentiation

The sections prepared from chondrogenic pellets were metachromatic as visualized by Toluidine Blue staining. Passaged-3 DPSCs succeeded in giving rise to bone cells since the culture treated by osteogenic medium tended to positively stain red with Alizarin Red. In the adipogenic culture, a lipid droplet appeared in the differentiating cell. Positive staining of the droplet by Oil Red O in the induced cells was proof of their adipogenic differentiation (Fig. 1C–E).

Flow cytometry

The majority of the DPSCs tended to express surface markers of MSCs such as CD90, CD73, and CD44. Endothelial and hematopoietic cell markers such as CD105, CD34, CD45, CD33, CD31, and CD11b were expressed in a very low percentage of the isolated cells (Fig. 1F).

LM and SEM

Based on the representative section prepared from the scaffold/cell construct, DPSCs appeared to occupy the scaffold pore spaces (Fig. 2A). In the SEM images, the cells were observed to establish an attachment to the scaffold surfaces (Fig. 2B).

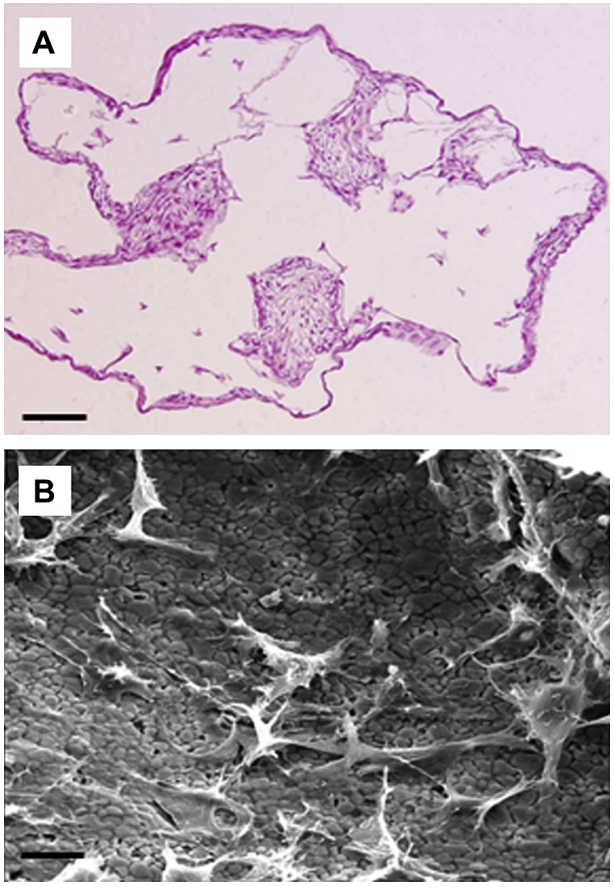

Figure 2 Light and scanning electron microscopy (SEM) of dental pulp stem cells (DPSCs) loaded onto tricalcium phosphate (TCP) scaffolds. (A) A representative section taken from the TCP–cell construct after its decalcification (H&E stain, bar = 200 μm). The internal pore occupied by the cells. (B) A representative SEM image taken from the surface of the TCP scaffold showing some adhered DPSCs (bar = 100 μm).

ALP activity

ALP activity tended to be significantly higher in the 3D cultures treated with vitamin D3 compared to those without vitamin D3 and monolayer cultures ($P < 0.05$; Fig. 3A).

Culture mineralization

The value of calcium deposition for the 3D cultures with vitamin D3 was 1.547 ± 0.141 mM. This was significantly different when compared to 3D cultures without vitamin D3 (0.947 ± 0.141 mM) and monolayer cultures (0.185 ± 0.029 mM; $P < 0.01$; Fig. 3B). Interestingly, the culture on TCP that lacked vitamin D3 was heavily mineralized compared to the monolayer culture treated with vitamin D3 ($P < 0.01$).

