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RESEARCH ARTICLE

Biosilica Porous Microspheres Promote the Osteogenic/Odontogenic Differentiation of Human Dental Pulp Cells

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Silicatein is a unique enzyme in sponges that serves as a template and catalyzes biosilica formation from silica and therefore may be useful for bone regeneration. In this study, we investigated the effects of novel biosilica porous microspheres consisting of poly(lactic-co-glycolic acid)/β-tricalcium phosphate coated with silica and silicatein on the growth and osteogenic/odontogenic differentiation of human dental pulp cells (hDPCs). Viability and morphology were assessed using Cell Counting Kit-8 assays, laser confocal microscopy and scanning electron microscopy. Marker expression was evaluated by immunofluorescence staining, quantitative reverse transcription-polymerase chain reaction (collagen type I and dentin sialophosphoprotein), and alkaline phosphatase activity quantification. Compared with poly(lactic-co-glycolic acid)/β-tricalcium phosphate (a control material), the novel biomicrospheres did not affect viability, supported the attachment and expansion of hDPCs, upregulated the expression of collagen type I and dentin sialophosphoprotein, and promoted alkaline phosphatase secretion in the osteoinductive environment. These findings demonstrate that these biosilica porous microspheres have potential clinical applications as novel carriers of inductive materials for dentin regeneration.

Keywords: Biosilica, Porous Scaffold, Human Dental Pulp Cell, Osteogenic Differentiation, Odontogenic Differentiation.

1. INTRODUCTION

Dentin is a calcified tissue that makes up most of the tooth; accordingly, it is a key component in tissue engineering for the tooth structure.[1] Tissue engineering is an emerging field that combines stem cells, growth factors, and scaffolds to regenerate diseased or lost tissues. Scaffolds are indispensable in tissue regeneration as they serve as carriers to facilitate the delivery of stem cells and/or growth factors to a local receptor site.[2] Ideally, scaffold materials should be biodegradable and biocompatible, promote cellular interactions and tissue development, and possess proper mechanical properties.[3]

To regenerate dentin, several types of scaffolds have been tested using dental pulp stem cells (DPSCs) both *in vitro* and *in vivo*, including those made of collagen, polylactic acid (PLA), polyglycolic acid (PGA), a copolymer of PGA and PLA, poly(lactic-co-glycolic acid) (PLGA), and bioceramics.[4–6] Composite scaffolds can be used to exploit the advantages of multiple individual materials.[2,7,8] In PLGA/tricalcium phosphate (TCP) composites, PLGA may cause a localized pH reduction due to the relative acidity of its hydrolytically degraded by-products, and the degradation products of TCP can potentially neutralize this acidic pH, thereby reducing the mild inflammation associated with polymer degradation.[2] Blaker et al.[9] demonstrated improved adhesion, spreading, and viability of cells grown on polymer–bioglass composites and further confirmed the high bioactivity and biocompatibility of the material for hard tissue repair. A PLGA/TCP composite scaffold is appropriate for the proliferation and differentiation of DPSCs, and was found to be superior to a pure PLGA scaffold for tooth tissue regeneration, especially for dentin formation.[10] PLGA/TCP containing silver can increase alkaline phosphatase (ALP) expression in human dental pulp cells (hDPCs).[11] Although these

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materials promote the proliferation and differentiation of DPCs, few are able to regenerate the complete dentin tissue,[12] and, in some cases, the newly formed dentin is very different from primary dentin.[13] Moreover, none of the materials developed to date has been tested in a clinical trial. Therefore, it is necessary to develop a suitable scaffold material for dentin regeneration.

Recently, Professor Müller and colleagues synthesized PLGA/β-TCP microspheres coated with silica and silicatein as a new type of bioactive and biodegradable material.[14] Silicateins are exclusively found in sponges (phylum Porifera) and serve as a template and catalyze the polymerization of nanoscale silicate to biosilica, the main mineral component of sponge skeletal elements (siliceous spicules). Both silicate and biosilica can induce bone regeneration.[15–17] The microspheres (diameter $\approx$ 800 μm) were prepared by encapsulation of β-TCP supplemented with silica and silicatein in PLGA. The PLGA/β-TCP microspheres coated with silica and silicatein showed no toxicity in a cell viability assay using human osteogenic sarcoma cells (SaOS-2 cells). Moreover, mineralization of SaOS-2 cells increased when β-TCP or silica was added to the microspheres, and mineralization was relatively weak in microspheres without β-TCP. However, the addition of silicatein to the spheres did not affect mineralization. Furthermore, an additional increase in mineralization was observed when silicatein-containing spheres were mixed at a 1:1 ratio with silica-containing spheres.[14]

hDPCs can differentiate toward the osteoblast lineage and contribute to the dentinal regeneration process;[18] accordingly, several studies have employed hDPCs as target cells to evaluate the hard tissue regeneration ability of various biomaterials.[19] Moreover, the environment of the transplantation site influences the fate of stem/progenitor cells *in vivo*. That is, the fate of implanted stem/progenitor cells may be determined by the site, rather than by the origin of cells, with a tendency to differentiate into the odontoblast phenotype when transplanted into the pulp chamber but into other cell lineages when transplanted to other target locations.[3] Since the bone and teeth are both apatite-like hard tissues, a material that promotes the osteogenic differentiation of hDPCs *in vitro* has potential benefits for the odontogenesis of hDPCs.

