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Transfection With Follicular Dendritic Cell Secreted Protein to Affect Phenotype Expression of Human Periodontal Ligament Cells

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ABSTRACT

Follicular dendritic cell secreted protein (FDC-SP), has been found to inhibit osteogenic differentiation of human periodontal ligament cells (hPDLCs) in recent studies. Based on these findings, we further investigate its effect on phenotype expression of hPDLCs in the present study, aiming to contribute to a better understanding of the biological functions governing FDC-SP-induced hPDLC differentiation. hPDLCs were firstly identified with immunocytochemical staining, followed by transfection with FDC-SP lentiviral vector. Western blot analysis was used to confirm the expression of FDC-SP. Then the influence of FDC-SP transfection on hPDLC proliferation, osteogenic and fibrogenic phenotype expression was evaluated at the mRNA and protein level. Procollagen type I c-peptide production was measured and alizarin red staining was then conducted to demonstrate effect of FDC-SP on functional differentiation. We found that hPDLCs could be successfully transfected with FDC-SP. Cell proliferation and cell cycle tests indicated that transfection with FDC-SP did not affect hPDLC proliferation. Moreover, according to real-time PCR and Western blot results, expression levels of type 1 collagen alpha 1, type 1 collagen alpha 2 and type 3 collagen were upregulated while that of osteocalcin, osteopontin, and bone sialoprotein were downregulated in FDC-SP transfected cells. In addition, hPDLCs overexpressing FDC-SP exhibited higher PIP production than the controls. Our findings demonstrate that transfection with FDC-SP has negligible adverse effect on proliferation of hPDLCs and imply the biological function of FDC-SP as a fibroblastic phenotype stabilizer by inhibiting hPDLCs differentiation into mineralized tissue-forming cells, thus regulating regeneration in periodontal tissue engineering. J. Cell. Biochem. 115: 940–948, 2014.

KEY WORDS: HUMAN PERIODONTAL LIGAMENT CELLS; FOLLICULAR DENDRITIC CELL SECRETED PROTEIN; TRANSFECTION; PHENOTYPE; PERIODONTAL TISSUE ENGINEERING

Periodontitis is considered as a chronic inflammation occurring in the deep periodontal tissues [Page et al., 1997]. Millions of people are afflicted with highly advanced periodontitis that causes extensive loss of the tooth-supporting periodontal tissues, such as alveolar bone, cementum and periodontal ligament (PDL) [Pihlstrom et al., 2005]. The ultimate goal of periodontal regeneration is the restoration of the lost periodontal tissues. Up to now, approaches to regenerate periodontal tissues have focused almost exclusively on

Abbreviations: PDL, periodontal ligament; PDLCs, periodontal ligament cells; FDC-SP, follicular dendritic cell secreted protein; MOI, multiplicity of infection; hPDLCs, human periodontal ligament cells; SPF, S-phase fraction; PI, proliferation index; COLIA1, type 1 collagen alpha 1; COLIA2, type 1 collagen alpha 2; COLIII, type 3 collagen; OCN, osteocalcin; OPN, osteopontin; BSP, bone sialoprotein.

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Lin Xiang and Li Ma contributed equally to this work.

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regenerating the lost alveolar bone [Akizuki et al., 2005; Hosoya et al., 2008; Hiraga et al., 2009]. However, reconstruction of the periodontal tissues is not a simple aspect just focusing one tissue but involves at least three quite specific and complicated tissues. Generally, restoration of the cementum is temporally and spatially regulated by cell signaling genes and proteins in a relatively precise manner [Ivanovski et al., 2007; Wu et al., 2009]. In addition, the PDL is a highly specialized connective tissue located between the alveolar bone and tooth root, once it is severely destructed by periodontal diseases, its regeneration is known to be a challenge to dentists and dental scientists [Fujii et al., 2006].

With its rapid development, periodontal tissue engineering has come into focus as a promising approach for the regeneration of periodontal tissues including alveolar bone, root cementum, and periodontal ligament [Bartold et al., 2006; Yamada et al., 2006; Healy and Guldberg, 2007; Pioletti et al., 2007]. Several studies have suggested that periodontal ligament cells (PDLCs) which consist of heterogeneous cell populations have the capacity to differentiate into osteoblasts, fibroblasts and cementoblasts due to their mesenchymal stem cell-like properties with multilineage differentiation potential [Seo et al., 2004; Nagatomo et al., 2006; Silvério et al., 2010]. It is demonstrated that, amongst periodontal tissue cells, only PDLCs have the potential to regenerate the lost periodontal attachment [Nyman et al., 1982a,b]. However, the characteristics and intrinsic functions of PDLCs are not yet fully understood.

Follicular dendritic cell secreted protein (FDC-SP), is a novel secretary protein that was first identified in primary follicular dendritic cells isolated from human tonsils [Marshall et al., 2002]. Previous studies have reported that FDC-SP had similar molecular properties to statherin, a protein exists in saliva which plays important roles in preventing Ca precipitation [Nakamura et al., 2005]. Both of the two molecules are small in size (43 and 68 residues for statherin and FDC-SP) and possess a proline-rich region in their C-terminal half. Moreover, these regions show obvious hydrophobicity in contrast to that of N-terminal hydrophilic regions. Considering these structural similarities, we hypothesized that specific roles for FDC-SP exist and that further experiments looking for functional similarities between FDC-SP and statherin would be very informative such as the inhibition of Ca precipitation. To our interest, a biomolecular study has suggested that the expression of FDC-SP may be associated with PDL phenotype expression [Nakamura et al., 2005], thus it is assumed that FDC-SP may promote differentiation of periodontal ligament cells towards a more fibrogenic phenotype while inhibiting their osteogenic differentiation. However, the intrinsic mechanism of FDC-SP in the expression of PDL phenotype remains still unclear.

