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Basic fibroblastic growth factor affects the osteogenic differentiation of dental pulp stem cells in a treatment-dependent manner

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Abstract

Qian J, Jiayuan W, Wenkai J, Peina W, Ansheng Z, Shukai S, Shafei Z, Jun L, Longxing N. Basic fibroblastic growth factor affects the osteogenic differentiation of dental pulp stem cells in a treatment-dependent manner. *International Endodontic Journal*, **48**, 690–700, 2015.

Aim To determine how basic fibroblastic growth factor (bFGF) affected the osteogenic differentiation of human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*.

Methodology Basic fibroblastic growth factor stimulation of DPSCs was divided into a pre-treatment period and an osteogenic differentiation period. Alizarin red quantification experiments and alkaline phosphatase activity quantification assay were performed to examine the osteogenic differentiation of DPSCs after different bFGF stimulation. Quantification reverse transcription polymerase chain reaction was used to analyze the osteogenic gene expression of DPSCs after different bFGF stimulation. In addition, DPSCs that received the 1 and 2 weeks bFGF pre-treatments as in the *in vitro* experiments were mineralized for 1 week and seeded into hydroxyapatite/tricalcium phosphate (HA/TCP) pills and subcutaneously transplanted into naked mice for 2 or 3 months. The transplants were removed, sliced and stained using Modified Ponceau Trichrome Stain to observe the formation of mineralized tissue.

Results Basic fibroblastic growth factor stimulation in the osteogenic differentiation period decreased the *in vitro* osteogenic differentiation ability of DPSCs. One week pre-treatment with bFGF increased the *in vitro* osteogenic differentiation ability of DPSCs, whereas 2 weeks pre-treatment with bFGF decreased the *in vitro* osteogenic differentiation ability of DPSCs. The pre-treatment period was vital for the osteogenic differentiation of DPSCs *in vitro*. The *in vivo* results were similar to the *in vitro* results.

Conclusions Basic fibroblastic growth factor affected the osteogenic differentiation of DPSCs in a treatment-dependent manner both *in vitro* and *in vivo*.

Keywords: basic fibroblast growth factor, dental pulp stem cells, osteogenic differentiation.

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Introduction

Oral stem cells include dental pulp stem cells (DPSCs), periodontal ligament stem cells and stem cells from the apical papilla, Gronthos *et al.* (2000) first reported that stem cells isolated from adult dental pulp could be cultured *in vitro*. In addition, this type of cell could differentiate into odontoblasts and could form dentine-like tissue. Since then, the concept of DPSCs has been proposed. DPSCs have been characterized as cells

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that are capable of self-regeneration, have a high degree of proliferation and have the potential to differentiate into multiple types of cells.

Basic fibroblastic growth factor (bFGF) is a mitogenic cationic peptide that consists of 155 amino acids that is extensively distributed throughout the human body. As a cell mitogen, it mainly impacts on mesoblasts and neuroderm-oriented cells, such as skeletal muscle cells, fibroblasts and osteocytes. Correspondingly, its receptor is expressed on these cells. In addition to its mitogenic effects, bFGF is a morphogenesis and differentiation inducer. The main biological effects of bFGF include (i) its role as an angiogenic factor, (ii) its promotion of wound healing and tissue restoration, (iii) its promotion of tissue regeneration, and (iv) its participation in neurogenesis (Davidson *et al.* 1985, Lobb *et al.* 1985, Abraham *et al.* 1986, Cuny *et al.* 1986, Canalis *et al.* 1987).

Many *in vivo* studies have shown that bFGF can induce bone regeneration (Mayahara *et al.* 1993, Nakamura *et al.* 1995, Zellin & Linde 2000). However, the *in vitro* effects of bFGF on cell osteogenic differentiation remain unclear. Some studies have shown that stimulation with bFGF inhibited the production of collagen and alkaline phosphatase (ALP) and the osteogenic differentiation of osteoblasts (Canalis *et al.* 1988, Varghese *et al.* 1995, Tang *et al.* 1996). In contrast, other reports have shown that bFGF enhanced the osteogenic differentiation of multipotent stem cells (MSCs), and osteoblasts if the cells were exposed to bFGF only during the late periods of cell osteogenic differentiation (Debiais *et al.* 1998, Tsutsumi *et al.* 2001, Fakhry *et al.* 2005). In addition, it has been demonstrated that bFGF could induce the dexamethasone-dependent osteogenic differentiation of MSCs and that combined treatment with bFGF and BMP-2 synergistically enhanced the osteogenic potency of bFGF in MSC cultures (Hanada *et al.* 1997).

