

Odontogenic differentiation of vascular endothelial growth factor-transfected human dental pulp stem cells *in vitro*

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Received September 24, 2013; Accepted May 23, 2014

DOI: 10.3892/mmr.2014.2481

Abstract. Dental pulp stem cells (DPSCs) can be induced towards odontogenic differentiation. Previous studies have shown that vascular endothelial growth factor (VEGF) is able to induce the osteogenic differentiation of cells, but the effectiveness of VEGF in the odontogenic differentiation of DPSCs remains unclear. This study aimed to investigate the effects of lentivirus-mediated human VEGF gene transfection on the proliferation and odontogenic differentiation of human DPSCs *in vitro*. DPSCs were transfected with either lentiviral pCDH-CMV-MCS-EFI-copGFP (pCDH) vector or recombinant pCDH-VEGF vector, and the growth characteristics of the resulting DPSCs/Vector and DPSCs/VEGF were subsequently assessed. The odontogenic differentiation genes of the two groups of cells, including alkaline phosphatase, osteocalcin, dentin sialophosphoprotein and dentin matrix protein 1 (DMP1), were evaluated by quantitative polymerase chain reaction (qPCR). The specific proteins of odontogenic differentiation, including dentin sialoprotein and DMP1, were analyzed by western blotting. DPSCs/VEGF showed similar proliferation characteristics to DPSCs/Vector during the observation period. qPCR results showed that the relative VEGF gene expression was significantly higher in DPSCs/VEGF than that in DPSCs/Vector two days after transfection ($P<0.01$). Similarly, western blot analysis showed that the protein expression levels of VEGF were higher in DPSCs/VEGF than those in DPSCs/Vector. On the first, fourth, eighth and 16th days after lentivirus-mediated transfection, the expression of odontogenic differentiation-specific genes and proteins was

higher in DPSCs/VEGF than that in DPSCs/Vector. These results indicated that lentivirus-mediated VEGF gene transfection promoted the odontogenic differentiation of human DPSCs *in vitro*.

Introduction

Dental pulp stem cells (DPSCs) are primarily isolated from dental pulp tissues (1,2). These cells exhibit multilineage differentiation potential, particularly odontogenic differentiation potential. Previous studies have indicated that DPSCs are capable of differentiating into an odontoblastic lineage *in vitro* and forming ectopic pulp-dentine-like tissue *in vivo* (2-4). However, limitations remain for the direct application of DPSCs in dental tissue regeneration, and optimized tissue engineering strategies are required to improve the odontogenic differentiation capacity of DPSCs (5).

The administration of cytokines is one of the most commonly utilized strategies to strengthen the osteogenic differentiation capacity of cells. Vascular endothelial growth factor (VEGF) is an important cytokine that can promote the osteogenic differentiation of cells. A previous study demonstrated that recombinant human VEGF improved the cell proliferation and alkaline phosphatase (ALP) expression of dental pulp cells (6). VEGF culture medium also promoted the osteogenic differentiation capacity of MC3T3 stem cells by increasing the expression of ALP and osteocalcin (OCN) *in vitro* (7). Compared with control cells, VEGF-treated human periodontal ligament stem cells exhibited a significantly higher ALP activity, which further led to the formation of increased mineralized tissue (8). Furthermore, VEGF culture medium increased ALP expression and calcium accumulation of DPSCs *in vitro* (9). However, there are limitations in the local delivery of cytokines, including the short half-life, large dose requirements, high costs, the necessity for repeated applications and poor expression distribution (10). Gene transfection technology may be a solution to some of these technical caveats. Transient transfection of the VEGF gene into human periosteal cells could promote the expression of ALP, OCN and Collagen I (11). VEGF gene-transfected rat bone marrow stromal cells (BMSCs) were reported to have higher ectopic osteogenesis *in vivo* (12). Another study also confirmed the

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Key words: dental pulp stem cells, vascular endothelial growth factor, gene transfection, odontogenic differentiation

improved osteogenic differentiation capacity of rat BMSCs by lentivirus-mediated VEGF gene transfection (13). Due to the similarity between osteogenic and odontogenic differentiation among DPSCs (14), lentiviral vector-mediated stable transfection of the VEGF gene may be an effective strategy to improve the odontogenic differentiation capacity of DPSCs.

This study aimed to explore the effects of lentivirus-mediated VEGF gene transfection on the odontogenic differentiation of human DPSCs *in vitro*. The proliferation and odontogenic differentiation capacities of VEGF gene-transfected DPSCs (DPSCs/VEGF) were analyzed. The results of this study could enhance the understanding of the biological characteristics of DPSCs/VEGF, and may provide theoretical evidence for the application of lentivirus-mediated VEGF gene transfection in dental tissue engineering.