Based on its characteristics and function in bone regeneration, we speculated that a 1:1 mixture of the novel biosilica microspheres (PLGA/β-TCP/silica-micro: PLGA/β-TCP/silicatein-micro) may be beneficial for the growth and differentiation of hDPCs, and is a potential new scaffold material for dentin regeneration. Therefore, the aim of this study was to investigate the effect of PLGA/β-TCP microspheres coated with both silica and silicatein on the growth and osteogenic/odontogenic differentiation of hDPCs in comparison with the effects of uncoated PLGA/β-TCP microspheres.

2. MATERIALS AND METHODS

2.1. Material Preparation

Microspheres were prepared by the encapsulation of β-TCP supplemented with silica and silicatein into PLGA. β-TCP ($\sim$10% w/w), $\sim$5% silica (w/w), and 0.5 μg/mg silicatein were entrapped into PLGA microspheres (diameter $\approx$ 800 μm) according to a previously reported procedure.[14] The average hardness of the microspheres was 0.05 GPa, and the average elastic modulus was 2.78 GPa. The PLGA/β-TCP/silica-silicatein microspheres were used as the experimental material and the PLGA/β-TCP microspheres were used as a control material. Each material was molded into disks (diameter = 10 mm, thickness = 1.3 mm, weight = 38 mg) to prepare the scaffolds used in subsequent cell experiments. Prior to use, all materials were treated with ultraviolet light for 1 h.

2.2. Cell Culture

Normal human dental pulp tissues were obtained from the third molars of three patients (18–25 years of age) at Peking University School and Hospital of Stomatology after obtaining informed consent from the donors and permission from the ethics committee (reference no. PKUSSLA2014193). The extracted pulp tissues were minced and digested in a solution containing 3 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ, USA) and 4 mg/ml dispase (Roche Molecular Biochemical, Mannheim, Germany) for 1 h at 37 °C. The hDPCs were then transferred to alpha-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (GIBCO, Waltham, MA, USA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 2 mM L-glutamine (Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. The culture medium was changed every 3 days and the cells were subcultured at 70% confluence. hDPCs at passages 4–6 were used for all cellular analyses. All experiments were repeated three times using hDPCs from the three patients.

2.3. Effects of PLGA/β-TCP/Silica-Silicatein on the Growth Behavior of hDPCs

2.3.1. Cell Viability Assay

The effect of PLGA/β-TCP/silica-silicatein on the viability of hDPCs was determined *in vitro* using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). PLGA/β-TCP/silica-silicatein and PLGA/β-TCP were pre-wetted in the culture medium for 24 h in a 24-well plate. Then, the hDPCs (2×10^4) were seeded onto each scaffold and incubated at 37 °C, 5% CO_2. For the negative control group, hDPCs were cultured without any materials. The culture medium was changed every 2 days. After 1, 3, 5, 7, and 9 days of cell culture, cells were further incubated with the counting reagent for 1 h, according to the manufacturer's instructions. The relative cell number was determined by measuring absorbance of the formazan

dye product in the cultures at a wavelength of 450 nm. The results are expressed as optical density values. The relative cell numbers in the PLGA/β-TCP/silica-silicatein group, PLGA/β-TCP group, and negative control group were compared.

2.3.2. *Confocal Laser-Scanning Microscopy*

The adhesion and growth of hDPCs on the scaffold surfaces were observed by confocal laser-scanning microscopy. The hDPCs (2×10^4) were seeded onto each scaffold and incubated at 37 °C, 5% CO_2. After 3, 5, 7, 9, and 11 days of culture, the cell-scaffold constructs were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 30 min. The constructs were then stained with 5 μg/ml fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma, St. Louis, MO, USA) for 1 h, incubated in 1 μg/ml 6-diamidino-2-phenylindole (DAPI; Sigma) solution for 5 min at room temperature (24–26 °C), and viewed under a confocal Zeiss Axiovert 650 microscope (Carl Zeiss Microimaging, Oberkochen, Germany) using a laser with wavelengths of 488 nm (green, FITC-labeled phalloidin) for F-actin and 405 nm (blue, DAPI) for nuclei.

2.3.3. *Scanning Electron Microscopy (SEM)*

The scaffold surfaces were examined by SEM to confirm the attachment of hDPCs. The hDPCs (2×10^4) were seeded onto each scaffold and incubated at 37 °C, 5% CO_2. Initially, the cell-scaffold constructs were washed with PBS and fixed in 4% paraformaldehyde for 30 min after culturing for 7 days. The specimens were dehydrated with a graded series of ethanol, mounted onto aluminum stubs, sputter-coated with gold, and viewed under field-emission SEM (EVO 18; Zeiss, Wetzlar, Germany). The microspheres without cells were also observed.

