# Three-dimensional simulated microgravity culture improves the proliferation and odontogenic differentiation of dental pulp stem cell in PLGA scaffolds implanted in mice

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**Abstract.** Tooth regeneration through stem cell-based therapy is a promising treatment for tooth decay and loss. Human dental pulp stem cells (hDPSCs) have been widely identified as the stem cells with the most potential for tooth tissue regeneration. However, the culture of hDPSCs *in vitro* for tissue engineering is challenging, as cells may proliferate slowly or/and differentiate poorly *in vivo*. Dynamic three-dimensional (3D) simulated microgravity (SMG) created using the rotary cell culture system is considered to an effective tool, which contributes to several cell functions. Thus, the present study aimed to investigate the effect of dynamic 3D SMG culture on the proliferation and odontogenic differentiation abilities of hDPSCs in poly (lactic-co-glycolic acid) (PLGA) scaffolds in nude mice. The hDPSCs on PLGA scaffolds were maintained separately in the 3D SMG culture system and static 3D cultures with osteogenic medium for 7 days *in vitro*. Subsequently, the cell-PLGA complexes were implanted subcutaneously on the backs of nude mice for 4 weeks. The results of histological and immunohistochemical examinations of Ki-67, type I collagen, dentin sialoprotein and DMP-1 indicated that the proliferation and odontogenic differentiation abilities of the hDPSCs prepared in the 3D SMG culture system were higher, compared with those prepared in the static culture system. These findings suggested that dynamic 3D SMG culture likely contributes to tissue engineering by improving the proliferation and odontogenic differentiation abilities of hDPSCs *in vivo*.

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## Introduction

Tooth loss due to periodontal disease, dental caries or trauma affects the quality of life of an individual. Attempts to successfully regenerate lost teeth or their components have long been an ambition of dentists. Based on stem cells, scaffolds and growth factors for regenerating missing or damaged tissues, tissue engineering is one of the latest emerging innovations, aimed at providing solutions for tissue creation and repair (1). Dental pulp stem cells (DPSCs) are characterized by their multipotent differentiation, self-renewal ability, clonogenic capacity and their odontogenic differentiation potential in particular. Previous studies have shown that DPSCs are capable of differentiating into odontoblast-like cells *in vitro*, and to form the dentin-pulp-like complex when transplanted into immunocompromised mice *in vivo* (2). However, despite the promising characteristics of hDPSCs, there are certain challenges, which require addressing prior to the routine use of regenerative techniques involving these cells in clinical applications, including improving the proliferation capacity and committed differentiation efficiency of the cells in biomaterials.

Scaffolds are indispensable in tissue engineering, as they serve as carriers to facilitate the delivery of stem cells and/or growth factors at a three-dimensional (3D) site to guide tissue formation by mediating cell survival and cell-scaffold interactions. Owing to their biodegradability, biocompatibility, and their nontoxic and nonimmunogenic properties (3-5), polymers are widely used in medical applications. Poly (lactic-co-glycolic acid) (PLGA) is a copolymer with desirable physical and mechanical properties. PLGA is a commonly used biomaterial for tissue engineering and is approved for clinical use (6). However, the diffusion of air and nutrient components in conventional 2D and 3D static culture is uneven, resulting in reduced cell growth, particularly within the 3D constructs (7,8).

In previous years, with its low hydrodynamic shear stress and low turbulence, the rotary cell culture system (RCCS) has been shown to allow the exchange of nutrients and transport of cellular secretions (9), contributing to the regulation of the differentiation and proliferation of stem cells. It has been suggested that RCCS provides a more controlled dynamic 3D

stimulated microgravity (SMG) environment, which qualifies for improved cell-cell interactions, and communication associated with proliferation and differentiation (10-12). Of note, several cell types and tissues have been successfully cultured under 3D SMG conditions, including the formation of living organoid-like tissue architecture, for example, cartilage and bone, *in vitro* (13-16). However, there have been few repots on the proliferation and differentiation of undifferentiated cells *in vivo* following culture in 3D SMG.

The present study investigated the proliferation and odontogenic differentiation of hDPSCs *in vivo* following the use of a 3D SMG culture system compared with static 3D culture. The isolated and identified hDPSCs seeded in PLGA scaffolds were maintained separately in the 3D SMG culture system and the static 3D culture system with osteogenic medium for 7 days *in vitro*. Subsequently, the differentiating cells with scaffolds were implanted subcutaneously on the backs of nude mice for 6 weeks. Histological and immunohistochemical analyses indicated that the proliferation and odontogenic differentiation abilities of the hDPSCs prepared in the 3D SMG culture system were higher, compared with those prepared in static culture. These results demonstrated the advantages of the 3D SMG culture system for improving the proliferation and odontogenic differentiation abilities of hDPSCs *in vivo*.