Several years ago, researchers began to focus on the relationship between bFGF and human dental pulp cells (HDPCs)/DPSCs (He *et al.* 2008, Kim *et al.* 2010, Osathanon *et al.* 2011). However, similar disagreement was found regarding the *in vitro* effects of bFGF on HDPCs/DPSCs. Some articles have reported that bFGF increased ALP activity and enhanced osteogenic differentiation in the HDPCs/DPSCs (He *et al.* 2008, Kim *et al.* 2010). However, other reports showed that bFGF inhibited the osteogenic differentiation of HDPCs/DPSCs (Shimabukuro *et al.* 2009, Osathanon *et al.* 2011). A review of these articles on bFGF and HDPCs/DPSCs revealed that the stature of the cells and the treatment protocol of bFGF were different. (Shiba *et al.* 1995, He *et al.* 2008, Shimabukuro *et al.* 2009, Kim *et al.* 2010, Osathanon *et al.* 2011). Therefore, an experiment was designed that was as comprehensive as possible to determine the relationship between bFGF and the osteogenic differentiation of DPSCs *in vitro* and *in vivo*.

Hydroxyapatite/tricalcium phosphate (HA/TCP) has been commonly used as a scaffold material for *in vivo* experiments in stem cell research. Its biological characteristics make it suitable for use in stem cell research, especially for *in vivo* osteogenic differentiation assays (Zhang *et al.* 2006). Shi *et al.* (2005) used this material to observe the osteogenic characteristics of MSCs (mesenchymal stem cells). Therefore, HA/TCP was used in the classic manner, which involves seeding DPSCs into HA/TCP and inserting the complex into the backs of naked mice to observe how the *in vivo* osteogenic differentiation of the DPSCs was affected by bFGF stimulation, according to the treatment method.

Materials and methods

Sample collection and cell culture

Human impacted third molars were collected from adults (19–29 years of age) at the dental hospital of the Fourth Military Medical University. The dental pulp was isolated as previously described (Gronthos *et al.* 2000). All the tooth extractions were conducted under the approval of the Ethical Committee of the hospital. Briefly, the pulp tissue was gently separated from the crown and root and then was digested in a solution of 3 mg mL^{-1} collagenase type I (Invitrogen, Carlsbad, CA, USA) and 4 mg mL^{-1} dispase (GIBCO/Invitrogen, Carlsbad, CA, USA) for 40 min at 37 °C. Single-cell suspensions of DPSCs were obtained by passing the solution through a 70-μm strainer and seeding 1×10^4 cells $well^{-1}$ onto six-well plate (Corning Costar, Cambridge, MA, USA). The standard medium was defined as: α-modification of Eagle's Medium (α-MEM; GIBCO/Invitrogen) supplemented with 10% foetal bovine serum (FBS; HyClone, Kerrville, TX, USA); 100 mol L^{-1} L-ascorbic acid 2-phosphate (Sigma, St Louis, MO, USA); 2 mmol L^{-1} L-glutamine (Sigma); 100 U mL^{-1} penicillin; and 100 μg mL^{-1} streptomycin (GIBCO) at 37 °C in 5% CO_2. The medium was changed every 2–3 days. Single cells were isolated using the limiting dilution

technique, and they were seeded onto a 96-well plate and were allowed to grow into a single colony. A number of these single-colony-derived strains of DPSCs were pooled, cultured and passaged at a 1 : 3 ratio when they reached 80% confluence. Next, the cells were stained with STRO-1, CD146, vimentin (Sigma), and keratin (Sigma) using immunofluorescence staining. Laser scanning confocal microscopy showed that the cells were STRO-1, CD146 and vimentin positive and keratin (negative control) negative. The experiments were performed using the cells from the third to sixth passages. bFGF (Calbiochem, San Diego, CA, USA) was prepared according to the manufacturer's instructions and was diluted to a concentration of 10 ng mL^{-1} in standard medium (α-MEM containing 10% FBS) or standard osteogenic differentiation medium [standard medium containing 50 mg mL^{-1} ascorbic acid (Sigma), 10 mmol L^{-1} beta-glycerophosphate (BGP; Sigma) and 10 ng mL^{-1} dexamethasone], as previously described (Osathanon *et al.* 2011).

In vitro osteogenic differentiation assay

The *in vitro* osteogenic differentiation assay was divided into five small assays to detect the effects of bFGF on the osteogenic differentiation of DPSCs. In assays 1, 2, 3 and 4, DPSCs were seeded into a 24-well plate at a density of approximately 1×10^5 cells per well and were divided into experimental and control groups. After reaching 80% confluence, the cells in the control and experimental groups were treated with or without bFGF according to Table 1. In assay 5, DPSCs were seeded into two 25-cm^2 cell culture flasks (Corning Incorporated, Corning, NY, USA) at a density of approximately 1×10^6 and were divided into experimental and control groups. After reaching 80% confluence, the cells in the experimental group were pre-cultured in standard medium with 10 ng mL^{-1} bFGF, and the cells in the control group were pre-cultured with standard medium for 1 week. Then, the cells were seeded into a 24-well plate at a density of approximately 1×10^5 cells per well after subcultivation. The experimental and control groups were cultured with standard osteogenic differentiation medium for 4 weeks according to Table 1. After cultivation, all of the cells were stained with Alizarin red for Alizarin red quantification experiments, according to the methods developed by Osathanon *et al.* (2011). All of the experiments were performed at least three times.