Materials and methods

Isolation of DPSCs. Patients were recruited for this study from the Guanghua Hospital of Stomatology (Guangzhou, China). All participants provided their informed consent. The study was approved by the Ethical Review Committee of the Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China). DPSCs were isolated from freshly extracted third molars without signs of decay (Fig. 1A). The extracted teeth were thoroughly cleaned, then cut at the cemento-enamel junction using a sterile dental fissure bur. The tissues in the pulp chamber were exposed and gently separated from the crown. The pulp tissues were subsequently minced and digested with 3 mg/ml collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/ml dispase (Boehringer Mannheim, Indianapolis, IN, USA) for 1 h at 37°C. The cells were then passed through a 70- μ m strainer (BD Biosciences, Bedford, MA, USA) to generate a single-cell suspension. The isolated DPSCs were seeded at densities of between 1×10^4 and 1×10^5 /well in six-well plates (Corning, New York, NY, USA) containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen Hong Kong Ltd., Hong Kong, China) supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), 100 μ M L-ascorbic acid-2-phosphate (Wako, Tokyo, Japan), 100 U/ml penicillin-G and 100 mg/ml streptomycin, and were cultured with 5% CO₂ at 37°C.

Differentiation stimulation. The multipotent differentiation potential of the cells was identified by osteogenic and adipogenic differentiation induction (14). Briefly, the cells were exposed to osteogenic medium (DMEM supplemented with 10 nmol/l dexamethasone, 10 mmol/l β -glycerophosphate, 50 μ g/ml ascorbate phosphate, 10 nmol/l 1,25 dihydroxyvitamin D₃ and 10% FBS) and adipogenic medium (DMEM supplemented with 1 μ mol/l dexamethasone, 1 μ g/ml insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine and 10% FBS) for 28 days. Alizarin Red S and Oil Red O reagent were used to visualize the calcium accumulation and oil droplets, respectively.

Flow cytometric analysis. Prior to the conduction of the experiments, the phenotype of the freshly isolated DPSCs was evaluated by flow cytometry (FCM) for the expression of STRO-1/Alexa Fluor 647 (BioLegend, San Diego, CA, USA), cluster of differentiation 146 (CD146)/phycoerythrin

(PE) (BD Pharmingen, San Diego, CA, USA), CD34/PE (BD Pharmingen), CD45/fluorescein isothiocyanate (FITC; BD Pharmingen) and CD24/FITC (BD Pharmingen).

Construction of lentivirus plasmid. The lentivirus vector pCDH-CMV-MCS-EF1-copGFP (pCDH; System Biosciences, Mountain View, CA, USA) with green fluorescent protein (GFP) label was used to visualize the recombinant plasmid expression. The human VEGF primers were designed with Oligo 7.0 software (Molecular Biology Insights, Plymouth, MN, USA) according to the National Center for Biotechnology Information GenBank no. AF486837.1, and were amplified by polymerase chain reaction (PCR). The PCR primers were designed as follows: Forward, GCCGAATTCATG AACTTTCTGCTGTCTTG (the underlined sequence indicates an *Eco*RI site); reverse, GCCGGATCCTCACCG CCTCGGCTTGTCAC (the underlined sequence indicates a *Bam*HI site). For PCR amplification, specific primers were used with following reaction conditions: Pre-denaturation, 95°C for 3 min; 30 cycles of denaturation, 95°C for 15 sec; primer annealing, 55°C for 30 sec; primer extension, 72°C for 1 min and final extension at 72°C for 7 min, prior to storage at 4°C for 10 min. The amplified products were digested using *Eco*RI and *Bam*HI restriction enzymes. The fragment containing human VEGF gene was cloned into the vector to produce the pCDH-CMV-MCS-EF1-copGFP-VEGF recombinant lentiviral plasmid, referred to as pCDH-VEGF.

Gene transfection. 293FT cells (System Biosciences) were cultured in DMEM with 10% FBS, prior to being seeded (1.2×10^6 cells) into a 10 cm dish one day before transfection. The recombinant plasmid pCDH-VEGF, packaging plasmid psPAX.2 (Cyagen, Guangzhou, China) and envelope plasmid pMD2.G were co-transfected into the 293FT cells using Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) (15,16). The liquid supernatant was collected after 48 h, centrifuged at $1,000 \times g$ at 37°C for 10 min and filtered using a 0.2- μ m syringe (Millipore, Bedford, MA, USA). The third-passage DPSCs were infected with the supernatant to acquire the recombined DPSCs/VEGF. By use of the same protocol, the blank pCDH vector was used to infect DPSCs to construct DPSCs/Vector as a negative control. Through analyzing the percentage of GFP fluorescence, the transfection ratio was identified by ImageJ software (NIH, Bethesda, MD, USA). VEGF expression from the DPSCs/Vector and DPSCs/VEGF was assessed using a quantitative PCR (qPCR) and western blot analysis two days after transfection. The DPSCs/Vector and DPSCs/VEGF were seeded in 25-cm² culture flasks containing DMEM supplemented with 10% FBS, and cultured with 5% CO₂ at 37°C. The culture medium was changed at 24-h intervals.