## Materials and methods

*Isolation and identification of hDPSCs*. All experiments performed in the present study were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University (Harbin, China). The isolation and culturing of the cells were performed as described previously (2). In brief, fresh dental pulp tissues were isolated from healthy impacted third molars of donors (age range, 18-29 years) from the department of the Oral and Maxillofacial Surgery of the First Affiliated Hospital of Harbin Medical University (Harbin, China) in July 2014, following the provision of written informed consent. The dental pulp tissues were digested with 3 mg/ml collagenase type I (Sigma-Aldrich; Merck Millipore, Darmstadt,) and 4 mg/ml dispase (BD Biosciences, San Jose, CA, USA) for 1 h at 37˚C, following which the solution was passed through a 70-$\mu$m strainer. The characterizations of the hDPSCs were based on a previous report (17). The cells between passages two and five were used in the following experiments.

*Cell-PLGA complex culture*. The PLGA scaffolds (Synthecon, Inc., Houston, TX, USA) were pretreated, as previously described (17). The hDPSCs ($2x10^6$) were seeded into each scaffold and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Chalfont, UK) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) for 72 h at 37˚C. Subsequently, the cell-scaffold composites were randomly divided into two groups: Static 3D culture and 3D SMG culture. The 3D SMG group was transferred into a 55-ml high-aspect-ratio vessel (Synthecon, Inc.) filled with osteogenic medium (DMEM supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate and 50 $\mu$g/ml ascorbic acid) in the RCCS (Synthecon, Inc.). The rotation speed of the vessel was adjusted throughout the period of cultivation to maintain the complexes at a relatively steady position within the vessel. In parallel, cells cultured in static culture with osteogenic medium were used as controls.

*Scanning electron microscopy (SEM) observation*. The cell-scaffold complexes of the static 3D culture and 3D SMG culture systems were gently rinsed three times with PBS. The samples were then fixed with 2% glutaraldehyde and dehydrated using a graded ethanol series of 30, 50, 70, 90 and 100%. Following being dipped into isoamyl acetate and dried in a critical-point dryer, the samples were observed under an SEM.

*In vivo transplantation*. A total of 20 female nude mice (6-8 weeks old) (Weitonglihua Experimental Animal Technology Co., Ltd., Beijing, China) were randomly assigned into the two groups. All the animals were housed under standard conditions of 12 h light/dark cycles and fed an autoclaved laboratory rodent diet. Following culture in the 3D static or 3D SMG rotating culture systems with osteogenic medium for 7 days *in vitro*, the cell-PLGA complexes were implanted subcutaneously onto the backs of the nude mice for 4 weeks. In each mouse, one control scaffold and one SMG scaffold was present on either side of the spine. At 4 weeks post-transplantation, the mice were sacrificed by cervical dislocation followed by extraction of the implants. The implants were fixed in formalin, embedded in paraffin and cut into sections measuring 5 $\mu$m in thickness for histological and immunohistochemical examinations.

*Histology*. The sample sections were deparaffinized in xylene, rehydrated through a gradient of ethanol solutions, stained with hematoxylin and eosin (H&E), Masson's trichrome staining and von Kossa staining, and viewed using a light microscope (Olympus Corporation, Tokyo, Japan).

*Immunohistochemistry*. Immunohistochemical analyses of the retrieved implants were performed using the streptavidin-biotin complex method, according to the manufacturer's recommended protocol. The deparaffinized sections were treated with 100 $\mu$l 3% $H_2O_2$ for 10 min at room temperature to suppress endogenous peroxidase activity. The sections were then blocked in 5% normal goat serum (Beijing Zhongshan Golden Bridge Biotechnology, Co.; OriGene Technologies, Inc., Rockville, MD, USA) for 1 h at room temperature and incubated with primary antibodies (1:100-1:500 dilutions) overnight at 4˚C. The following primary monoclonal antibodies were used: Ki-67 and type I collagen (rabbit anti-mouse, cat. no. ab16667, diluted 1:500 and goat anti-mouse, cat. no. ab34710, diluted 1:100; Abcam, Cambridge, MA USA), and dentin sialoprotein (DSP) and DMP-1 (goat anti-mouse, cat. nos. sc-18328 and sc-54181, diluted 1:100 and 1:200; Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Incubation in PBS alone instead of primary antibodies served as negative controls. The sections were rinsed in PBST and incubated in biotinylated secondary antibodies (anti-goat IgG, cat. no. sc-2042 and anti-rabbit IgG, cat. no. sc-2040, all purchased from Santa Cruz Biotechnology, Inc.; diluted 1:400) for 45 min at room temperature. The sections were then washed three times in PBST, incubated in streptavidin-biotin complex