ALP activity quantification

The DPSCs (200 cells $well^{-1}$) were seeded onto a 96-well plate and were divided into three groups. Each group had five wells. In this assay and the following assays, the cells pre-treated with standard media with bFGF (10 ng mL^{-1}) for 1 week were designated as group 1, the cells pre-treated with standard media

Table 1 *In vitro* osteogenic differentiation assay

Assay	Group	Cell condition	Pre-treatment	Osteogenic differentiation for 4 weeks
Assay 1	EXP	Confluence	–/–	Osteogenic differentiation medium with bFGF
	CTR	Confluence	–/–	Osteogenic differentiation medium
Assay 2	EXP	Confluence	Standard medium for 1 week	Osteogenic differentiation medium with bFGF
	CTR	Confluence	Standard medium for 1 week	Osteogenic differentiation medium
Assay 3	EXP1	Confluence	Standard medium with bFGF for 1 week	Osteogenic differentiation medium with bFGF
	EXP2	Confluence	Standard medium with bFGF for 1 week	Osteogenic differentiation medium
	CTR	Confluence	Standard medium for 1 week	Osteogenic differentiation medium
Assay 4	EXP1	Confluence	Standard medium with bFGF for 2 weeks	Osteogenic differentiation medium with bFGF
	EXP2	Confluence	Standard medium with bFGF for 2 weeks	Osteogenic differentiation medium
	CTR	Confluence	Standard medium for 2 weeks	Osteogenic differentiation medium
Assay 5	EXP	Confluence	Standard medium with bFGF for 1 week and subcultivation	Osteogenic differentiation medium
	CTR	Confluence	Standard medium for 1 week and subcultivation	Osteogenic differentiation medium

EXP, experimental; CTR, control; bFGF, basic fibroblastic growth factor.

with bFGF (10 ng mL^{-1}) for 2 weeks were designated as group 2, and the cells pre-treated with standard media without bFGF for 1 week were designated as the control group. After pre-treatment, the culture media in all of the groups were all changed to standard osteogenic differentiation medium. The ALP activity assay was performed on days 2, 4, 6, 8, 10, 12 and 14 after osteogenic differentiation, using an ALP assay kit (JianCheng Co., Nanjing, China) according to the manufacturer's instruction. The results were measured spectrophotometrically at 520 nm.

Quantification Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The DPSCs (1×10^4 cells per dish) were seeded onto 30 mm dishes and were also divided into group 1, group 2 and the control group. After pre-treatment, the culture media in all of the groups were all changed to standard osteogenic differentiation medium. One week later, total RNA was extracted from the DPSCs using RNAiso Plus (Takala, Osaka, Japan) and was reverse transcribed using a Takala reverse transcriptase kit (Takala). Real-time PCR was performed using a standard SYBR Green PCR kit (Takala) and the Applied Biosystems 7500 Real-Time PCR System (ABI, Foster City, CA, USA), according to the respective manufacturers' instructions. β-actin was used as an internal control. The expressions of osteogenic differentiation marker mRNA, ALP, dentine sialophosphoprotein (DSPP), osteocalcin (OCN) and bone sialoprotein (BSP) were analyzed. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative levels of gene expression. The results represent the ratio between the expression in the control group and that in the bFGF pre-treatment groups ($\log_{10}$ scale). The primer sequences are presented in Table 2.

Table 2 Primer sequences

Primer	Sequence (5′–3′)
Actin-forward	TGGCACCCAGCACAATGAA
Actin-reverse	CTAAGTCATAGTCCGCCTAGAAGCA
ALP-forward	CCACGTCTTCACATTTGGTG
ALP-reverse	AGACTGCGCCTGGTAGTTGT
DSPP-forward	TCACAAGGGAGAAGGGAATG
DSPP-reverse	TGCCATTTGCTGTCAGTTT
OCN-forward	GGCAGCGAGGTAGTGAAGAG
OCN-reverse	CTGGAGAGGAGCAGAACTGG
BSP-forward	AAAGTGAGAACGGGGAACCT
BSP-reverse	GATGCAAAGCCAGAATGGAT

DSPP, dentine sialophosphoprotein; OCN, osteocalcin; BSP, bone sialoprotein; ALP, alkaline phosphatase.

In vivo osteogenic differentiation assay

Cell culture

The DPSCs (1×10^6 cells per dish) were seeded in 32 10-cm dishes and were divided into group 1, group 2, a control group and a group called group 3, in which cells were pre-treated with bFGF (10 ng mL^{-1}) for 1 week and were treated with standard medium for another week (Table 3). After pre-treatment, the cells in group 1, group 2 and the control group were treated with standard osteogenic differentiation medium for 1 week.

Cell seeding and transplantation

After cultivation, the DPSCs (1×10^7 cells) in each dish were seeded into HA/TCP (60/40) pills (diameter = 5 mm, thickness = 1.5 mm) and were transplanted subcutaneously into 16 naked mice, which had been averagely and randomly divided into four groups. Incision were made in the backs of each animal and then bilateral blunt dissection created two pouches. The pills were placed into each side of the back, which was then sutured. These procedures were performed in accordance with the specifications of an animal protocol approved by the Ethics Committee of the Fourth Military Medical University.