Cell proliferation assessment. DPSCs/Vector and DPSCs/VEGF were seeded onto 96-well plates at the density of 2×10^3 cells/well and cultured in DMEM with 10% FBS. The cell proliferation was evaluated using a Cell Counting kit 8 (CCK8) according to the manufacturer's instructions (Dojindo Molecular Technologies, Tokyo, Japan). The CCK8 reduction/attenuation values of the wells were measured by spectrophotometer at an optical density (OD) of 520 nm

Table I. Human-specific primer sequences used for quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	Size (bp)
VEGF	Forward: CTACCTCCACCATGCCAAGT Reverse: AGCTGCGCTGATAGACATCC	104
ALP	Forward: CTATCCTGGCTCCGTGCTC Reverse: GCTGGCAGTGGTCAGATGTT	100
OCN	Forward: CTCACACTCCTCGCCCTATT Reverse: TTGGACACAAAGGCTGCAC	107
DSPP	Forward: GCCACTTTCAGTCTTCAAAGAGA Reverse: GCCCAAATGCAAAAATATGTAA	130
DMP1	Forward: AAAATTCTTTGTGAACCTACGGAGG Reverse: GAGCACAGGATAATCCCCAA	94
GAPDH	Forward: AAGGTGAAGGTCGGAGTCAA Reverse: AATGAAGGGGTCATTGATGG	108

VEGF, vascular endothelial growth factor; ALP, alkaline phosphatase; OCN, osteocalcin, DSPP, dentin sialophosphoprotein; DMP1, dentin matrix acidic phosphoprotein 1; bp, base pairs.

(BioPhotometer Plus; Eppendorf, Hamburg, Germany). The tests were performed on the first, second, fourth and eighth days after seeding. The assay of each well was repeated in triplicate.

qPCR analysis. On the first, second, fourth, eighth and 16th days after transfection, the total RNA of the DPSCs/Vector and DPSCs/VEGF was isolated using TRIzol® (Invitrogen Life Technologies), and the quantity of extracted RNA was evaluated by a spectrophotometer (BioPhotometer Plus; Eppendorf). For each sample, 2 µg RNA was used to synthesize the cDNA of each gene using the RevertAid™ First Strand cDNA Synthesis kit (Thermo Scientific™ Molecular Biology, Shenzhen, China). The qPCR reaction mix used was iQSYBR Green Supermix (BioRad, Hercules, CA, USA) and the reaction was controlled by the spectrofluorimetric thermal iCycler iQ5 (Bio-Rad). VEGF gene and four odontogenic differentiation genes, ALP, OCN, dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1), were assessed. For PCR amplification of the cDNA of each gene, an initial amplification using gene-specific primers (Table I) was performed with a denaturation step (95°C for 3 min), followed by 39 cycles of denaturation (95°C for 10 sec), primer annealing (55°C for 10 sec) and primer extension (72°C for 30 sec). The amplification efficiency of these genes was determined relative to the housekeeping gene, GAPDH. Each sample was performed in triplicate.

Western blot analysis. DPSCs/Vector and DPSCs/VEGF were seeded on six-well plates with 5x10⁴/well density. On the first, second, fourth, eighth and 16th days after transfection, the cells were harvested for western blot analysis. The culture media of the DPSCs/Vector and DPSCs/VEGF were removed from the six-well plates and the cells were washed with 2 ml phosphate-buffered saline at 4°C for 1 min. The total proteins of these cells were extracted with 100 µl radio-immunoprecipitation assay buffer (BioTeke, Beijing, China) and 1 µl phenylmethanesulfonyl fluoride phosphatase inhibitor, and then collected by centrifugation

at 12,000 rpm for 5 min. A bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to detect the total protein concentrations of the two groups of cells. Equal quantities of protein from the samples were then subjected to 12% SDS-PAGE for electrophoresis. Thereafter, the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) non-fat milk for 2 h at room temperature, and incubated respectively with 1:500 mouse anti-human VEGF antibody (Abcam, MA, USA), rabbit anti-human DMP1 antibody and goat anti-human dentin sialoprotein (DSP) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. The membranes were subsequently incubated with secondary antibody at 37°C for 2 h. GAPDH was used as the internal control. The resultant films were visualized by an Enhanced Chemiluminescence Western Blotting Detection system (Millipore).