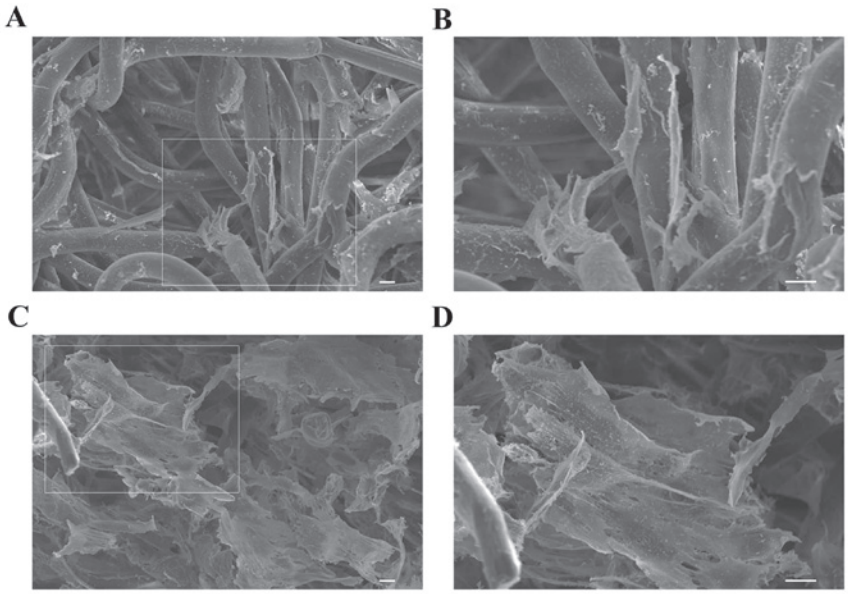


Figure 1. Scanning electron microscopic evaluation of hDPSCs in the poly (lactic-co-glycolic acid) scaffold. (A and B) At 7 days post-seeding of hDPSCs onto the scaffold under static 3D cultures, the cells were attached to the inner surface of the scaffold. (C and D) At 7 days post-seeding, the cells in the 3D SMG culture system grew tightly to each other with abundant extracellular matrix deposited on the scaffolds. Scale bar=100 $\mu$m. hDPSCs, human dental pulp stem cells.

for 30 min at room temperature and stained with 100 $\mu$l DAB solution. When brown coloration was detected, the slides were rinsed and then counterstained with hematoxylin for 1 min and observed under a light microscope.

*Statistical analysis*. The numbers of hDPSCs were counted three times (n=3) in each field of view and section, with three samples for each group. Immunohistochemical analyses were performed using three samples for each group, and calculated three times with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Values are presented as the means ± standard deviation. Statistical analyses were performed using Student's t-test with SPSS version 16.0 software. (SPSS, Inc., Chicago, IL, USA) $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Cell morphology of hDPSCs in PLGA under static 3D culture and 3D SMG culture in vitro*. To investigate the cellular interaction of hDPSC within PLGA scaffolds under static 3D culture and 3D SMG culture, cell growth and morphology were observed using an electron microscope. As shown in Fig. 1A and B, the cells under static 3D culture were attached to the inner surface of the scaffold *in vitro*. The cells in the 3D SMG culture system grew tightly to each other with abundant extracellular matrix deposited on the scaffolds (Fig. 1C and D). These results indicated that the scaffolds were suitable for the following *in vivo* experiments.

*3D SMG culture promotes the growth of hDPSCs in vivo*. Following 3D static or 3D SMG culture for 7 days with osteogenic medium *in vitro*, the differentiating cells within the scaffolds were implanted subcutaneously on the backs of nude mice. Subsequent H&E staining showed that the number of cells cultured in the SMG system was higher, compared with that in the static culture system (Fig. 2A and B). Immunohistochemical analysis of the endogenous proliferation marker, Ki-67, showed an increase in cell proliferation in the SMG group (Fig. 2C and D).