In the present study, we first constructed a recombinant lentiviral vector with stable and effective expression of FDC-SP. To further investigate the effect of this lentiviral vector on the phenotype expression of human periodontal ligament cells (hPDLCs), we evaluated biological characteristics including osteogenic and fibrogenic potentials of the transfected hPDLCs, aiming to provide valuable cell source for the regeneration of periodontal tissues in periodontal tissue engineering.

MATERIALS AND METHODS

Informed consent of these studies had been obtained from patients, and the study protocol was approved by the Ethics Committee of Sichuan University.

CELL CULTURE

Premolars extracted from healthy voluntary donors that between 12 and 14 years of age were used. Briefly, PDL tissues were separated from the mid-third of the roots and were minced into pieces of small size. After a 30-min enzymatic digestion (0.05% trypsin and 0.15% collagenase; Sigma, St Louis, MO) and centrifugation, single cells in suspension were obtained and then cultured in Dulbecco′s modified Eagle′s medium (DMEM; Gibco, Carlsbad, CA), containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 200 mM L-glutamine (Invitrogen, Life Technologies Co.), 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate, at 37°C with 5% CO_2. Cell culture was continued with medium changes every 3 days until PDL cells reached subconfluency. hPDLCs at passage 2–4 were used for following experiments.

IDENTIFICATION OF HUMAN PERIODONTAL LIGAMENT CELLS

Cells obtained following the third passage were identified with immunocytochemical staining for vimentin and cytokeratin. hPDLCs were fixed with 4% paraformaldehyde for 15 min at room temperature, and then permeabilized with 0.25% Triton X-100 for 8 min followed by blocking with 1% bovine serum albumin (BSA, Sigma). After that, cells were incubated with primary antibody to vimentin or cytokeratin overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. Finally, the diaminobenzidine (DAB) kit was used to develop the color and the slides counterstained with hematoxylin. Secondary antibody was incubated in the absence of primary antibody as negative controls.

FDC-SP GENES TRANSFECTION

Firstly, we retrieved the human FDC-SP gene sequence from GeneBank (NM_152997) and then designed the following primers: FDC-SP–F: CGGGATCCCGCCACCATGAAGAAAGTTCTCCTCC and FDC-SP–R: CGGGTACCGGTACCTTTTCGCTAGGAAGGGGAG (Jierui Bio Co., China). At the same time, we used restriction endonucleases Age I (NEB, USA) and BamH I (NEB, USA) to digest the pGC-FU-3FLAG-IRES-Puromycin vector (GENECHEM Co., Shanghai, China). After that, the FDC-SP insert and linearized plasmid were joined by T4-DNA ligase (NEB, USA) to construct pGC-FU-FDC-SP. The recombinant pGC-FU-FDC-SP was then characterized by restriction endonuclease digest, PCR, and sequencing analysis.

Lentiviral vector containing FDC-SP was firstly transfected into the packaging cells 293T (GENECHEM Co.) to obtain high-level lentiviral particles packed in culture supernatant. After that, 2×10^5 hPDLCs were incubated in a 25 cm^2 culture flask with 1:1 mix of viral supernatant/growth medium with 5 μg/ml polybrene (Sigma) for 10 h. After transfection, the cells were cultured in normal DMEM medium containing 10% FBS for 96 h. FDC-SP expression levels were detected by Western blot analysis. Three different groups were designed in the following tests, including negative control

(untransfected cells), empty vector control and FDC-SP overexpression groups. Cells in these groups were applied to further research experiments.

EXPRESSION OF FDC-SP IN TRANSFECTED CELLS

hPDLCs collected in the three groups were subjected to lysis buffer (Keygen total protein extraction kit, Keygen Biotech, China). After centrifugation, the cytosolic fraction was collected as the supernatant, followed by a bicinchoninic acid (BCA) assay to detect the samples' concentration. After boiling for 5 min, 50 μg protein of each group was applied to SDS–PAGE (10% polyacrylamide gel) at 80 V for 40 min and 100 V for 80 min. Then the target gel bands were cut and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA). After blocking, the membranes were probed with 1:200 dilutions of the anti-FDC-SP antibody (Santa Cruz Biotechnology) overnight, followed by the addition of HRP-conjugated secondary antibody (diluted 1:5,000). Reactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA). The density of the bands was computer analyzed by a densitometer (Quantity One, Bio-Rad, USA).

CELL PROLIFERATION ASSAY

Cell proliferation was analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay according to the cell proliferation kit manual (Sigma). Optical density (OD) values of the cells were measured on a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA) at 492 nm. Three parallel replicates were read for each sample.