Statistical analysis. The data are presented as the mean ± standard deviation. The two-way analysis of variance (ANOVA) test was used to analyze the differences between the DPSCs/Vector and DPSCs/VEGF (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Morphological, multipotent differentiation and phenotypic characteristics of DPSCs. DPSCs were isolated from third molars and a cellular suspension was obtained (Fig. 1A and B). The majority of the adherent DPSCs exhibited a spindle-like appearance with extending cytoplasmic processes (Fig. 1C). Following *in vitro* culture for 10 days, the first passage of DPSCs reached complete confluence (Fig. 1D). Calcium accumulation (Fig. 1E) and oil droplets (Fig. 1F) were observed in DPSCs subsequent to differentiation induction for 28 days. The

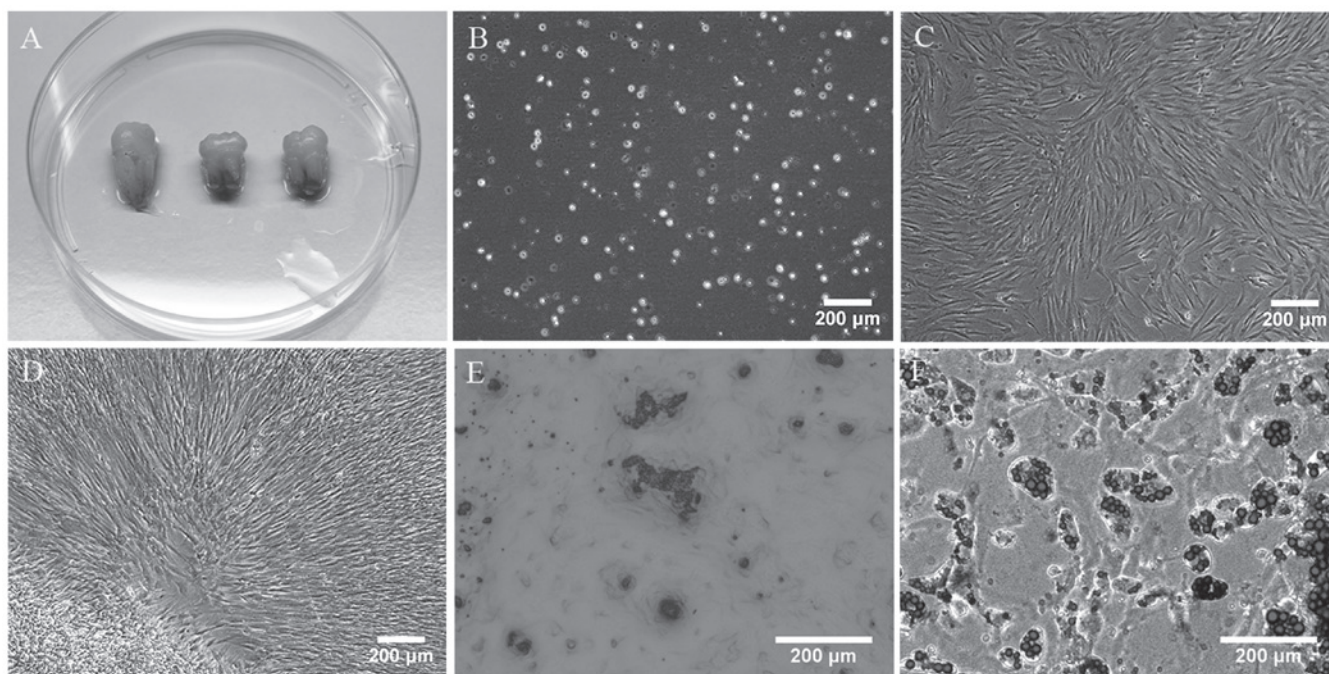


Figure 1. Isolation and identification of DPSCs. (A) DPSCs were isolated from healthy third molars. (B) Dental pulp tissue was digested, and a DPSC suspension was obtained. (C) Following three days in culture, DPSCs exhibited a spindle-like appearance with 2-4 extending cytoplasmic processes. (D) Following 10 days in culture, DPSCs reached ~100% confluency. (E) Alizarin Red S staining of DPSC cultures on the 28th day of osteogenic induction. (F) Oil red O staining of DPSC cultures on the 28th day of adipogenic induction. DPSCs, dental pulp stem cells.

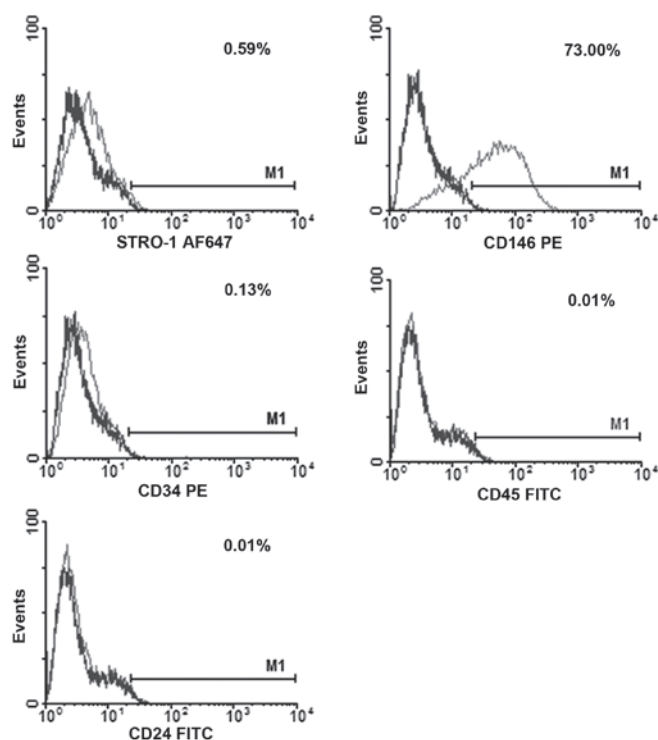


Figure 2. Flow cytometry of DPSCs. Representative flow cytometry samples for DPSC characterization are shown. The expression levels of STRO-1, CD146 and CD34 were 0.59, 73.00 and 0.13%, respectively. CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; DPSCs, dental pulp stem cells.