*3D SMG culture induces increased collagen fibrils, calcium phosphate formation and the expression of DMP-1 and dentin sialoprotein (DSP) in vivo*. The tissue sections were stained with Masson's trichrome and von Kossa to identify evidence of collagen fiber formation and mineralization, respectively. The Masson's trichrome staining showed a higher number of collagen fibers stained blue in the SMG culture, compared with the static culture (Fig. 3A). An increase of von Kossa staining was observed in the SMG culture, suggesting that the 3D SMG culture enhanced matrix mineralization. The positive staining of type I collagen observed in the static culture was also reduced, compared with the SMG culture, consistent with the results of the Masson's trichrome staining (Fig. 3A). The immunohistochemical data showed a marginal increase in the expression of DMP-1 (Fig. 3B). However, a significant increase in the expression of DSP was observed in the 3D SMG culture, compared with the static culture (Fig. 3C).

## Discussion

In the present study, a 3D dynamic system consisting of an SMG rotary bioreactor, biodegradable polymer scaffolds and osteogenic medium, was successfully established. Following the culture of DPSCs in this system for 7 days *in vitro*, post-transplantation analysis indicated that the proliferation and odontogenic differentiation abilities of the hDPSCs were increased compared with those of cells cultured in the static culture system. These findings indicated that the 3D SMG

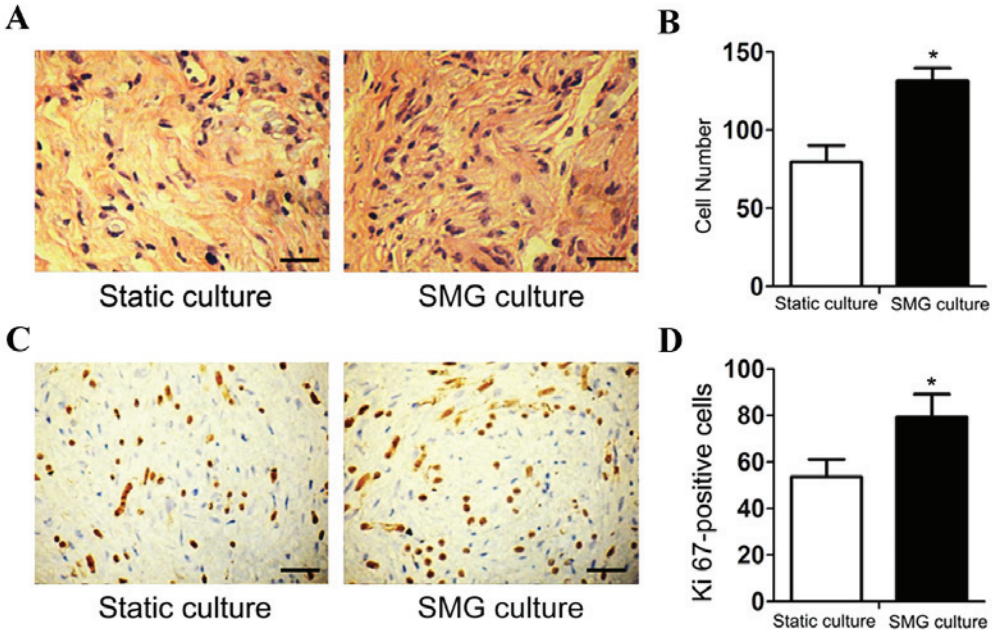


Figure 2. Effects of 3D SMG culture on the growth of hDPSCs in vivo. (A) Hematoxylin and eosin staining of cells grown on scaffolds transplanted on the back of nude mice for 72 h. Following 3D static or 3D SMG culture for 7 days with osteogenic medium, the (B) number of cells in the SMG culture was higher, compared with that in the static culture. (C) Images and (D) quantification of the immunohistochemical analysis of the Ki-67 showed increased positive staining in the SMG cultures, compared with the static cultures (*P<0.05). Scale bar=10 $\mu$m. SMG, simulated microgravity; hDPSCs, human dental pulp stem cells.