CELL CYCLE ASSAY

Cells in different groups were collected with trypsin. Then, a 5-min centrifugation at 2,000*g* was conducted twice to wash the collected cells with PBS, followed by fixation with 70% (w/v) ice-cold ethanol at 4°C overnight. hPDLCs were washed with PBS and then centrifuged at 1,000*g* for 8 min. To stain the cells, 100 μl of RNAase A (Keygen Cell Cycle Detection Kit, Keygen Biotech) was added at 37°C for 30 min, followed by incubation with 400 μl of propidium iodide (PI) (Keygen Cell Cycle Detection Kit, Keygen Biotech). Then a flow cytometry (Beckman Coulter, Indianapolis, IN) was used to analyze cell cycle in each sample.

The S-phase fraction of total cells (SPF) in each sample was calculated according to the formula:

$$\mathrm{SPF}(\%) = \frac{\mathrm{S}}{\mathrm{G_0/G_1 + S + G_2/M}} \times 100.$$

The proliferation index (PI) was calculated according to the formula:

$$\mathrm{PI}(\%) = \frac{\mathrm{S + G_2/M}}{\mathrm{G_0/G_1 + S + G_2/M}} \times 100.$$

RNA ISOLATION AND REAL-TIME PCR ANALYSIS

Total RNA from each incubation was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) at days 4, 7, and 14, according to the manufacturer's instructions. The RNA was then treated with DNase and cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara Bio, Inc., Shiga, Japan). Each real-time PCR was conducted in triplicate in a 20 μl reaction mixture and performed using an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, USA). Calculations of relative gene expression levels were performed according to the $2^{-\Delta\Delta Ct}$ method, and presented as fold increase relative to the control group. The primer sequences for type 1 collagen alpha 1 (COLIA1), type 1 collagen alpha 2 (COLIA2), type 3 collagen (COLIII), osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) are listed in Table I and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

WESTERN BLOT ANALYSIS

Whole-cell protein extracts were obtained as the method described previously. After boiling for 5 min, 20–25 μl of the lysate (50 μg of protein) was applied to 10% SDS–PAGE at 80 V for 40 min and 100 V for 80 min. Then the separated proteins were transferred electrophoretically onto PVDF membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1 h in 0.1% Tween-20 TBS (TBS-T) containing 5% skim milk, then incubated overnight at 4°C with anti-COLIA1 (1:400, MyBioSource), anti-COLIA2 (1:400, MyBioSource), anti-COLIII (1:200, Enzo Life Sciences), anti-OCN (1:1,000, Abcam), anti-OPN (1:200, Santa Cruz Biotechnology) and anti-BSP antibody (1:1,000, Cell Signaling Technology). The membranes were washed with TBS-T three times for 5 min, and then incubated for 1 h with HRP-conjugated secondary antibody (1:5,000). Reactive bands were visualized using an ECL kit (Millipore, Billerica, MA). The density of the bands was computer analyzed by a densitometer (Quantity One, Bio-Rad).

PROCOLLAGEN TYPE I C-PEPTIDE (PIP) MEASUREMENT

To investigate the effect of FDC-SP overexpression on hPDLC fibroblast activity, we conducted procollagen type I C-peptide (PIP) production assay. PIP is the C-terminal peptide of procollagen, which

TABLE I. Specific Primers for Real-Time PCR

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
Type 1 collagen alpha 1	CACTGGTGCTAAGGGAGAGC	CTCCAGCCTCTCCATCTTTG
Type 1 collagen alpha 2	TAATGGGGAAGCTGGATCTG	GTCCCTGAGCACCATTGTTT
Type 3 collagen	CCTCCAACTGCTCCTACTCG	CGGGTCTACCTGATTCTCCA
OCN	GAGGGCAGCGAGGTAGTGAA	GATGTGGTCAGCCAACTCGTC
OPN	TTCCAAGTAAGTCCAACGAAAG	GTGACCAGTTCATCAGATTCAT
BSP	AGGAGGAGGAAGAAGAGGAGACT	CATAGCCCAGTGTTGTAGCAGA
GAPDH	CTTTGGTATCGTGGAAGGACTC	GTAGAGGCAGGGATGATGTTCT

is cleaved away at the collagen triple helix formation step and the amount of PIP is known to represent type 1 collagen production. According to the manufacture's instruction, 10^5 cells were seeded in six-well plates and then cultured in serum free DMEM medium at subconfluence. After 24 h, culture supernatants were collected for procollagen PIP measurement. PIP enzyme immunoassay (EIA) kit (Takara Bio, Inc.) was used to detect PIP concentration. All assays were performed in triplicate and each experiment was repeated at least three times. The production of PIP was adjusted as its concentration (ng/ml) per 10^5 cells.

ALIZARIN RED STAINING

The presence of mineral nodule formation in the cell cultures was determined by alizarin red (Sigma-Aldrich Co.) staining after 3 weeks of osteogenic induction. Briefly, the hPDLCs in six-well culture dishes were rinsed with cold PBS and then fixed with 70% ethanol for 1 h. Following a rinse with PBS, the cells were stained with 40 mM alizarin red (pH 4.2) for 30 min at room temperature. The density of mineral nodules was quantified using QUANTITY ONE software (Bio-Rad). This experiment was repeated three times.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS 17.0 (SPSS, Inc., Chicago, IL). All assays were performed in triplicate and each experiment was repeated at least three times. The results were presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) and Newman–Keuls Student's *t*-test. *P*-Value of <0.05 was considered statistically significant.