Real-time PCR analysis

Both odontoblast-specific genes (*DMP1* and *DSPP*) tended to be upregulated in 3D cultures (both with and without vitamin

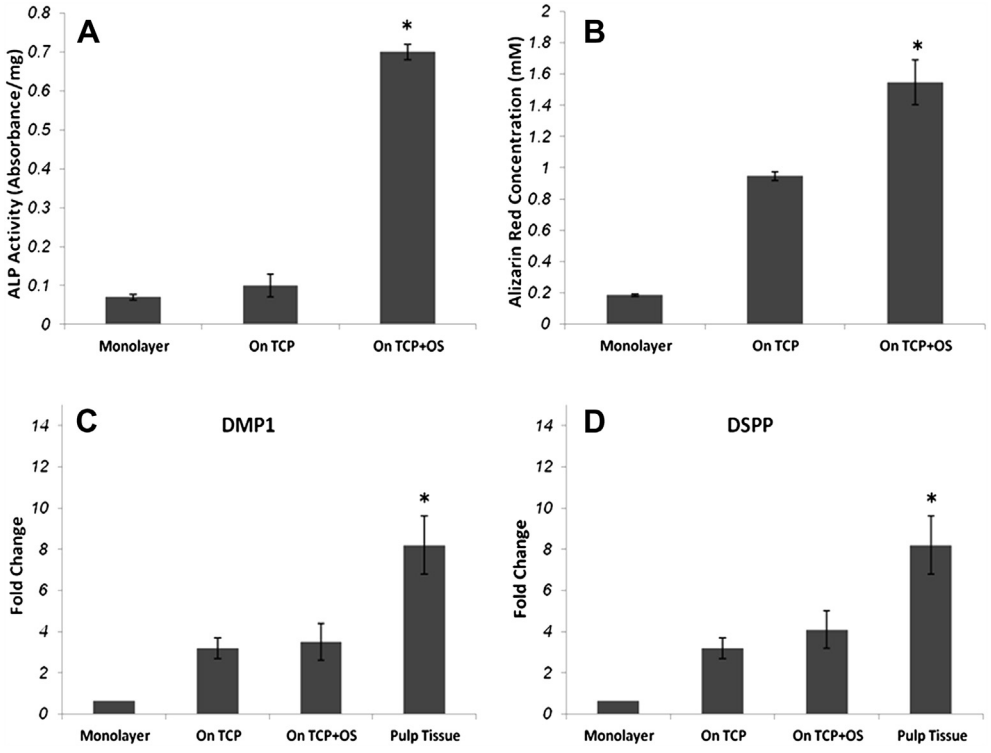

Figure 3 Quantification of odontogenic differentiation. (A) Analysis of alkaline phosphatase (ALP) activity. ALP activity was relatively high in the three-dimensional (3D) culture with odontogenic supplements (OS). (B) Evaluation of culture mineralization. Alizarin Red concentration (as the index of mineralization) was significantly higher in the 3D culture with OS. (C,D) Comparative analysis of the fold change of DMP1 and DSPP expression using quantitative polymerase chain reaction. The level of DMP1 expression in the 3D culture with OS was significantly higher than in the monolayer culture and lower than in the odontoblast in pulp tissue. DSPP was expressed in a similar fashion to DMP1.* Significant difference, $P < 0.05$. TCP = tricalcium phosphate.

D3) compared to those in monolayer cultures. In 3D cultures, the level of gene expression was significantly lower than that in the pulp tissue ($P < 0.05$; Fig. 3C and D). Interestingly, the expression level of odontoblast-specific genes in DPSCs cultivated on TCP in the absence of vitamin D3 was comparable to that of cultures with vitamin D3.

Discussion

In the present study, human pulp-derived MSCs were loaded onto TCP scaffolds and directed into an odontoblastic differentiation. According to our findings, the cultures tended to become heavily mineralized, and dentin-related genes (including *DMP1* and DSPP) appeared to become significantly upregulated. These were indicative of odontogenesis that occurred on the TCP surfaces. Based on our results, the level of gene expression on TCP was significantly lower than that of pulp tissue. This implies that the culture conditions remain far from the ideal microenvironment that favors optimal cell differentiation. These data would be useful for those involved in the regeneration of dental-related tissue.