Sectioning and staining

The transplants were removed 2 and 3 months after transplantation, were fixed with 4% paraformaldehyde, and then cut into slices and stained using a Modified Ponceau Trichrome Stain (Oh *et al.* 2013). For histometric analysis of cartilage-like and bone-like tissue formation, computer-assisted histometric measurements were acquired with an automated image analysis, as previously described (Zhang *et al.* 2012).

Statistical analysis

The data are expressed as means ± SDs. The statistical significance was evaluated using unpaired or paired Student's *t*-tests for comparisons between two means, whereas MANOVA was used to evaluate multiple means. A value of $P < 0.05$ was assumed to denote statistical significance.

Table 3 Culture method

Group	Cell condition	Pre-culture	Osteogenic differentiation
Negative control	Confluence	Standard medium for 1 week	Standard osteogenic differentiation medium for 1 week
Positive control	Confluence	Standard medium with bFGF for 1 week	Standard medium for 1 week
Group1	Confluence	Standard medium with bFGF for 1 week	Standard osteogenic differentiation medium for 1 week
Group2	Confluence	Standard medium with bFGF for 2 weeks	Standard osteogenic differentiation medium for 1 week

bFGF, basic fibroblastic growth factor.

Results

bFGF affected the osteogenic differentiation of DPSCs in a treatment-dependent manner

Basic fibroblastic growth factor affected the osteogenic differentiation of DPSCs in a treatment-dependent manner. In assay 1, bFGF stimulation during the osteogenic differentiation period could upregulate the osteogenic differentiation ability of DPSCs (Fig. 1a). In assay 2, bFGF stimulation during the osteogenic differentiation period could upregulate the osteogenic differentiation ability of DPSCs, regardless of 1-week pre-treatment with standard medium (Fig. 1b). In assay 3, after 1 week of pre-treatment with bFGF, the osteogenic differentiation ability of DPSCs was increased, regardless of bFGF stimulation during the osteogenic differentiation period (Fig. 1c). In assay 4, after 2 weeks of pre-treatment with bFGF, the osteogenic differentiation ability of DPSCs was decreased, regardless of bFGF stimulation during the osteogenic differentiation period (Fig. 1d). In assay 5, after 1 week of pre-treatment with bFGF, the osteogenic differentiation ability of DPSCs was increased after subcultivation (Fig. 1e). According to the Alizarin red quantification results, the pre-treatment dura-

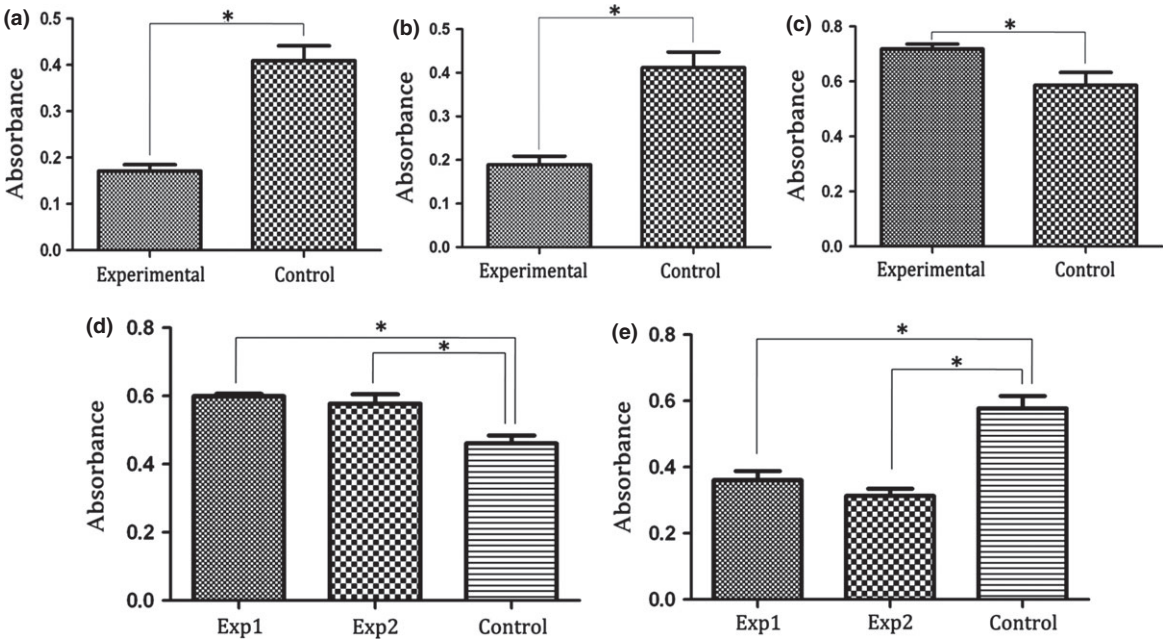

Figure 1 Alizarin red quantification assay results. Different basic fibroblastic growth factor (bFGF) treatment manners lead to different results of osteogenic differentiation of dental pulp stem cells (DPSCs) in alizarin red quantification assay 1 (a), 2 (b), 3 (c), 4 (d), 5 (e). The means and the standard errors of the means (SEMs) of three identical experiments are shown. *Indicates $P < 0.05$ compared to the control group.