RESULTS

ISOLATION, CULTURE, AND IDENTIFICATION OF hPDLCs

Primary cultured hPDLCs displayed a representative fibroblast-like morphology (Fig. 1A). After passage 3, the plasma dominant staining for vimentin, and a negative staining for cytokeratin confirmed the mesenchymal origin of the cells (Fig. 1B,C).

EXPRESSION OF FDC-SP IN TRANSFECTED CELLS

To verify the expression of FDC-SP in transfected hPDLCs, Western blot analysis was performed. After 72 h transfection via MOI 20, FDC-SP overexpression group showed obvious expression of FDC-SP protein. However, FDC-SP expressions in other two control groups (negative control and empty vector control) were almost undetectable (Fig. 2).

EFFECT OF FDC-SP TRANSFECTION ON CELL PROLIFERATION

The cell proliferation evaluated by MTT method, was shown in Figure 3. At day 1, we observed no significant difference between the experimental and two control groups ($P > 0.05$). To each group, the OD value increased with time. However, there were no significant differences among the three groups at days 3, 5, and 7 ($P > 0.05$). To further confirm the effect of FDC-SP transfection on cell proliferation, its impact on cell-cycle was assessed by flow cytometry. As shown in Figure 4, the SPF parameter (Fig. 4A) of untransfected hPDLCs was a little higher than that of empty vector transfected cells, followed by hPDLCs transfected with FDC-SP. As for PI value, FDC-SP overexpression group was slightly lower than the two controls (Fig. 4B). But there were no significant differences among the three groups concerning the two cell-cycle parameters ($P > 0.05$). Taken together, these observations were in accordance with MTT results, which indicated that transfection with FDC-SP via MOI 20 had negligible adverse effect on cell proliferation. For reference purposes, the DNA content histograms of different groups were shown in Figure 4C. Proliferation activity was reflected by the cell cycle distribution in the logarithmic growth phase.

REAL-TIME PCR RESULTS

To evaluate the effect of FDC-SP transfection, the expression of specific fibrogenic markers (COLIA1, COLIA2, COLIII) and osteogenic phenotypes (OCN, OPN, BSP) were examined with real-time PCR at days 4, 7, and 14. We noted that from 4 to 14 days, the COLIA1 gene expression was higher in FDC-SP transfected hPDLCs than in the untransfected and empty vector transfected cells ($P < 0.05$; Fig. 5A), while for COLIA2, there were no significant differences among the

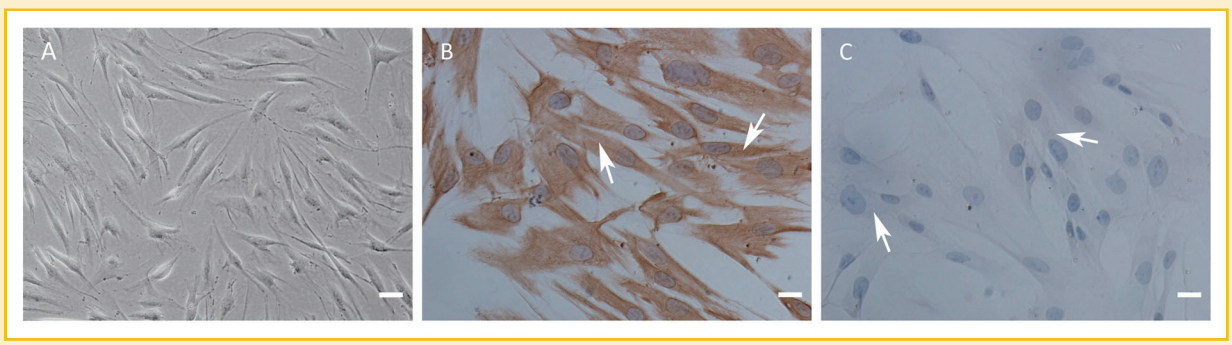

Fig. 1. Primary cultures and identification of hPDLCs. A: hPDLC primary cultures maintained in basic culture medium were observed by phase-contrast microscopy. B,C: Immunocytochemical staining results. The cells after passage 3 illustrated positive for vimentin (B) (arrows) and negative for cytokeratin (C) (arrows), confirming that they came from mesoderm. Scale bar = 200 μm for (A), 50 μm for (B) and (C). Representative photomicrographs are shown for three independent experiments.

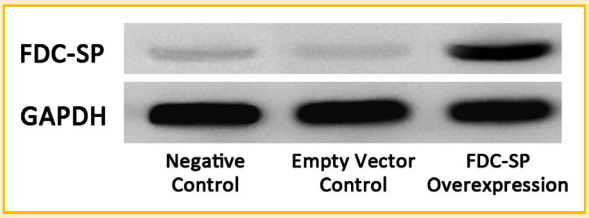

Fig. 2. Expression of FDC-SP after 72 h transfection via MOI level of 20. Cells in FDC-SP overexpression group showed obvious expression of FDC-SP protein. However, FDC-SP expressions in other two groups (negative control and empty vector control) were almost undetectable. Similar data were obtained from three independent experiments.

three groups at each time point ($P > 0.05$; Fig. 5B). Transfection with FDC-SP also enhanced the expression level of COLIII gene at 14 days ($P < 0.05$; Fig. 5C). For osteogenic phenotypes, dramatically lower expression of these genes was observed in FDC-SP overexpression group. For example, OCN, a marker of terminal osteogenic differentiation, demonstrated decreased mRNA expression at days 7 and 14 ($P < 0.05$; Fig. 5D). The FDC-SP transfection also promoted a significant decrease of OPN (Fig. 5E) and BSP (Fig. 5F) gene expression over the time ($P < 0.05$). For each phenotype, there was no significant difference between the two control groups ($P > 0.05$). Collectively, these data indicated that transfection with FDC-SP upregulated fibrogenic phenotype expression, and down-regulated the expression of osteogenic markers in hPDLCs.