2.4. Effects of PLGA/β-TCP/Silica-Silicatein on the Osteogenic/Odontogenic Differentiation of hDPCs

The hDPCs (2×10^4) were seeded onto the two scaffolds and incubated at 37 °C, 5% CO_2 in regular culture medium for 2 days, followed by incubation in osteogenic-inducing medium for 14–28 days, which comprised fresh α-MEM containing 10% fetal bovine serum, 100 U/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 100 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 0.2 mM L-ascorbic acid (Sigma). The effects of PLGA/β-TCP/silica-silicatein on the osteogenic/odontogenic differentiation of hDPCs were evaluated by immunofluorescence staining to observe the expression of osteogenic/odontogenic-related proteins. Immunofluorescence staining to detect collagen type I (COLI) was performed on the 14th day and dentin sialophosphoprotein (DSPP) staining was performed on the 28th day after osteoinduction. The protocol was as follows: cell-scaffold constructs were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by post-fixation in 0.1% Triton X-100 for 15 min. The constructs were washed three times in PBS and incubated with 1:100 primary antibodies (COLI: Proteintech, Rosemont, IL, USA; DSPP: Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The constructs were then washed again and incubated in 1:200 secondary antibodies (Proteintech) for 1 h at room temperature, followed by staining with 5 μg/ml FITC-labeled phalloidin for 1 h and incubation in 1 μg/ml DAPI solution for 5 min at 37 °C. Following three additional washings with PBS, the specimens were viewed under the confocal Zeiss Axiovert 650 microscope using a laser with wavelengths of 594 nm (red, COLI and DSPP), 488 nm (green, F-actin), and 405 nm (blue, nuclei).

2.5. Effects of Biosilica on the Osteogenic/Odontogenic Differentiation of hDPCs

To evaluate the effect of biosilica released from the microspheres on cell differentiation, hDPCs were co-cultured with the scaffolds rather than being seeded directly on the scaffolds. The ALP activity and gene expression levels of *COLI* and *DSPP* in adherent cells were analyzed. The hDPCs (5×10^4) were seeded in 12-well plates with regular culture medium for 2–3 days. Upon reaching 70%–80% confluence, the regular culture medium was replaced with osteogenic-inducing medium, the scaffolds (PLGA/β-TCP/silica-silicatein and PLGA/β-TCP) were added to each well simultaneously, and co-culturing was conducted for 7–21 days. In the negative control group, the hDPCs were cultured in the osteogenic-inducing medium, without any additional materials.

2.5.1. *ALP Activity Quantification*

The ALP activity and protein levels of hDPCs were measured on the 7th, 14th, and 21st day of osteoinduction using the ALP Kit (JianCheng Bioengineering Institute, Nanjing, China) and the BCA Protein Assay Kit (ComWin Biotech Co. Ltd., Beijing, China), respectively, according to the manufacturers' instructions. Absorbance was measured at 520 nm, and ALP levels were normalized against the total protein content.

2.5.2. *Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)*

The expression levels of osteogenic/odontogenic-related genes in hDPCs were evaluated by qRT-PCR. Total cellular RNAs were isolated at 7, 14, and 21 days after osteoinduction using the TRIzol reagent (Invitrogen) and used for first-strand cDNA synthesis using the PrimeScript RT Reagent Kit (TaKaRa Bio, Otsu, Japan). Gene transcripts were quantified by qRT-PCR using a SYBR® Premix Ex Taq II Kit (TaKaRa) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primer sets used to detect *COLI, DSPP* and

Table I. Primer sequences for qRT-PCR.

Genes	Primer sequence
COLI	(F) 5′-AAAAGGAAGCTTGGTCCACT-3′
	(R) 5′-GTGTGGAGAAAGGAGCAGAA-3′
DSPP	(F) 5′-ATATTGAGGGCTGGAATGGGGA-3′
	(R) 5′-TTTGTGGCTCCAGCATTGTCA-3′
GAPDH	(F) 5′-GAGTCAACGGATTTGGTCGT-3′
	(R) 5′-GACAAGCTTCCCGTTCTGAG-3′

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) are listed in Table I. *GAPDH* was used as a housekeeping gene to normalize the mRNA expression levels. The cycle threshold (Ct) values were used to calculate the fold differences by the $\Delta\Delta Ct$ method. The qRT-PCR conditions were as follows: denaturation at 95 °C (10 min), and 40 cycles at 95 °C (15 s) and 60 °C (1 min, annealing).