FCM results showed that the expression of STRO-1, CD146, CD34, CD45 and CD24 was 0.59, 73.00, 0.13, 0.01 and 0.01%, respectively (Fig. 2).

Transfection efficiency. The DPSCs/Vector and DPSCs/VEGF were successfully constructed (Fig. 3). The DPSCs/Vector and DPSCs/VEGF exhibited a spindle-like shape with extending cytoplasmic process, as observed in untransfected DPSCs (Fig. 3A and D). The green fluorescence was detected in the majority of the transfected cells (Fig. 3B and E), and the transfection ratio was ~90% (Fig. 3C and F), analyzed using ImageJ software. The VEGF gene expression was higher in the DPSCs/VEGF (8.22 ± 0.59) than that in the DPSCs/Vector (1.00 ± 0.06) ($P < 0.01$) two days after transfection (Fig. 3G). The western blot analysis also showed that VEGF expression in the DPSCs/VEGF was significantly upregulated, compared with that in the DPSCs/Vector (Fig. 3H).

DPSC proliferation characteristics. A similar proliferation profile was observed for the DPSCs/Vector and DPSCs/VEGF (Fig. 4). The average OD values of the two groups of cells significantly increased from 0.2 to 0.5 during the observation period of eight days ($P < 0.05$). However, larger standard deviations in the OD readings from the DPSCs/VEGF were observed when compared with those from the DPSCs/Vector over time.

Odontogenic differentiation gene expression. The relative ALP gene expression of the DPSCs/Vector and DPSCs/VEGF increased during the first eight days (Fig. 5). The relative ALP gene expression in the DPSCs/VEGF was significantly higher than that in the DPSCs/Vector on the first, fourth, eighth and 16th days after gene transfection ($P < 0.05$). The increasing trend in the relative OCN expression in the DPSCs/Vector was no longer observed on the 16th day after transfection; however, the increasing trend remained in the DPSCs/VEGF. The relative expression of OCN was statistically higher in the