WESTERN BLOT ANALYSIS

To examine the effect of FDC-SP on fibrogenic and osteogenic phenotype expression of hPDLCs, Western blot analysis was performed. The results suggested that cells transfected with lentiviral vector containing FDC-SP showed increased expression of fibrogenic phenotypes, including an increase of COLIA2, COLIII, and a significant elevation of COLIA1. However, osteogenic phenotype expression of OCN, OPN, and BSP were downregulated compared with other two groups (Fig. 6).

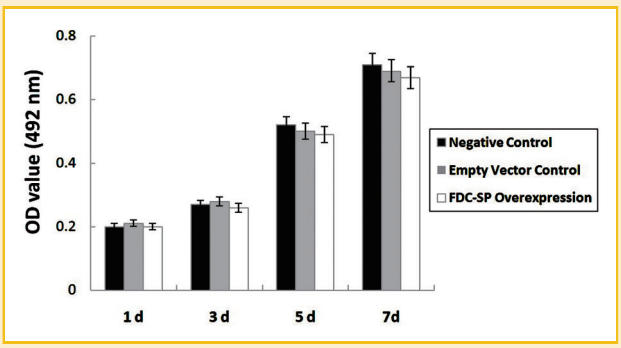

Fig. 3. Effects of FDC-SP transfection on cell proliferation. At day 1, we observed no significant difference among cells transfected with FDC-SP, empty vector or nothing ($P > 0.05$). To each group, the OD value increased with time. However, there were no significant differences among the three groups at days 3, 5, and 7 ($P > 0.05$). The data are shown as the mean ± SD from three independent experiments. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test.

PIP MEASUREMENT

To confirm the difference in hPDLC fibroblast activity, we chose PIP, which is the c-terminal peptide of procollagen and cleaved away at the collagen triple helix formation step, to represent type 1 collagen production. As demonstrated in Figure 7, cells in FDC-SP over-expression group showed significantly higher levels of PIP than that of negative control and empty vector control groups ($P < 0.05$). There were no significant difference between the two controls ($P > 0.05$).

ALIZARIN RED STAINING

In order to understand more clearly the effect of FDC-SP on the mineralization activity of hPDLCs in vitro, we carried out alizarin red staining experiment after 3 weeks of osteogenic induction to measure calcium nodule formation of hPDLCs in different groups. The results indicated that compared with the two controls, FDC-SP transfection group showed a decreased mineral nodule formation (Fig. 8A), which was in accordance with the quantitative analysis (Fig. 8B). We observed no significant difference between the two control groups ($P > 0.05$).

DISCUSSION

The treatment of periodontal diseases is a major challenge due to difficulties in periodontal tissue regeneration. As the reconstruction of periodontal tissues including at least three quite specific and complicated tissues [Bartold et al., 2006; Yamada et al., 2006; Healy and Guldberg, 2007; Pioletti et al., 2007], an increasing number of researchers have focused on cell-based regeneration, and their studies have reported that PDLCs seem to be a promising cell source that can differentiate into cementoblasts, fibroblasts, and osteoblast [McCulloch and Bordin, 1991; Nohutcu et al., 1997; Bartold et al., 2000; Ishikawa et al., 2009]. Taken together, PDLCs play an important role, not only in the maintenance of periodontium, but also in promoting periodontal regeneration including the reconstruction of alveolar bone, periodontal ligament, and root cementum. Thus, the isolation and characterization of cell phenotypes in the PDLC population is definitely required.

To date, gene therapy has been widely used as treatment of diseases or medical disorders via transfecting therapeutic genes into cells [Wolff et al., 1990; Escors and Breckpot, 2010]. Meanwhile, with its rapid development, gene therapy for periodontal regeneration has attracted a great deal of attention in this field. Jin et al. [2003] utilized ex vivo bone morphogenetic protein-7 (BMP-7) gene transfer to stimulate tissue engineering of alveolar bone wounds, and they found that the osseous lesions treated by target gene delivery demonstrated osteogenesis, cementogenesis, and predictable bridging of the periodontal bone defects. Javed et al. [2011] reviewed that platelet derived growth factor (PGDF) played a significant role in periodontal bone and tissue regeneration via PGDF gene transfection technology. However, the success of gene