2.6. Statistical Analysis

Quantitative results are reported as means ± standard deviations ($n = 3$). Results were evaluated by one-way analysis of variance for the CCK-8 assay and ALP activity analysis and by t-tests for qRT-PCR data using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Structures of Microspheres

SEM shows that microspheres were bonded together. The surfaces of PLGA/β-TCP microspheres were fairly smooth (Figs. 1(A–C)). The surfaces of PLGA/β-TCP/silica-silicatein microspheres were covered with micropores, with diameters ranging from 5 μm to 8 μm, and nanopores (Figs. 1(D–F)). Compare to the smooth surface, porous surface are favorable for the adhesion and growth of DPCs.[20]

3.2. Effects of PLGA/β-TCP/Silica-Silicatein on hDPC Growth

3.2.1. PLGA/β-TCP/Silica-Silicatein Does Not Affect the Viability of hDPCs

The effect of PLGA/β-TCP/silica-silicatein microspheres on the viability of hDPCs was assessed by CCK-8 assays. As shown in Figure 2, there were showed no statistically significant differences ($P > 0.05$) in the relative number of hDPCs among the PLGA/β-TCP/silica-silicatein group, PLGA/β-TCP group, and negative control group throughout the experimental period (0–9 days). The relative cell count increased by 2.5-fold on the 5th day and by 5.3-fold on the 9th day after cell seeding (Fig. 2).

The cytotoxicity of materials used in dentinal regeneration is an important practical concern. Materials should either improve cell viability or be biologically neutral.[21] Given that PLGA/β-TCP/silica-silicatein did not cause any significant changes in the viability of hDPCs compared to the viability of control cells at the early stage of cell culture, this material could provide a suitable substrate for the initial adherence, spreading, and further multiplication of cells. Thus, these results indicated that PLGA/β-TCP/silica-silicatein is a biocompatible scaffold material without cytotoxicity, consistent with previous

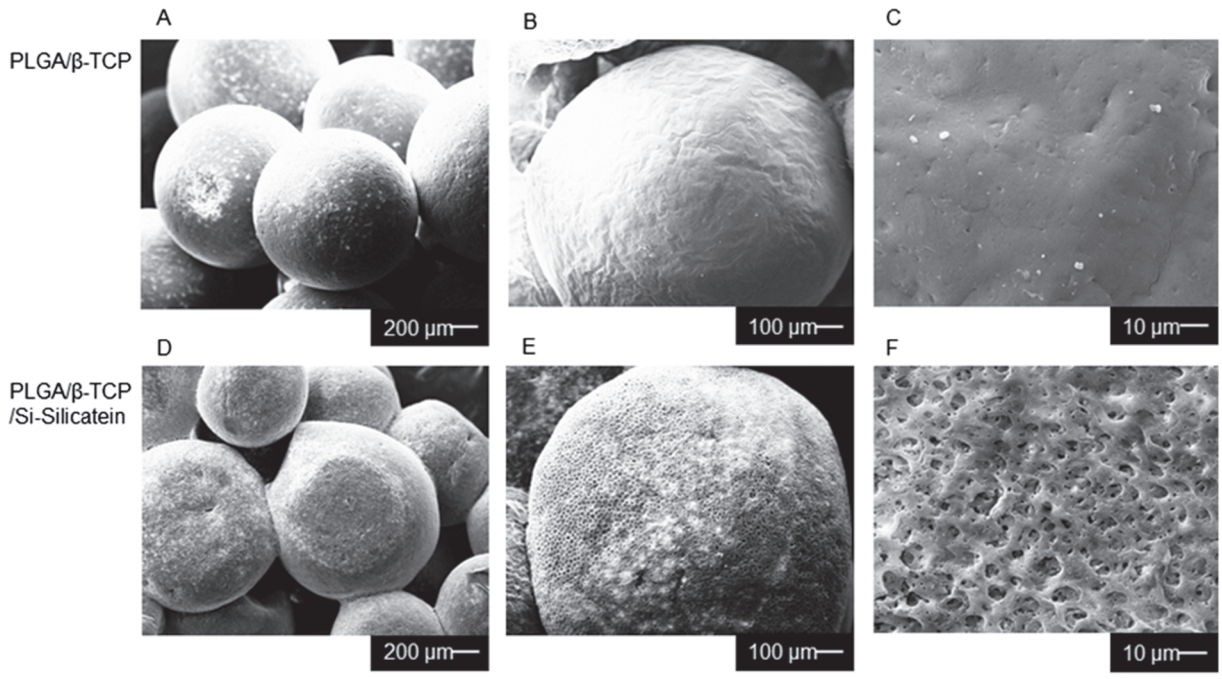

Fig. 1. Scanning electron microscopy images of microspheres. The surfaces of PLGA/β-TCP microspheres were smooth (A–C), and those of PLGA/β-TCP/silica-silicatein microspheres were microporous (D–F).

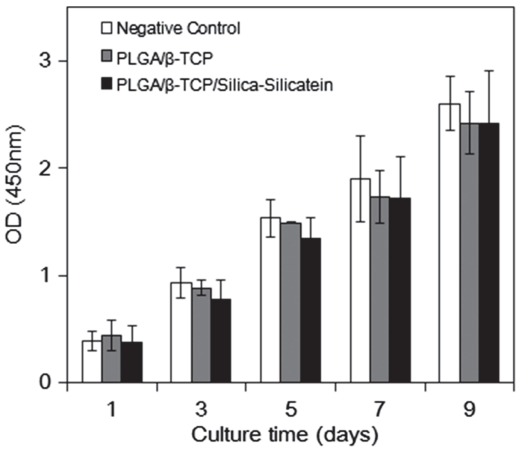

Fig. 2. A CCK-8 assay showed no statistically significant differences ($P > 0.05$) in the viability of hDPCs among the PLGA/β-TCP/silica-silicatein group, PLGA/β-TCP group and negative control group at days 0–9.

analyses of the effects of this material on the viability of SaOS-2 cells.[14]

3.2.2. PLGA/β-TCP/Silica-Silicatein Supports the Attachment and Expansion of hDPCs

Based on observations of microspheres by confocal laser-scanning microscopy, as shown in Figures 3(A) and (C), hDPCs grew well on the surfaces of both kinds of scaffolds. The hDPCs gradually covered the microsphere surface, reaching 70%–80% confluence at the end of the 7 days. The microspheres were completely covered with hDPCs by the 11th day after cell seeding.

