Osteo-/odontogenic differentiation of BMP2 and VEGF gene-co-transfected human stem cells from apical papilla

WEN ZHANG, XIAOLEI ZHANG, JUNQI LING, XI WEI and YUTAO JIAN

Guangdong Key Laboratory of Stomatology, Department of Operative Dentistry and Endodontics, Guanghua School and Hospital of Stomatology, Sun Yat-sen University, Guangzhou, Guangdong 510055, P.R. China

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Abstract. Stem cells from apical papilla (SCAP) possess clear osteo-/odontogenic differentiation capabilities, and are regarded as the major cellular source for root dentin development. Bone morphogenetic protein 2 (BMP2) and vascular endothelial growth factor (VEGF) serve pivotal roles in the modulation of tooth development and dentin formation. However, the synergistic effects of BMP2 and VEGF on osteo-/odontogenic differentiation of SCAP remain unclear. The current study aimed to investigate the proliferative and osteo-/odontogenic differentiating capabilities of BMP2 and VEGF gene-co-transfected SCAP (SCAP-BMP2-VEGF) in vitro. The basic characteristics of the isolated SCAP were identified by the induction of multipotent differentiation and by flow cytometry. Lentiviral vector-mediated gene transfection was conducted with SCAP in order to construct blank vector-transfected SCAP (SCAP-green fluorescent protein), BMP2 gene-transfected SCAP (SCAP-BMP2), VEGF gene-transfected SCAP (SCAP-VEGF) and SCAP-BMP2-VEGF. The Cell Counting Kit 8 assay was used to analyze the proliferative capacities of the four groups of cells. The expression of osteo-/odontogenic genes and proteins in the cells were evaluated by reverse transcription-quantitative polymerase chain reaction and western blotting. The mineralized nodules formed by the four group cells were visualized by alkaline phosphatase (ALP) staining. Among the four groups of cells, SCAP-VEGF was demonstrated to exhibit increased proliferation, and SCAP-BMP2-VEGF exhibited reduced proliferation during eight days observation. SCAP-BMP2-VEGF exhibited significantly increased expression levels of ALP, osteocalcin, dentin sialophosphoprotein, dentin matrix acidic phosphoprotein gene 1 and dentin sialo-protein than the other three groups at the majority of the time points. Furthermore, the SCAP-BMP2-VEGF group exhibited a significantly greater number of ALP-positive mineralized nodules than the other groups following 16 days culture in vitro. In conclusion, lentiviral vector-mediated BMP2 and VEGF gene co-transfection significantly activated the osteo-/odontogenic differentiation of human SCAP.

Introduction

Stem cells from apical papilla (SCAP) are a type of multipotent mesenchymal stem cells, which can be isolated from the apical papilla of immature teeth (1,2). SCAP possess the potential to differentiate into odontoblasts, which may contribute to dentin-pulp complex formation. SCAP also exhibit clear proliferative activity (3-5). Due to the clear osteo-/odontogenic potential and proliferative capacity, SCAP are regarded as valuable seed cells, and may be used in dentin regeneration.

SCAP have specific phenotypic markers, including STRO-1, CD146 and CD24 (3). STRO-1 and CD146 are specific markers of mesenchymal stem cells (3,6). STRO-1- and CD146-positive SCAP have previously been revealed to exhibit osteo-/odontogenic potential (7). CD24, which had not been detected in dental pulp stem cells (DPSCs) (3), has been reported to be a specific marker of SCAP (2,7).

Certain cytokines and growth factors participate in regulation of proliferation, differentiation and mineralization of SCAP, including bone morphogenetic protein (BMP) and vascular endothelial growth factor (VEGF) (8-12). A previous study has indicated that BMP2 gene transfection is an effective method to enhance the osteo-/odontogenic differentiation capacity of DPSCs by increasing osteo-/odontogenic gene and protein expression levels (12). Another previous study reported that lentiviral-mediated BMP2 gene transfection was effective in improving the odontogenic differentiation capacity of human SCAP in vitro (11). VEGF is also known to exhibit osteogenetic activity (13-15), and has been widely used in the regulation of proliferation and osteo-/odontogenic differentiation in stem cells derived from dental tissue (16-18).