tion was one of the key factors that determined how bFGF affected the osteogenic differentiation of the DPSCs. In assays 1, 2 and 4, bFGF inhibited the osteogenic differentiation of DPSCs. Assays 1 and 2 showed that bFGF stimulation in the osteogenic differentiation period decreased the *in vitro* osteogenic differentiation ability of DPSCs. In addition, no significant differences were found between cells that had just reached confluence and cells cultured for 1 week. There was no significant difference between cells that were not pre-treated with bFGF, and cells that were pre-treated with bFGF for 2 weeks in assay 4. However, in assays 3 and 5, in which the DPSCs were pre-treated with bFGF for 1 week, the osteogenic differentiation of the DPSCs was enhanced. Assay 5 also showed that the impact of bFGF on DPSCs could be transferred to the next passage because assay 5 showed similar results to assay 3 after subculture.

Based on these results, ALP activity quantification and qRT-PCR were performed, which showed supplemental results. In both assays, group 1 refers to 1 week of bFGF pre-treatment, group 2 refers to 2 weeks of bFGF pre-treatment, and control group refers to 1 week of standard medium pre-treatment. ALP activity quantification was performed every 2 days from the initiation of osteogenic differentiation. Interestingly, the absorbance value (wavelength = 520 nm) of group 1 was higher than that in the control group beginning on day 6, whereas the absorbance value of group 2 was lower than that in the control group beginning on day 6 (Fig. 2a). qRT-PCR results showed that the expressions of ALP, DSPP, OCN and BSP of the DPSCs in group 1, in which DPSCs were pre-treated with bFGF for 1 week, were higher than in the control group, and the expressions of ALP, DSPP, OCN and BSP of the DPSCs in group 2, in which DPSCs were pre-treated with bFGF for 2 week, were lower than in the control group (Fig. 2b).

bFGF affected the *in vivo* osteogenic differentiation of DPSCs depending on the duration of pre-treatment

Two months after transplantation

After 2 months, cartilage-like tissues (stained in blue) were formed in the edge of the hole in the control group (Fig. 3a), and there were cartilage-like tissues formed in the middle of the hole in group 1 (Fig. 3b). Obvious cartilage- or bone-like tissue formation was not observed in group 2 (Fig. 3c) or group 3 (Fig. 3d). However, well-developed vascular tissue was only observed in group 3. Histometric measurements showed that there were more cartilage-like tissues formed in group 1 than in the control group (Fig. 5a). In addition, little bone formation was observed in any of the groups (Fig. 5b).

Three months after transplantation

After 3 months, cartilage (stained in blue) – and bone-like (stained in red) tissue formation was observed in the control group (Fig. 4a) and in group 1(Fig. 4b). Obvious cartilage- or bone-like tissues

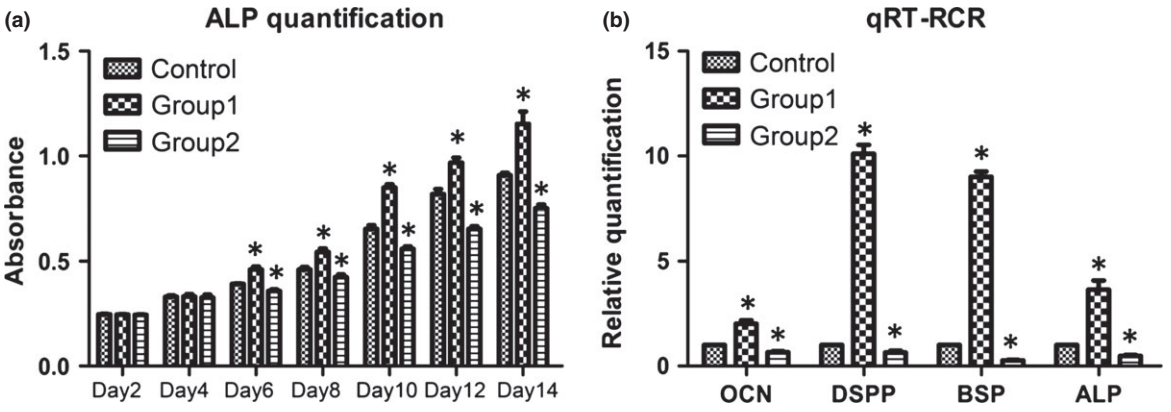

Figure 2 Alkaline phosphatase (ALP) activity quantification and qRT-PCR results. ALP activity quantification (a) and qRT-PCR (b) results showed that 1 week/2 weeks of pre-treatment with basic fibroblastic growth factor (bFGF) increased/decreased the osteogenic differentiation ability of dental pulp stem cells (DPSCs). The means and the standard errors of the means (SEMs) of three identical experiments are shown. *Indicates $P < 0.05$ compared to the control group.