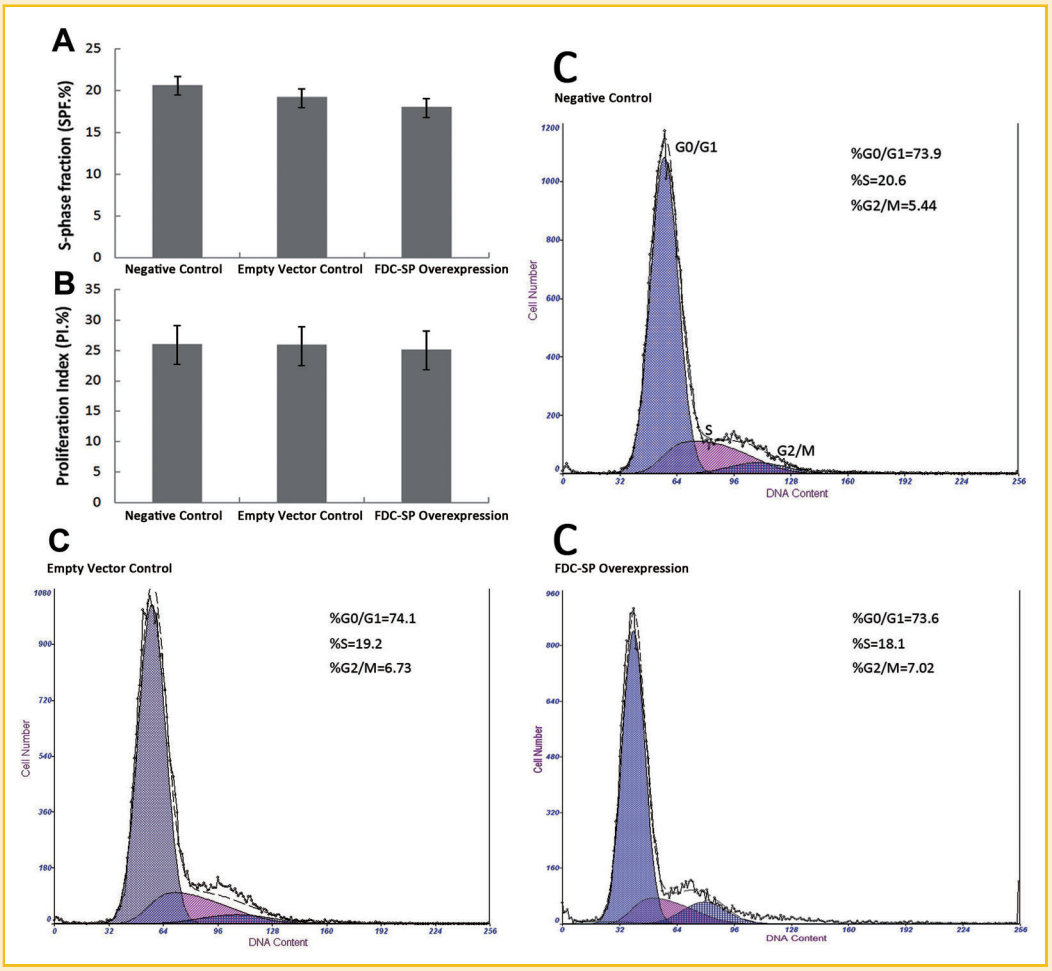

Fig. 4. Results of flow cytometry assay. The results showed that the SPF (A) parameter of untransfected hPDLCs was a little higher than that of empty vector transfected cells, followed by hPDLCs overexpressing FDC-SP. As for PI value, FDC-SP overexpression group was slightly lower than that of the controls (B). But there were no significant differences among the three groups concerning the two cell-cycle parameters ($P > 0.05$). The data are shown as the mean ± SD from three independent experiments. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. The DNA content histograms of different groups were shown. Proliferation activity was reflected by the cell cycle distribution in the logarithmic growth phase (C).

therapy is largely dependent on the development of the gene delivery vector. Lentiviral vector has been considered as an ideal genetic vector system which affords both efficient and stable gene delivery capacity in fundamental biological research, functional genomics and gene therapy [Logan et al., 2002; Katzourakis et al., 2007; Hargrove et al., 2008]. The present study focused on the effect of transfection with FDC-SP on phenotype expression of hPDLCs, aiming to regulate periodontal regeneration in a safe, appropriate and precise manner. Firstly, we constructed a FDC-SP expression recombinant lentiviral vector system and stably transfected it into hPDLCs via MOI 20. As expected, the experimental group has higher FDC-SP protein expression than the empty vector and untransfected control groups, indicating that we successfully overexpressed the target gene in hPDLCs. In addition, FDC-SP expression did not differ between the two controls, which demonstrates that transfection with the empty vector alone does not affect FDC-SP expression in hPDLCs.

For a successful gene therapy, exogenous genes have to be safely, specifically, and efficiently transferred into cellular targets [Niidome and Huang, 2002]. Moreover, delivery systems that confer higher levels of gene expression with minimal toxicity in vivo should be considered by researchers in this field [Rols et al., 1998]. Wen et al. [2012] have applied a lentiviral vector with high and stable expression of enhanced green fluorescent protein (eGFP) to label human periodontal ligament stem cells (PDLSCs). In their study, they conducted MTT assay to evaluate the transfection effect on PDLSCs. Then, based on the results that transfection under appropriate condition had negligible adverse effect on cell proliferation, they performed the follow-up tests at genetic levels. Besides, Guo et al. [2009] have used both MTT and flow cytometry to investigate the impact of a eukaryotic expression vector of HCCR-2 on proliferation status of HepG2 hepatocellular carcinoma cells, as proliferation constitutes one of the most important aspects of cell biological behaviors. According to the previous studies, we performed both the