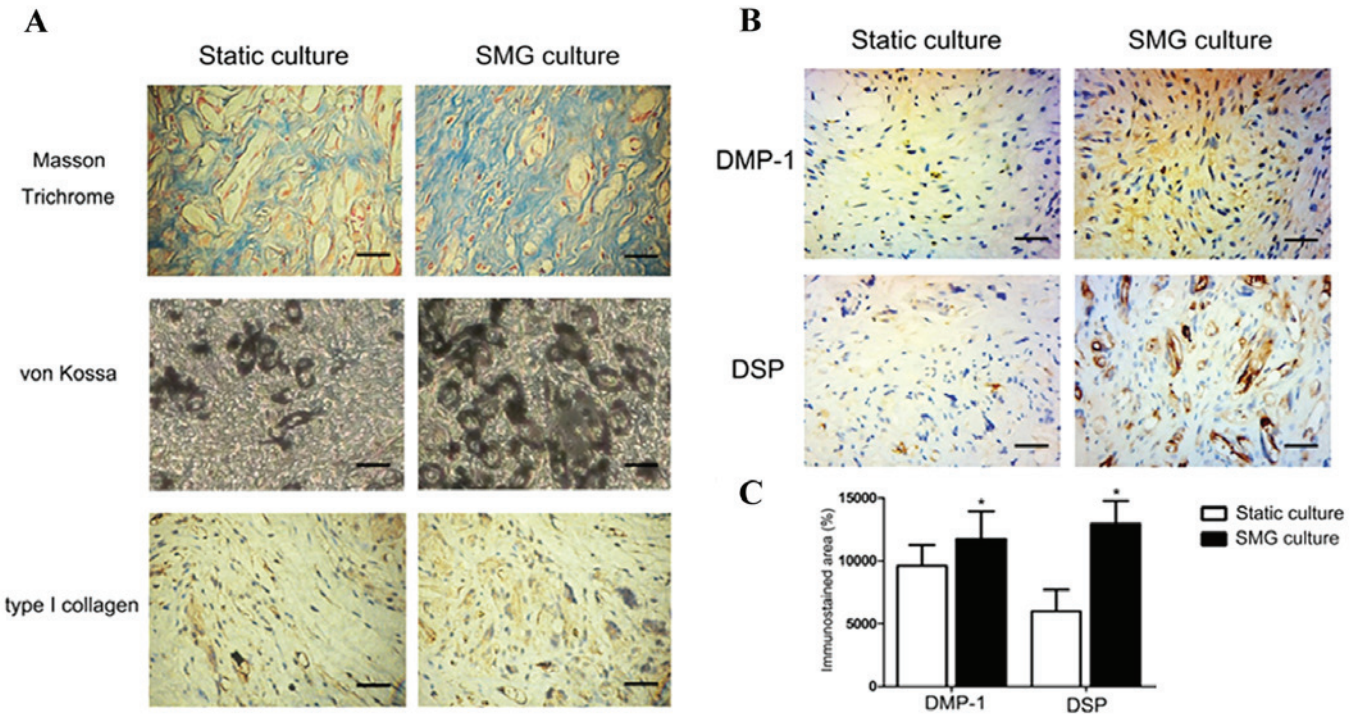


Figure 3. Effects of 3D SMG culture on the odontogenic differentiation of hDPSCs *in vivo*. (A) Masson's trichrome staining revealed increased collagen fibers stained blue in the SMG culture, compared with the static culture. Increased von Kossa staining was observed in the SMG culture. Positive staining of type I collagen in the static cultures was lower, compared with than that in the SMG cultures. (B) Immunohistochemical data showed a marginal increase in the expression of DMP-1. The expression of DSP was significantly higher in the 3D SMG culture, compared to static cultures. (C) Areas of immunostaining of each protein were detected (*P<0.05). Scale bar=10 $\mu$m. SMG, simulated microgravity; hDPSCs, human dental pulp stem cells; DMP-1, dentin matrix acidic phosphoprotein 1; DSP, dentin sialoprotein.

dynamic system offers potential for use as a potent method for tooth tissue regeneration.

Due to their odontogenic differentiation potential, DPSCs been used as effective seed cells for dental tissue engineering

and regeneration. The application of DPSCs in dental tissue engineering provides a significant enhancement to dental regeneration; however, sufficient cell numbers are required, leading to the formation of 3D mineralized, dentin tissue-like constructs. However, limitations in the quantity and committed differentiation efficiency of DPSCs inevitably introduce challenges to dental regeneration. Microgravity and polymer scaffolds have been confirmed to offer significant advantages in cell culture by providing a dynamic 3D microenvironment with low-shear forces and high-mass transfer (18-23).