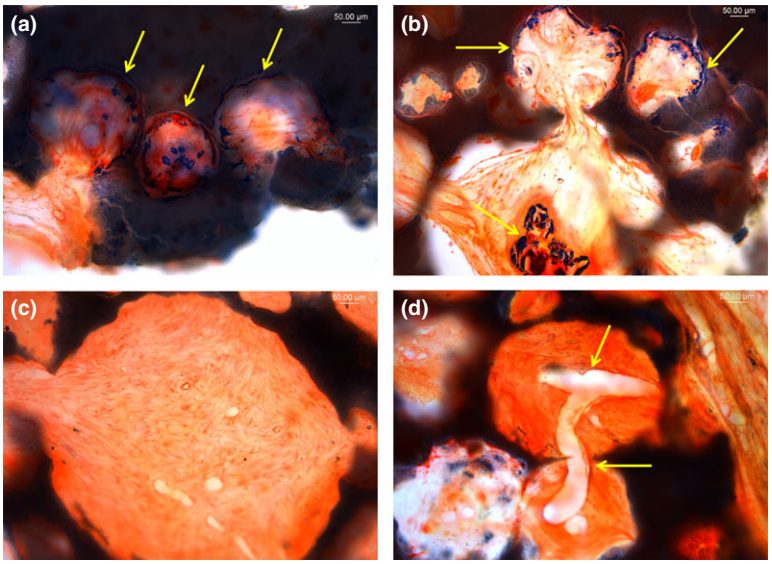

Figure 3 Two-month *in vivo* osteogenic differentiation assay. Different basic fibroblastic growth factor (bFGF) treatment manners lead to different results of osteogenic differentiation of dental pulp stem cells (DPSCs) in the *in vivo* osteogenic differentiation assay after 2 months transplantation.

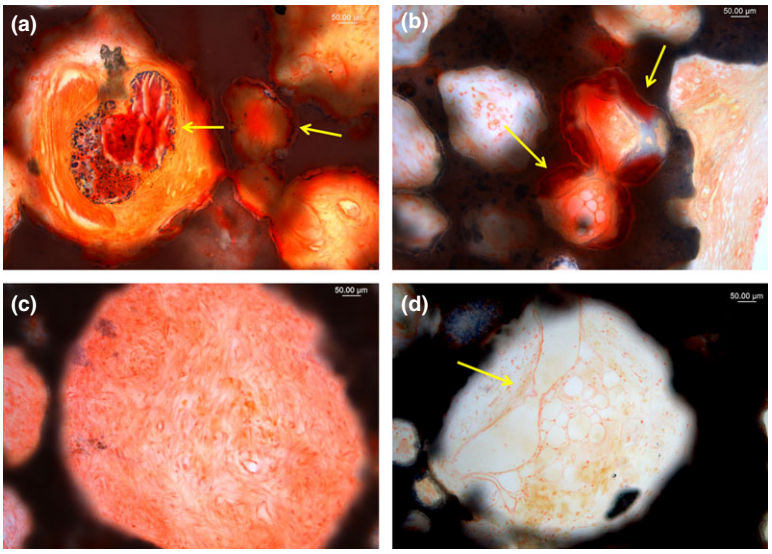

Figure 4 Three-month *in vivo* osteogenic differentiation assay. Different basic fibroblastic growth factor (bFGF) treatment manners lead to different results of osteogenic differentiation of dental pulp stem cells (DPSCs) in the *in vivo* osteogenic differentiation assay after 3 months transplantation.

formation was also not observed in group 2 (Fig. 4c) or group 3 (Fig. 4d). There was well-developed vascular tissue formation in group 3, which was not observed in the other groups. Histometric measurements showed that there were more bone-like tissues formed in group 1 than in the control group (Fig. 5d), but there were no significant differences in the cartilage-like tissue formation between group 1 and the control group (Fig. 5c).

The *in vivo* results were similar to the *in vitro* results. Thus, this experiment confirmed the results of the *in vitro* experiments.