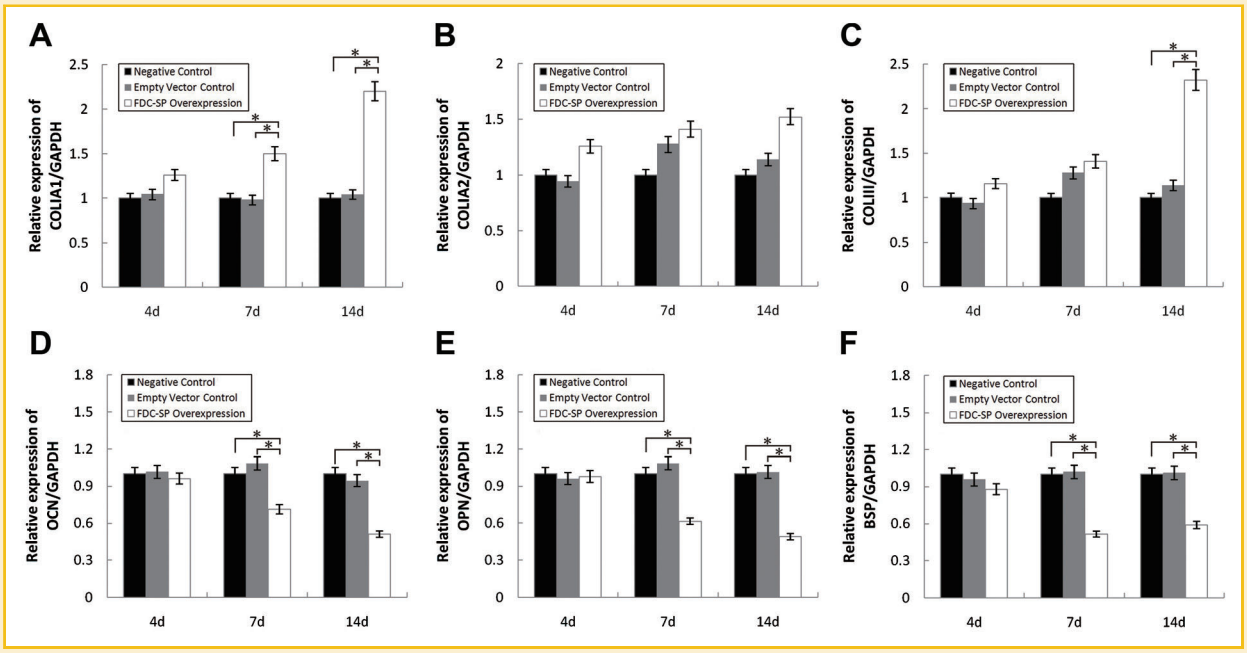

Fig. 5. Real-time PCR results of relative expression levels of COLIA1 (A), COLIA2 (B), COLIII (C), OCN (D), OPN (E), and BSP (F). The expression levels of COLIA1 and COLIII in FDC-SP transfected cells were higher than in untransfected and empty vector transfected cells during the entire observation period ($P < 0.05$). Moreover, transfection with FDC-SP promoted a significant decrease of OCN, OPN, and BSP gene expression over the time ($P < 0.05$). The data are shown as the mean ± SD from three independent experiments. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. $^{*}P < 0.05$ versus control.

MTT test and cell-cycle assay to detect effects of FDC-SP transfection before the following mRNA and protein tests. It was demonstrated that proliferation status of hPDLCs transfected with FDC-SP had no significant difference from the two controls, thus confirming the safety of this transfection method.

It is known that type 1 collagen alpha and type 3 collagen are the main extracellular matrix components of PDL [Romanos et al., 1992], and we found a higher extent of these fibroblastic phenotypes in FDC-SP overexpression group, compared with the cells untransfected or transfected with empty vectors. As for osteobastic markers, OCN is

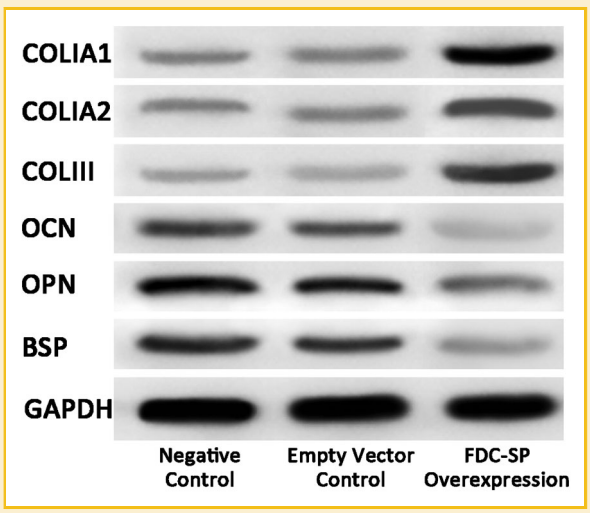

Fig. 6. Effect of FDC-SP transfection on phenotype expression of hPDLCs. The results suggested that transfection with FDC-SP increased expression of fibrogenic phenotypes, including an increase of COLIA2, COLIII, and a significant elevation of COLIA1. However, osteogenic phenotype expression of OCN, OPN, and BSP were downregulated compared with the controls. Similar data were obtained from three independent experiments.