SEM was used to observe the fine structures of cells to assess the effect on hDPC attachment. As shown in Figures 3(B) and (D), the porous topography of the PLGA/β-TCP/silica-silicatein surface allowed hDPCs to form a blanket and flourish. The cells bridged microspheres via their pseudopodia (Figs. 3(B, D), arrows). Moreover, the hDPCs cultured on PLGA/β-TCP/silica-silicatein exhibited more clearly defined cytoplasmic extensions, which projected from the cells to the surrounding surface or adjacent cells, than those on the surface of PLGA/β-TCP.

The attachment and spread of hDPCs on material surfaces are important for subsequent cellular function, including differentiation.[22] The results described above indicated that the silicon and porous microspheres are appropriate for the growth of hDPCs, consistent with previous findings.[19, 20, 23, 24] The geometry of porous scaffolds used for tissue engineering has recently been shown to significantly influence the cellular response. Surface (nano-) topography could determine stem cell fate, facilitating stem cell differentiation towards the osteogenic lineage.[25] Small pores may be beneficial *in vitro* because a higher fluid velocity means that cells have less time for attachment to the surface of the scaffold. The cell seeding efficiency may therefore decrease as pore size increases.[26] The pores on the surface of PLGA/β-TCP/silica-silicatein were smaller than the cellular dimensions, providing a channel for the release of silicon, while simultaneously increasing the roughness of the microspheres. Microscaffolds aid in the rapid expansion of cells.[20] Owing to their geometry, porous microscaffolds provide a large surface area, thereby minimizing the utilization of media, and can effectively mimic the *in vivo* microenvironment for cell growth.[20, 23] The pores formed between the bonded microspheres and the nanopores on the surface of microspheres result in the formation of multiple three-dimensional porous structures that can transport oxygen, nutrients, and metabolites, which facilitate cell attachment and growth inside the structures.

Biosilica is composed of nanoparticles of 50–70 nm in diameter, which is suitable for uptake by cells via endocytosis. Indeed, biosilica has been shown to promote the proliferation of SaOS-2 cells.[16] The positive effects of silicon on cell proliferation and adhesion are achieved by altering the functional presentation of the major integrin-binding domains of adsorbed proteins, and consequently by modulating integrin binding, localization, and specificity.[24] Moreover, a high silicon content can trigger the enhancement of total integrin and COLI expression, thereby affecting integrin expression profiles via collagen-binding and fibronectin-binding subintegrin on human primary cells.[19]

Given this background, the PLGA/β-TCP/silica-silicatein microspheres appear to provide a suitable microenvironment for the growth and subsequent differentiation of hDPCs.

3.3. PLGA/β-TCP/Silica-Silicatein Promotes the Expression of COLI and DSPP in hDPCs

As shown in Figure 4, hDPCs seeded on PLGA/β-TCP/silica-silicatein exhibited COLI-positive staining after 14 days of osteoinduction and DSPP-positive staining after 28 days of osteoinduction. However, no positive staining for either marker was detected in the cells seeded on PLGA/β-TCP (Fig. 4).

A high porosity of a scaffold is thought to aid in osteoconductivity, since the increased surface area supports enhanced bone growth and exposes cells to greater surface reactivity.[27] When mesenchymal stem cells were seeded on a three-dimensional PLA porous scaffold in a previous study, the expression levels of osteoblast-related genes, including collagen type I, osteocalcin, and bone sialoprotein, were upregulated after 14 days of culture. The authors attribute this to the adherence of cells to a surface with a complex three-dimensional pore geometry and the beneficial effect of the surrounding curved surface for their subsequent spread and migration, leading to further osteogenic differentiation.[23]

In addition, a previous study has shown that biosilica could induce SaOS-2 cells to generate more