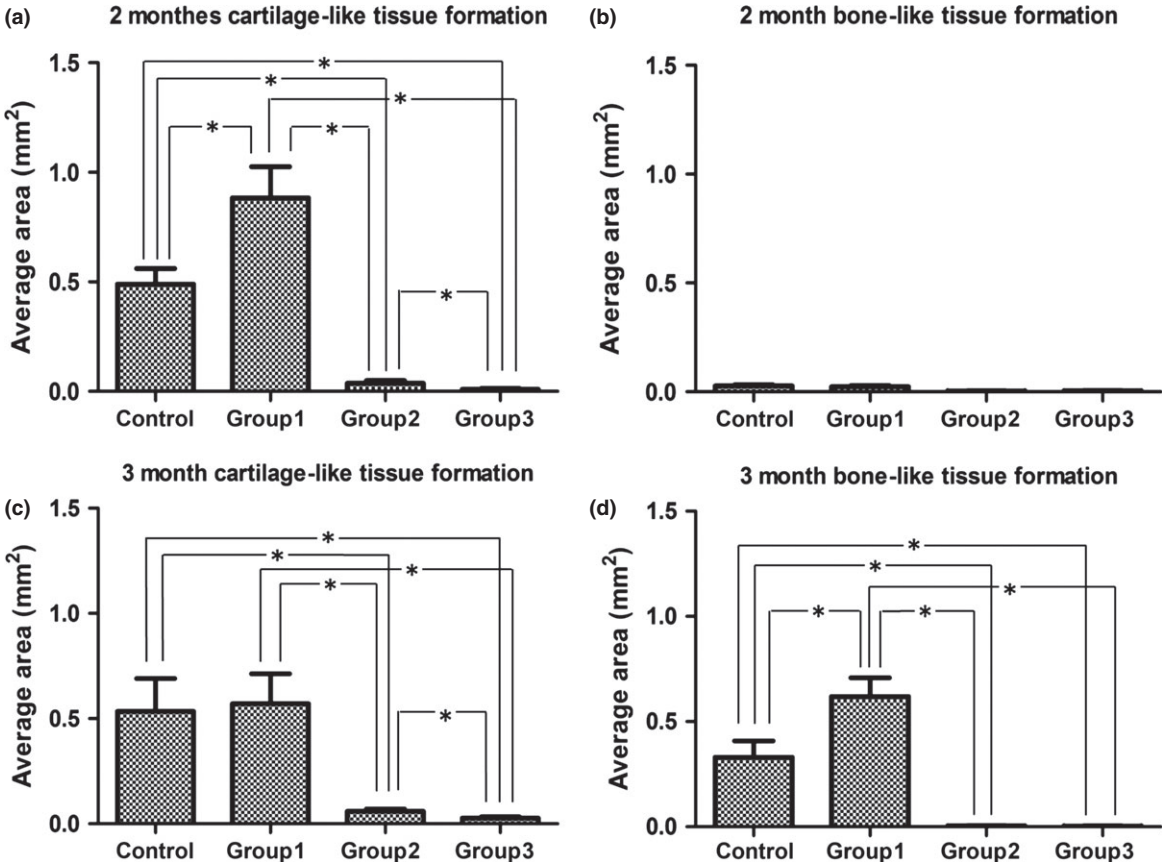

Figure 5 Quantitative analysis of the *in vivo* osteogenic differentiation assay. The results of the *in vivo* osteogenic differentiation assays were statistically confirmed by histometric measurements. *Indicates $P < 0.05$.

Discussion

Several interesting and confusing results appear in the literature, which indicate that the nature of bFGF action is complex and that the biological effects of bFGF could depend on the differentiation stage of osteoblasts and the length and mode of exposure to certain factors (Fakhry *et al.* 2005). In the present study, an experiment was performed on how different bFGF stimulation conditions affected the osteogenic differentiation of DPSCs, and so, 1 and 2 weeks of stimulation of bFGF was chosen as the treatment protocol because these durations have been frequently used in previous studies (Shiba *et al.* 1995, He *et al.* 2008, Shimabukuro *et al.* 2009, Osathanon *et al.* 2011). The Alizarin red quantification assay was divided into five small assays. The main assay division indicated the differences between the stimulation time and order. The bFGF stimulation of DPSCs was divided into the pre-treatment period and the osteogenic differentiation period. As observed from the results, the difference in the stimulation time and order led to different osteogenic differentiation abilities of DPSCs. bFGF treatment did not stimulate proliferation of human calvarial bone cells that had been grown in mineralizing medium for several weeks (Debiais *et al.* 1998). As shown in assays 1 and 2, bFGF stimulation during the osteogenic differentiation period decreased the osteogenic differentiation of DPSCs, regardless of the cell condition (confluence or multilayer). Fakhry *et al.* (2005) found that the mitogenic effects of bFGF were more evident in maturing osteoblasts than in osteogenic pre-cursor cells. Assay 4 showed that 2 weeks of pre-treatment with bFGF decreased the osteogenic differentiation ability of DPSCs regardless of bFGF stimulation during the osteogenic differentiation period. However, the lack of mitogenic effect might have resulted from the onset of mineralization (Fakhry *et al.* 2005). With 1 week of pre-treatment with bFGF, the osteogenic differentiation ability of the

DPSCs was increased regardless of bFGF stimulation during osteogenic differentiation period. Assays 3 and 4 indicated that the pre-treatment period was vital for the osteogenic differentiation of DPSCs *in vitro*. Assay 5 showed that the impact of bFGF could be inherited. In other words, these phenomena indicated that the impact of bFGF on DPSCs occurred at the genetic level and was not abolished by subcultivation. According to these results, it can be concluded that 1 week of pre-treatment with bFGF increased the *in vitro* osteogenic differentiation ability of DPSCs. However, these effects were not time dependent: 2 weeks of pre-treatment with bFGF decreased the *in vitro* osteogenic differentiation ability of DPSCs. This finding could have been due to bFGF playing different roles depending on the differentiation stage of DPSCs into osteoblasts (Fakhry *et al.* 2005). In addition, the ALP activity quantification assay and qRT-PCR results also confirmed that the pre-treatment period was vital for the osteogenic differentiation of DPSCs *in vitro*.