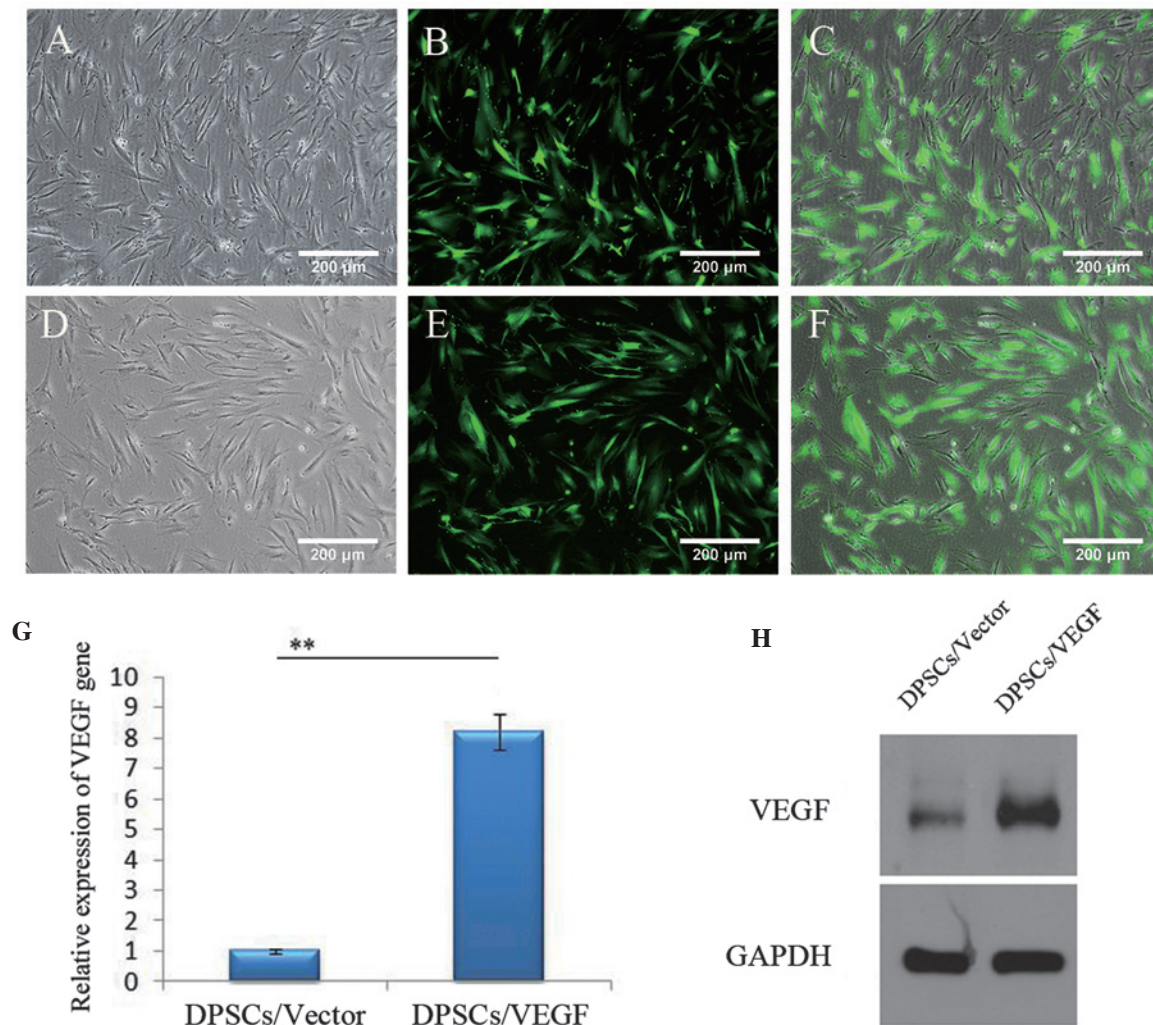


Figure 3. Lentiviral transfection efficiency. (A) DPSCs/Vector. (B) GFP fluorescence in DPSCs/Vector. (C) Merged image of (A) and (B). GFP was expressed in 89% of DPSCs/Vector. (D) DPSCs/VEGF. (E) GFP fluorescence in DPSCs/VEGF. (F) Merged image of (D) and (E). GFP was observed in 91% of DPSCs/VEGF. (G) The relative expression of VEGF gene in DPSCs/Vector and DPSCs/VEGF was assessed by quantitative polymerase chain reaction two days after transfection. The relative expression of VEGF was statistically higher in DPSCs/VEGF than in DPSCs/Vector (** $P<0.01$). (H) VEGF expression in DPSCs/Vector and DPSCs/VEGF was analyzed by western blotting two days after transfection. Error bars represent the mean \pm standard deviation. VEGF, vascular endothelial growth factor; DPSCs, dental pulp stem cells; GFP, green fluorescent protein.

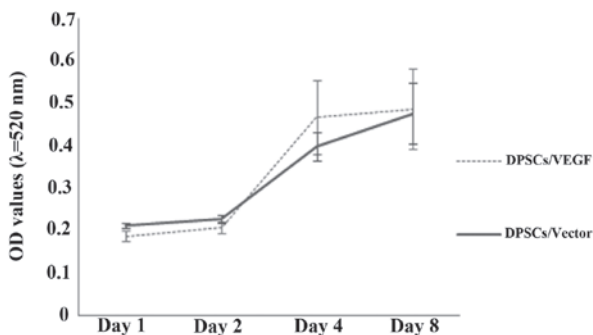


Figure 4. Cell proliferation. The proliferation of DPSCs/Vector and DPSCs/VEGF was analyzed by cell counting kit 8 on the first, second, fourth and eighth days of lentivirus-mediated gene transfection. The OD values of the two groups of cells were comparable during the eight-day observation period. Error bars represent the mean \pm standard deviation. VEGF, vascular endothelial growth factor; DPSCs, dental pulp stem cells; OD, optical density.

DPSCs/VEGF as compared with that in the DPSCs/Vector on the fourth, eighth and 16th days after gene transfection

($P<0.05$). Both the DPSCs/Vector and the DPSCs/VEGF showed an enhancement in DSPP expression over time. The DSPP expression levels were statistically higher in the DPSCs/VEGF than those in DPSCs/Vector at the four time-points ($P<0.01$). Furthermore, the relative expression levels of DMP1 gene in the DPSCs/VEGF were significantly higher than those in the DPSCs/Vector on the first, fourth and 16th days after gene transfection ($P<0.05$). For the expression of ALP, OCN, DSPP and DMP1 genes, the two-way ANOVA showed a significant main effect for the cell types and culture times ($P<0.05$), and the interaction between the cell types and culture times was also significant ($P<0.05$).