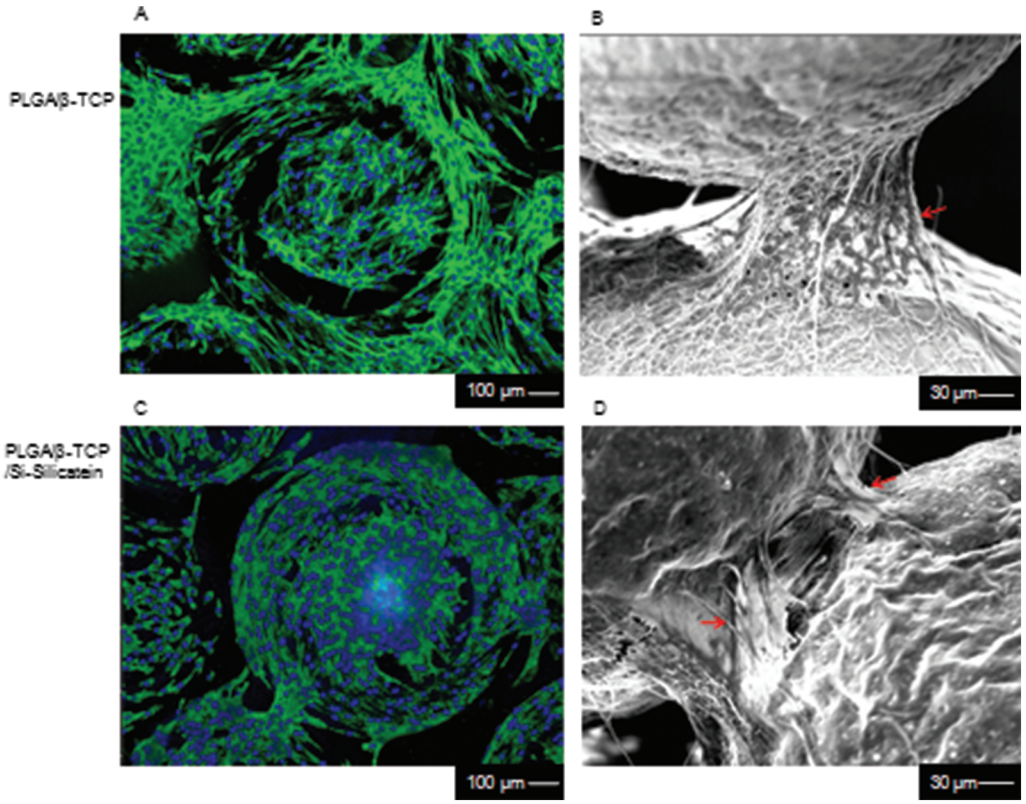

Fig. 3. Confocal laser-scanning microscopy and scanning electron microscopy images of the hDPC-microsphere construct. The whole microspheres of PLGA/β-TCP (A) and PLGA/β-TCP/silica-silicatein (C) were completely covered with hDPCs on the 11th day after cell seeding (magnification 40×). Nuclei were stained blue, and F-actin was green. SEM showed that hDPCs cultured on PLGA/β-TCP/silica-silicatein (D) exhibited better defined cytoplasmic extensions (arrows) that projected from the cells to the surrounding surface or adjacent cells than those on the surface of PLGA/β-TCP (B) (magnification 200×).

hydroxyapatite (HA) nodules and express higher levels of bone morphogenetic protein 2 (BMP2), which induces bone formation by directing the differentiation of bone-forming progenitor cells to mature osteoblasts while simultaneously inhibiting the function of osteoclasts.[16] BMP2 can also promote postnatal pulp stem cells to differentiate into odontoblast-like cells, and induces reparative dentin formation *in vivo*.[28] Most studies of the effects of biosilica on tooth mineralization have focused on enamel formation. For example, the biosilica-regulated expression levels of amelogenin and enamelin, which are both involved in enamel formation, were investigated in SaOS-2 cells.[29] During the reaction of silicatein with the substrate sodium metasilicate, nanoscale biosilica layers (50–150 nm) form on the dental surface to generate protective biosilica layers on the teeth, which could in turn reduce the risk of bacterial-induced caries/cavities.[30] However, no studies have evaluated the role of biosilica in dentin mineralization. In the present study, DSPP, an odontogenic-related protein, was selected to confirm the effect of PLGA/β-TCP/silica-silicatein on hDPC odontoblastic differentiation. DSPP is highly expressed in odontoblasts but is expressed at very low levels in other tissues, such as the bone and kidney. DSPP is also one of the most important non-collagenous proteins involved in the nucleation and control of the HA mineral phase during dentin calcification.[31] Therefore, DSPP expression is a useful marker of mature odontoblasts. Thus, the finding of positive DSPP expression in hDPCs on PLGA/β-TCP/silica-silicatein after 28 days of osteoinduction, with no such expression detected in the PLGA/β-TCP group, provides the first demonstration that biosilica can promote the odontogenesis of hDPCs. However, further evaluation *in vivo* is needed to confirm this result.