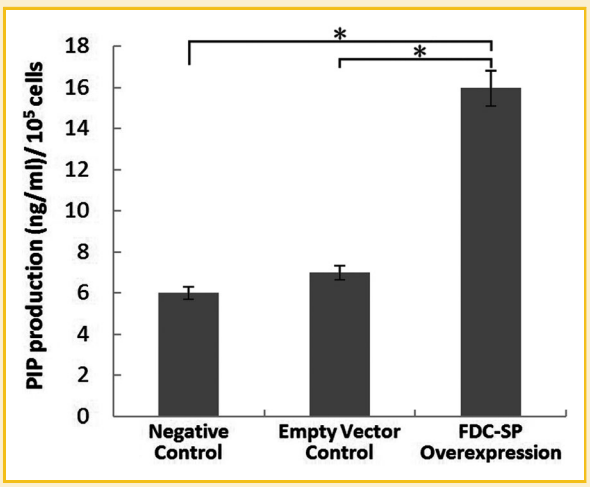

Fig. 7. Effect of FDC-SP transfection on PIP production. Cells in FDC-SP overexpression group showed significantly higher levels of PIP than that of negative control and empty vector control groups ($P < 0.05$). There were no significant difference between the controls ($P > 0.05$). The data are shown as the mean ± SD from experiment performed triplicate on three independent cultures. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. $^{*}P < 0.05$ versus control.

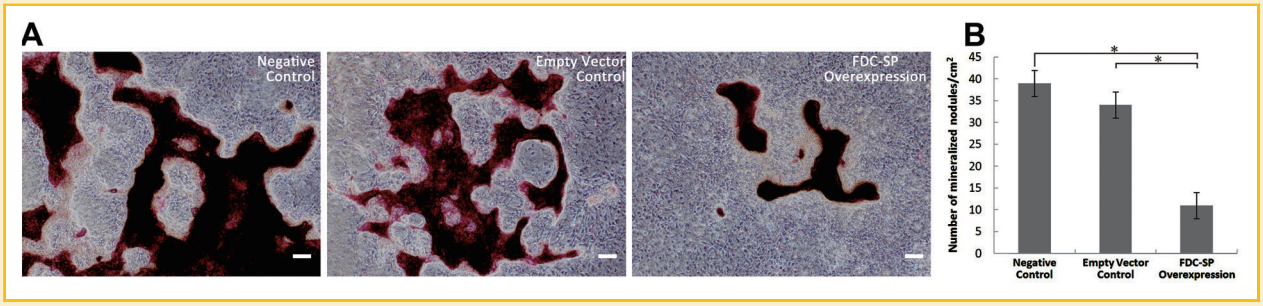

Fig. 8. Results of alizarin red staining. A: Microscopy observation of mineral nodule formation in negative control, empty vector control, and FDC-SP overexpression groups, respectively. Alizarin red-positive nodules appeared more evenly distributed in the two control cultures, whereas blank regions lacking calcium deposits were the most apparent in FDC-SP overexpressing group (Alizarin red staining, $\times 40$, scale bars $= 500\ \mu$m). B: Quantitation of the density of mineral nodules. The results were in accordance with microscopy observation. Each bar represents the mean value of the number of mineralized nodules obtained from three independent experiments carried out in parallel. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. $^{*}P < 0.05$ versus control.

considered as a later marker of bone formation and related to matrix deposition and mineralization [Ducy et al., 1996]; OPN is mainly considered as a relatively earlier marker of osteogenic differentiation, associated with the maturation stage of osteoblasts [Jiang et al., 2009]; whereas BSP, an early marker during osteogenic differentiation process, is considered to participate in the biological behaviors of osteogenic cell populations including migration, proliferation, and differentiation [Bianco et al., 1991]. In the present study, we found the experimental group showed lower expression of osteoblastic markers as a result of FDC-SP transfection. Taken together, the downregulation of osteogenic phenotypes (OCN, OPN, and BSP) expression was accompanied by an upregulated expression of those fibrogenic markers (COLIA1, COLIA2, and COLIII). Similar phenomena were also observed in PIP production assay and alizarin red staining results. FDC-SP overexpressing group showed higher collagen synthesis with a decreased formation of mineralized nodules, which indicated that FDC-SP transfection may be a promotion of fibrogenicity and a remarkable inhibition of osteogenic capability of hPDLCs.

Our study is unique in that few studies focused on the effect of FDC-SP transfection on the heterogeneity of hPDLCs. A recent study has identified this novel protein in human PDL tissue, and suggested that the expression of FDC-SP might be associated with the expression of the PDL phenotype, but the specific influence of FDC-SP on hPDLC phenotype expression remains still unclear [Nakamura et al., 2005]. The present research will help verify if such transfection system could become a suitable method to regulate periodontal regeneration in periodontal tissue engineering. And we believe more studies are necessary to confirm our findings in the next step.

In conclusion, the findings observed in this study suggest that hPDLCs transfected with FDC-SP maintained their basic biological characteristics. FDC-SP transfection did not affect the proliferation of hPDLCs and it may act as a fibroblastic phenotype stabilizer by inhibiting hPDLCs differentiation into mineralized tissue-forming cells. It is expected that hPDLCs transfected with FDC-SP may have a potential application in periodontal regenerative medicine for treatment of periodontal diseases in a safe, appropriate and precise manner.

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