In the *in vivo* experiments, the same results were obtained. One week of pre-treatment with bFGF increased the formation of cartilage- and bone-like tissues, whereas 2 weeks of pre-treatment with bFGF decreased cartilage- and bone-like tissue formation. In the experiments, cell-scaffold complexes were collected after 2 and 3 months. After 2 months, the cartilage- and bone-like tissue formation of the DPSCs was not obvious, even in the control group and in group 1. After 3 months, cartilage- and bone-like tissues were remarkably formed in the control group and in group 1. However, group 2 and group 3 still exhibited no significant cartilage- or bone-like tissue formation. Therefore, it was concluded that obvious cartilage- and bone-like tissue formation could be observed after 3 months; thus, the *in vivo* experiment should last 3 months. It was also concluded that 1 week of pre-treatment with bFGF could increase the *in vivo* osteogenic differentiation ability of DPSCs, whereas 2 weeks of pre-treatment with bFGF would decrease the *in vivo* osteogenic differentiation ability of DPSCs.

The wound healing process is activated when dentine injury occurs. The wound healing process could offer another explanation for the results. Previous research has shown that bFGF played an important role in the wound healing process (Smith 2002, Tran-Hung *et al.* 2008). The pre-treatment period in the present study could be considered the preliminary stage of wound healing. During this stage, there was little osteogenic differentiation induction component in the cellular matrix. The main function of bFGF was the promotion of the proliferation and migration of DPSCs (Shimabukuro *et al.* 2009). According to the results, 1 week of pre-treatment with bFGF could promote the osteogenic differentiation of DPSCs, and this result indicated that DPSCs could differentiate into osteoblast-like cells more easily with stimulation by bFGF and participate in the preliminary stages of wound healing. Previous research showed that 2 weeks of stimulation with bFGF could attenuate the osteogenic differentiation-related mRNA expression of collagen type I, osteonectin, BSP and DSPP (Shimabukuro *et al.* 2009). These results were similar to those of the present study. Furthermore, Osathanon *et al.* (2011) reported that 2 weeks of bFGF stimulation would enhance the stemness of DPSCs by upregulating OCT4, NANOG1 and REX1, which was an additional finding that indicated that 2 weeks of bFGF stimulation could attenuate the osteogenic differentiation of DPSCs. Thus, it can be deduced that bFGF can activate DPSCs by upregulating the proliferation, migration and osteogenic differentiation of DPSCs during the preliminary stage of wound healing. In contrast, bFGF attenuates the osteogenic differentiation and maintains the stemness of the DPSCs, which is not activated in the preliminary stages of wound healing. This mechanism will maintain the population of DPSCs in the dental pulp during the wound healing process.

The osteogenic differentiation of dental pulp tissue is a double-edged sword. When dental pulp is subjected to harmful stimulation, the osteogenic differentiation of the odontoblasts or the odontoblast-like cells can form reactive or reparative dentine, which protects the dental pulp from further damage. It is known that DPSCs can differentiate into odontoblasts or odontoblast-like cells. However, during age-related changes and chronic inflammation of the dental pulp, the osteogenic differentiation of dental pulp cells will produce pulp stones and will form calcifications in the dental pulp cavity and root canal, which will make root canal treatment more complicated. It is known that DPSCs are mostly located in multicellular layers, and that these cells are relevant to the calcification obtained after calcium hydroxide pulp capping and pulpotomy. Therefore, the present study found a new method to control the osteogenic differentiation of dental pulp tissue with bFGF, to obtain the desired osteogenic differentiation state, which could bring great changes to the field of endodontic tissue engineering and endodontic therapy.

Conclusion

One week of pre-treatment with bFGF increased the *in vitro* and *in vivo* osteogenic differentiation ability of DPSCs, whereas 2 weeks of pre-treatment with bFGF decreased the *in vitro* and *in vivo* osteogenic differentiation ability of DPSCs. Furthermore, the pre-treatment duration was vital for the osteogenic differentiation of DPSCs *in vitro* and *in vivo*. bFGF affected the osteogenic differentiation of DPSCs in a treatment-dependent manner both *in vitro* and *in vivo*. The data suggest that different treatment periods and durations could have opposite effects when the same cells were treated with the same cytokines. For future studies of DPSCs and bFGF, the stature of the DPSCs and treatment protocol with bFGF must be the primary considerations for all of the variables because a slight change in the stature of the DPSCs or the treatment protocol with bFGF could result in great changes to the result when DPSCs were treated by bFGF.

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