Collagen, particularly COLI, has potent effects on the proliferation and mineralization capacity of hDPCs.[32] COLI is associated with the extracellular matrix; it is highly expressed during the proliferation stage and then gradually declines prior to differentiation.[33] Cement with a certain amount of silicon is beneficial for COLI adsorption and secretion.[34] Moreover, COLI was found to be preferable to fibronectin for hDPC adhesion.[24] When SaOS-2 cells were cultured with biosilica, *COLI* was upregulated, along with the upregulation of *BMP2* expression.[35] Similarly, in the present study, hDPCs seeded on PLGA/β-TCP/silica-silicatein microspheres showed strong COLI protein expression, indicating that the porous structure of the microspheres along with the properties of

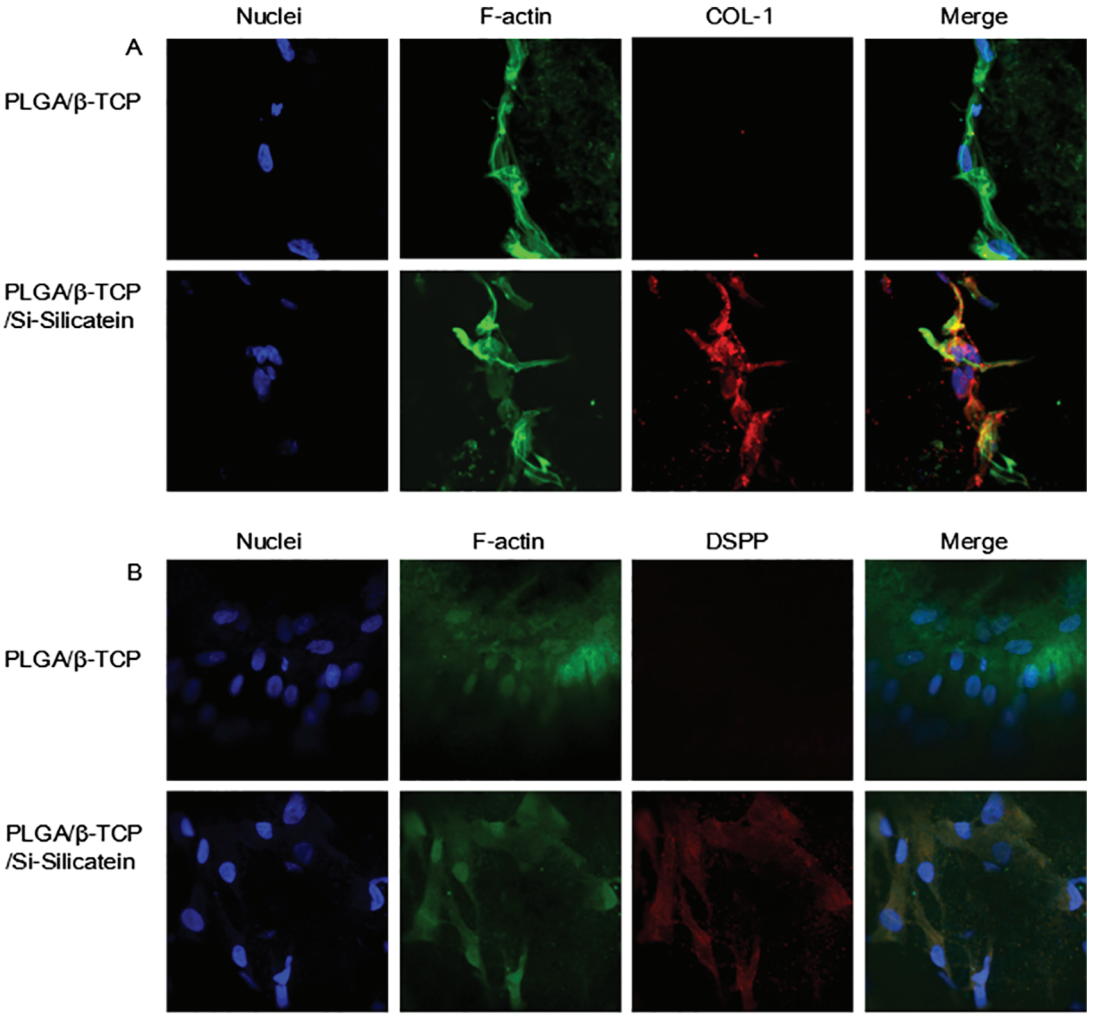

Fig. 4. Immunofluorescent staining for COLI (A) and DSPP (B) in hDPCs cultured on PLGA/β-TCP/silica-silicatein and PLGA/β-TCP (magnification 400×). COLI and DSPP were stained red, nuclei were blue, and F-actin was green. hDPCs seeded on PLGA/β-TCP/silica-silicatein exhibited COLI-positive staining after 14 days of osteoinduction and DSPP-positive staining after 28 days of osteoinduction.

biosilica promoted the expression of COLI to enhance cell proliferation and attachment.

3.4. Biosilica Promotes ALP Secretion and DSPP Gene Expression in hDPCs

Early osteoblastic differentiation can be assessed by estimating the expression and activity of ALP.[36] Therefore, to assess the specific effect of biosilica released from PLGA/β-TCP/silica-silicatein on the osteogenic/odontogenic differentiation of hDPCs, ALP activity and the expression levels of *COLI* and *DSPP* on adherent hDPCs co-cultured with the scaffolds were analyzed. ALP activity in the three groups (PLGA/β-TCP/silica-silicatein, PLGA/β-TCP, and negative control groups) increased continuously over time after osteoinduction, reaching peak levels on the 14th day, and then began to decline on the 21st day (Fig. 5(A)). There were no significant differences in ALP activity among the three groups ($P > 0.05$) on days 7 and 14. However, the PLGA/β-TCP/silica-silicatein group showed significantly greater ALP activity than that of the other two groups on the 21st day ($P < 0.05$). ALP plays an important role in mineral deposition and is also present in the early differentiation stage.[37] Earlier studies have shown that solutions containing a certain concentration of silicon promote collagen production and osteogenesis in osteoblasts.[38–40] Although PLGA/β-TCP/silica-silicatein did not promote ALP secretion during the early stage of cell differentiation, it resulted in a gradual and less substantial decrease in ALP over time compared with ALP secretion in the other two groups, and relatively strong activity was observed at the later stage of cell differentiation.