Previous studies have indicated that a variety of 3D biomaterials are suitable for the proliferation and differentiation of DPSCs (24-26). However, due to the effect of gravity, cells seeded in 3D static culture systems preferentially fall down to the base of the scaffolds, rather than scattering evenly (27). In addition, air, nutrient components and metabolic wastes are also distributed unevenly. Cellular metabolic waste is difficult to transport out of the scaffolds, and the concentration of growth/differentiation factors is usually confined to the surface of the scaffold, resulting in decreased cell proliferation and lineage-specific differentiation (28,29). Fortunately, these problems can be overcome by the dynamic system of the SMG rotary bioreactor, which creates a suspension culture environment contributing to supply of oxygen and nutrients, and the transport of metabolic waste from the cells. In the present study, a 3D dynamic system of SMG was prepared for 7 days *in vitro*, in which the DPSCs grew tightly to each other with abundant extracellular matrix, and a higher number of cells were observed *in vivo* (Fig. 2). As previous experiments have reported, undergoing 3D dynamic SMG culture leads to the promotion of cell proliferation (18,30-32). Ki-67, used as a biomarker for the proliferation of cells, showed an increase in cell proliferation in the dynamic system (Fig. 2) as a result of DPSCs obtaining sufficient nutrition and the prompt delivery of metabolic waste in the suspension culture environment. Following transplantation *in vivo*, the optimal viability and state of the DPSCs were observed. The increased proliferation of the DPSCs *in vivo* is important for dental tissue engineering and regeneration, as this is limited in autologous or allogenic seeding of cells.

The most notable feature of hDPSCs is their odontogenic differentiation potential for dental tissue engineering (33). In the present study, Masson's trichrome staining and the immunohistochemical analysis of type I collagen were applied to determine the collagen fibers in the tissue sections. Type I collagen is the most important constituent of the extracellular matrix of dental pulp connective tissue (34). It has been suggested that the synthesis of type I collagen is an important step in the odontoblast differentiation process (35). Previous studies have shown that type I collagen may be a component of the predentin secreted by polarized odontoblasts (36), and it has been found to be associated with the production and mineralization of dentine (37). In the present study, increased collagen was produced in the dynamic system group, which indicated that dynamic culture triggered the deposition of oriented collagen fibers, which in turn suggested the possibility of the formation of dentin. Von Kossa staining is usually used to identify the existence and formation of calcium phosphate (38). As the primary component of teeth is calcium phosphate, the results indicating a higher level of calcium phosphate formation in the implanted cells from the dynamic SMG system, compared with that in static culture 4 weeks post-transplantation *ex vivo* suggested that the dynamic SMG system promoted the mineralization of DPSCs. DSP and DMP-1, the major noncollagenous proteins synthesized by odontoblasts, are well-known markers of odontogenic differentiation. DSP, which is expressed at high levels in odontoblasts, is essential to the formation and calcification of dentin (39,40). Expressed prior to DSP, DMP-1 regulates the mineralization of dentin (41) and is involved in the differentiation of odontoblasts (42-44). Thus DSP and DMP-1 are usually selected as specific markers of differentiation to detect the odontogenic potential of DPSCs. The upregulation of DSP and DMP-1 in the DPSCs induced by the dynamic SMG system, indicated the promotion of odontogenesis of the DPSCs. The dynamic system of SMG upregulated the mineralization capacity and expression levels of DSP and DMP-1 in the DPSCs, which supported the idea that the dynamic SMG system was more suitable for odontogenic differentiation of DPSCs. There is substantial evidence, which shows that SMG promotes the differentiation of stem cells *in vitro* (18,30-32) and, consistent with these reports, the present study found that the dynamic SMG system increased the odontogenic differentiation of DPSCs *in vivo*. This may also be due, in part, to the prompt delivery of factors in osteogenic medium and interactions with the microenvironment in the nude mice. The DPSCs under the dynamic culture system, which contributed to the sufficient transfer of nutrients and factors in osteogenic medium, were maintained in good condition throughout the entire treatment process prior to *in vivo* implantation. With DPSCs in a preferable condition, the interaction between cells and the microenvironment *in vivo* may be improved, which may have a positive effect on the committed differentiation of stem cells.

In conclusion, the present study showed that the dynamic system combining SMG with scaffolds and osteogenic medium significantly improved the proliferation and odontogenic differentiation of DPSCs by improving their metabolism and microenvironment. These results further indicated the potential of the dynamic SMG system in dentin regeneration, and provided novel insight into tooth engineering.

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