Odontogenic differentiation protein expression. Western blot analysis confirmed that the odontogenic differentiation-specific marker DSP was significantly upregulated ($P<0.05$) in the DPSCs/VEGF as compared with the DPSCs/Vector on the first, fourth, eighth and 16th days after transfection, and the odontogenic differentiation-specific marker DMP1 was also significantly upregulated ($P<0.05$) in the DPSCs/VEGF as

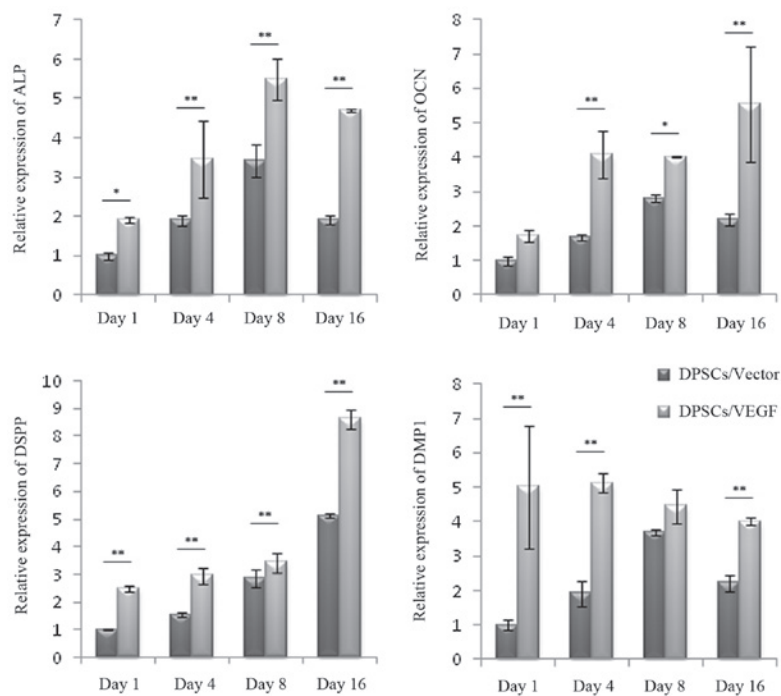


Figure 5. Odontogenic differentiation gene expression. The gene expression of DPSCs/Vector and DPSCs/VEGF was assessed by quantitative polymerase chain reaction on the first, fourth, eighth and 16th days of lentivirus-mediated gene transfection. The relative expression levels of ALP, OCN, DSPP and DMP1 genes were generally upregulated in DPSCs/VEGF as compared with those in DPSCs/Vector. * $P < 0.05$ and ** $P < 0.01$. Error bars represent the mean \pm standard deviation. VEGF, vascular endothelial growth factor; ALP, alkaline phosphatase; OCN, osteocalcin, DSPP, dentin sialophosphoprotein; DMP1, dentin matrix acidic phosphoprotein 1; DPSCs, dental pulp stem cells.

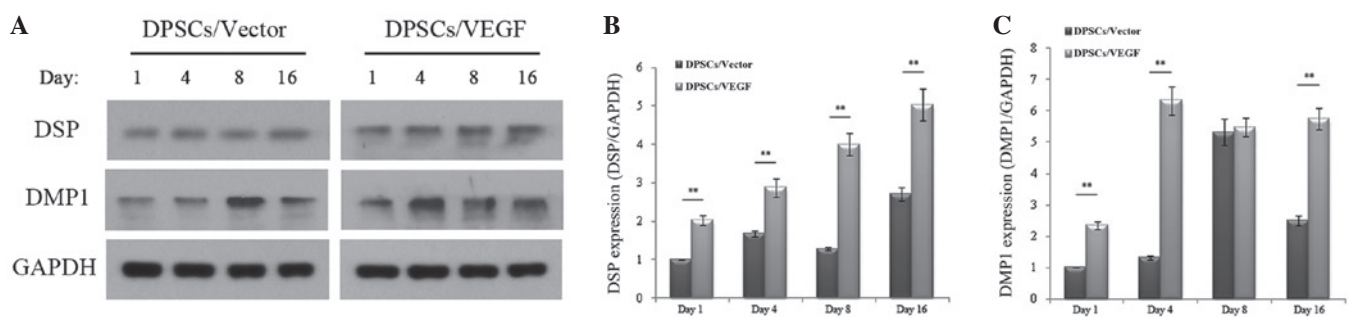


Figure 6. Odontogenic differentiation protein expression. (A) The expression levels of DSP and DMP1 in DPSCs/Vector and DPSCs/VEGF were analyzed by western blotting on the first, fourth, eighth and 16th days of gene transfection. (B) DSP and (C) DMP1 expression was significantly upregulated in DPSCs/VEGF as compared with that in DPSCs/Vector at the four time-points. ** $P < 0.01$. Error bars represent the mean \pm standard deviation. VEGF, vascular endothelial growth factor; DPSCs, dental pulp stem cells; DSP, dentin sialoprotein; DMP1, dentin matrix acidic phosphoprotein 1.

compared with the DPSCs/Vector on the first, fourth and 16th days after transfection (Fig. 6). For the expression of DSP and DMP1, two-way ANOVA showed a significant main effect for the cell types and culture times ($P < 0.05$), and the interaction between the cell types and culture times was also significant ($P < 0.05$).