Similar to the results for ALP activity, the PLGA/β-TCP/silica-silicatein group showed significantly higher (1.5-fold) *DSPP* expression levels than those of the PLGA/β-TCP and negative control groups ($P < 0.05$) on the 21st day, whereas there were no statistically significant differences among the three groups on the 7th and

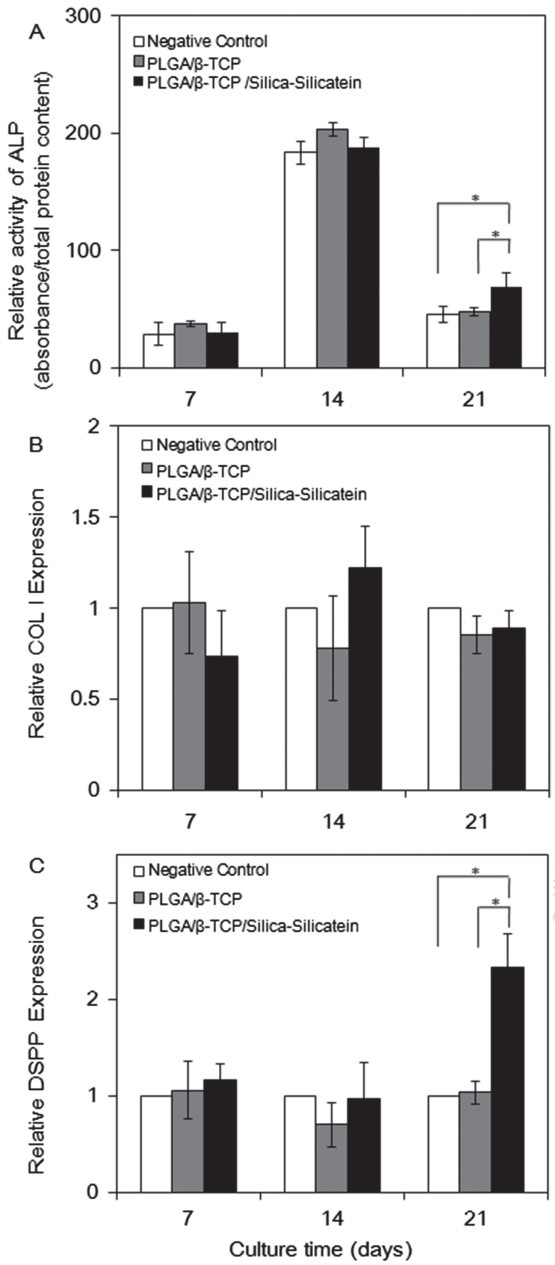

Fig. 5. (A) ALP activity of hDPCs cultured with and without scaffolds in osteogenic-inducing medium for 7, 14, and 21 days. (B and C) Relative gene expression levels (*COLI* and *DSPP*) of hDPCs cultured with and without scaffolds in osteogenic-inducing medium for 7, 14, and 21 days. *Significant difference at $P < 0.05$.

14th days ($P > 0.05$; Fig. 5(C)). The significant upregulation of *DSPP* expression at 21 days after cell osteoinduction may be related to the mechanism of action of biosilica, which is readily taken up by cells by endocytosis and may be degraded to orthosilicate.[16] Therefore, biosilica can promote the osteogenic/odontogenic differentiation of hDPCs in the early stage of cell culture; however, further studies are needed to elucidate the underlying signal transduction pathways.

There were no significant differences in the relative expression levels of the *COLI* gene among the three groups at any time point ($P > 0.05$; Fig. 5(B)). However, hDPCs on PLGA/β-TCP/silica-silicatein microspheres had stronger COLI protein expression after 14 days of osteoinduction compared with expression levels in the negative control groups. This result indicated that the porous structure of PLGA/β-TCP/silica-silicatein in combination with the release of biosilica, rather than the biosilica alone, promoted the expression of COLI.

4. CONCLUSIONS

A PLGA/β-TCP/silica-silicatein nanoporous microsphere scaffold can provide a suitable environment to support the growth of hDPCs. The released biosilica can promote the osteogenic/odontogenic differentiation of hDPCs in the osteoinduction environment, and the interaction between the porous structure and biosilica can further enhance this effect. This novel material thus shows good potential as a carrier of inductive materials for dentin regeneration. Further *in vivo* experiments are needed to confirm its ability to promote dentin formation.

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