To investigate the level of odontogenic differentiation of the DPSCs on TCP compared to that of odontoblasts that normally exist in the dental pulp chamber, the pulp tissue was collected from the human third molars. To release the odontoblasts, the chamber wall was gently scraped using a 1-mL insulin syringe with a fine needle. Based on the real-time PCR analysis, the expression level of odontoblast-specific genes in dental pulp was significantly higher than that in DPSCs on TCP 3D cultures. It should be mentioned that the pulp cells prepared in this study were not purely composed of odontoblasts but contained other cells such as hematopoietic, fibroblastic, and endothelial cells that are normally present in pulp tissue. Even this fraction of odontoblasts within the pulp tissue expressed a significantly higher level of odontoblast-specific genes than did DPSCs on TCP 3D culture.

Our data indicated that in 3D cultures not treated with vitamin D3, there was still a significant upregulation of *DMP1* and *DSPP* genes and heavy culture mineralization compared to the monolayer cultures with vitamin D3. These results indicated that TCP could induce pulp-derived MSCs into odontoblastic differentiation. This property of TCP has not yet been reported in previous investigations. TCP is a totally biocompatible and resorbable material that, for several decades, has been proven to be reliable in orthopedic, maxillofacial, and preimplantation surgery.[21–23] This commercially available material is entirely synthetic; risks with regard to immune response or infection are thus eliminated.

Human tooth pulp tissue was enzymatically digested and the released cells cultivated under standard culture conditions. Emerging fibroblastic cells were then evaluated according to the criteria proposed by the Tissue Specific Stem Cell Committee of the International Society for Cell Therapy in order to verify the MSC nature of the isolated cells.[24] The cells were examined in terms of their

multilineage differentiation into bone, cartilage, and adipose cells. We also investigated the expression of certain surface epitopes on the cells. Since fibroblastic cells from human pulp tissue have successfully differentiated along skeletal cell lineages and positively expressed certain mesenchymal antigens, we are convinced that they belong to the MSC family that occurs in pulp tissue.

One major challenge in establishing 3D cultures using a scaffold is the loading of cells onto the scaffold's internal pores. The most recommended, most successful method is to apply perfusion bioreactors.[25] In the absence of a bioreactor system, we suspended the cells in a small drop of culture medium and placed this on the top surface of scaffold that was presoaked in this culture medium. The scaffold—cell complex was incubated for a few hours to allow for cell penetration. To ensure that the cells were loaded onto the scaffold, we collected the culture medium and performed a cell count. LM and SEM were used to observe the cells within the scaffold's internal spaces.

ALP has been shown to be present in different calcifying tissues, including odontoblasts and enamel.[26] It is among the first functional gene expressed in the process of calcification. A clue to the role of ALP in calcification has arisen from studies of patients with hypophosphatasia, whose disease results from missense mutations in the gene that codes for tissue nonspecific ALP, which leads to decreased or absent ALP activity.[27] In the present study, ALP activity was significantly higher in 3D cultures treated with vitamin D3 compared to those without vitamin D3. This indicated that active mineralization occurred in the presence of vitamin D3.

In the present study, DMP1 and DSPP have been used as odontoblast differentiation markers. DMP1 is an extracellular matrix glycoprotein critical for the proper mineralization of dentin. During odontoblast maturation, the protein becomes phosphorylated and is exported to the extracellular matrix where it orchestrates mineralized matrix formation.[28,29] *DSPP* is a human gene that encodes for a pre-proprotein of the same name. The pre-proprotein is secreted by odontoblasts and cleaved into dentin sialoprotein and dentin phosphoprotein, which are the two principal proteins of the dentin extracellular matrix. Dentin phosphoprotein is thought to be involved in the biomineralization process of dentin.[30,31]

In conclusion, a 3D culture system improves the odontogenic differentiation of DPSCs. The cells differentiated in such a system show a gene expression level much lower than those odontoblastic cells present in pulp tissue. Furthermore, TCP appears to possess an odontogenic property, promoting the odontoblastic differentiation of pulp-derived MSCs in the absence of an odontogenic inducer.

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