Discussion

In the present study, evidence from the formation of calcium accumulation and oil droplets following osteogenic and adipogenic induction showed that the isolated DPSCs were multipotent. The expression of STRO-1 and CD146 in the DPSCs was 0.59 and 73.00% respectively, indicating that the isolated cells conformed to the phenotypic characteristics of

DPSCs. STRO-1 has been previously observed in pulp tissues and is regarded as a marker for stromal stem cells, recognizing the trypsin-insensitive epitope on perivascular cells (4). In the majority of previous studies, STRO-1 expression has been observed in DPSCs, with an expression level ranging between 0.46 and 26.14% (4,14,17). CD146 is a stem cell marker identified in adult bone marrow and dental pulp tissue, associated with blood vessels (18). Studies have shown positive expression of CD146 among DPSCs, with a range in expression of between 20.4 and 98.0% (2,14,19,20). CD34 is a marker expressed in the majority of stem and progenitor cells, involved in the maintenance of the plastic state of undifferentiated cells (21). The expression of CD34 in this study was 0.13%, suggesting that undifferentiated cells were present in the DPSC culture. CD45 and CD24 are markers of hematopoietic/endothelial stem

cells (14). Consistent with discussions in a previous review (4), no CD45 and CD24 expression was observed in this study.

In the present study, cell-culture conditions without L-ascorbic acid-2-phosphate and other external supplements following lentivirus-mediated transfection were selected. Cells that are cultured with L-ascorbic acid-2-phosphate have been shown to exhibit a significant improvement in cell proliferation, ALP expression and osteogenic differentiation capacity (22-25). Culturing the DPSCs with L-ascorbic acid-2-phosphate may cause hybrid bias in the analysis and confound the interpretation of the role of the VEGF expressed in the described genetically modified DPSCs.

ALP has been widely used as a marker for differentiated cells producing mineralized matrix (7,14). OCN, a non-collagenous protein, is usually expressed during the late stages of osteogenic/odontogenic differentiation (7,14). In this study, the expression of ALP and OCN was significantly enhanced in the DPSCs/VEGF as compared with that in the DPSCs/Vector. This result was consistent with that from other reports on cells with VEGF overexpression *in vitro* (11,13). The expression of odontogenic differentiation-specific markers, such as DSPP and DMP1 genes, was also enhanced among the DPSCs/VEGF in this study. DSPP is a dentine non-collagenous protein, mainly expressed by odontoblasts in the mineralized nodules and organized structures (14). DMP1 plays an important role in the maturation of ameloblasts, osteoblasts and odontoblasts, as well as in the mineralization of these cells (26). The increased expression of odontogenic differentiation genes identified in this study is consistent with an improvement in the odontogenic differentiation capacity of the DPSCs due to the overexpression of VEGF.

DSP, the amino-terminal part of DSPP, is a specific protein involved in the odontogenic differentiation of DPSCs, and it is always expressed by newly-formed odontoblasts associated with the secretion of pre-dentin matrix (27,28). The expression of DSP occurs prior to the initiation of the dentin mineralization, not yet present in pre-odontoblasts (27). An *in vivo* study has additionally shown that DSP is involved in the initiation of dentin mineralization, but not in the maturation of dentin (29). DSP appeared not only in the odontoblasts of the primary dentin but also in the odontoblast-like cells of reparative dentin. The function of DSP could be associated with the synthesis of the dentin matrix and the conversion of pre-dentin to mineralized dentin (28). In the present study, western blot analysis indicated that DSP expression in the DPSCs/VEGF was significantly higher than that in the DPSCs/Vector at the four selected time-points. Furthermore, DMP1 expression in the DPSCs/VEGF was also significantly higher than that in the DPSCs/Vector at three time-points. These results were consistent with the qPCR data, and therefore further confirmed that VEGF gene transfection improved the odontogenic differentiation of DPSCs.

In conclusion, DPSCs/VEGF were successfully constructed in the present study by lentivirus-mediated VEGF gene transfection of human DPSCs. No significant difference was observed between the DPSCs/VEGF and the DPSCs/Vector with regard to the proliferation characteristics of the cells; however, the DPSCs/VEGF showed a significantly enhanced expression of odontogenic differentiation genes and proteins. The results demonstrate that VEGF gene transfection may be

a strategy to improve the efficacy of DPSCs for odontogenic differentiation. Further evaluation on the effectiveness of the VEGF gene in promoting the odontogenic differentiation of DPSCs *in vivo* is now required.

Acknowledgements

The authors would like to thank Dr Chenfei Zhang (Clinical Associate Professor in Endodontics, The University of Hong Kong) for his help in the initial phases of this study and the staff and postgraduate students of the Guanghua School of Stomatology, Sun Yat-sen University, who helped in the collection of clinical samples and the laboratory analysis. This study was supported by the Guangdong Medical Science Research Fund (B2012142) and the National Natural Science Foundation of China (81170932).

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