The application of BMP2 or VEGF individually has been confirmed to be effective in improving osteo-/odontogenic differentiation of certain stem cells, however, simultaneous delivery of BMP2 and VEGF resulted in markedly improved...
results for osteogenesis (19-22). However, one study contradictorily reported that VEGF inhibited the expression of BMP2 and osteogenesis in rat bone marrow mesenchymal stem cells (BMMSCs) (23). The effects of BMP2 and VEGF gene co-transfection on the osteo-/odontogenic differentiation of SCAP require further elucidation. The aim of the current study was to analyze osteo-/odontogenic differentiation and the mineralization characteristics of human SCAP with lentiviral-mediated BMP2 and VEGF gene co-transfection.

Materials and methods

Isolation and identification of SCAP. Human SCAP were isolated from an extracted immature mandibular third molar of a male patient aged 18 years old by a method used in a previous study (11). All protocols were reviewed and approved by the Ethics Committee of Guanghua School and Hospital of Stomatology, Sun Yat-sen University (Guangzhou, China). The osteogenic and adipogenic differentiation capacities of SCAP were identified by Alizarin Red staining and Oil Red O staining (Cyagen Biosciences, Inc., Guangzhou, China), respectively (11). The typical phenotypes, including STRO-1/Alexa Fluor 647-Allophycocyanin (BioLegend, Inc., San Diego, CA, USA), CD146/Phycoerythrin (BD Pharmingen, San Diego, CA, USA), CD24/Fluorescein Isothiocyanate (FITC) (BD Pharmingen) and CD45/FITC (BD Pharmingen), using 2nd passage of SCAP were assessed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Construction of lentivirus plasmid and cell infection. Human BMP2 and VEGF gene primers were designed and amplified as described previously (11,16). The digonucleotides were combined into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (pCDH; System Biosciences, Mountain View, CA, USA) to build the recombinant plasmids, pCDH-BMP2 and pCDH-VEGF. The recombinant plasmids and packaging plasmids were then coinfectated into 293FT cells. The recombinant plasmids, pCDH-BMP2 and pCDH-VEGF gene primers were designed and amplified as the reference gene (Table I).

Cell proliferation Cell Counting Kit 8 (CCK8) assay. Four groups of cells, SCAP-GFP, SCAP-BMP2, SCAP-VEGF and SCAP-BMP2-VEGF, were seeded into 96-well plates at a density of 2x10^3 cells/well and were cultured in α-minimum essential medium (MEM) with 15% fetal bovine serum (FBS) (Cyagen Biosciences, Inc.). The proliferation rate of the cells was analyzed using the CCK8 assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) on the 1st, 2nd, 4th and 8th days subsequent to gene transfection.

RT-qPCR. Total RNA was extracted from SCAP-GFP, SCAP-BMP2, SCAP-VEGF and SCAP-BMP2-VEGF using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) on days 1, 4, 8 and 16 subsequent to transfection. The synthesis of complementary DNA (cDNA) was conducted using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. For each sample 2 µg RNA was used to synthesize the cDNA. The RT-qPCR reaction was conducted using iQ SYBR Green Supermix (BioRad Laboratories, Inc., Hercules, CA, USA) and regulated by the spectrophotometric iQ5 Thermal iCycler (BioRad Laboratories, Inc.). For PCR amplification, specific primers were used, as indicated in Table I. Initial denaturation was conducted at 95˚C for 3 min. This was succeeded by 30 cycles as follows: Denaturation, 95˚C for 15 sec; annealing, 55˚C for 30 sec; extension, 72˚C for 1 min. This was followed by a final extension at 72˚C for 7 min, prior to storage at 4˚C for 10 min. The mRNA expression of VEGF (GenBank accession no. MIM 612369), BMP2 (GenBank accession no. MIM 612369) and four osteo-/odontogenic genes, including alkaline phosphatase (ALP; GenBank accession no. MIM 612369), osteocalcin (OCN; GenBank accession no. MIM 612369), dentin sialophosphoprotein (DSP; GenBank accession no. MIM 612369) and dentin matrix acidic phosphoprotein gene 1 (DMP1; GenBank accession no. MIM 612369), were analyzed by RT-qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the reference gene (Table I).

Table I. Reverse transcription-quantitative polymerase chain reaction primers.

| Gene          | Primer sequence                      | Product size (bp) |
|---------------|--------------------------------------|------------------|
| BMP2          | F: 5'-CAGTCTGGCGACGCTTCC-3'          | 107              |
|               | R: 5'-CCTCCGTGGGATAGAATCTT-3'        |                  |
| VEGF          | F: 5'-CTACTGCTCACATGGAAGT-3'         | 104              |
|               | R: 5'-AGCTGCCTGATGACATCC-3'          |                  |
| ALP           | F: 5'-CTATCCTGGCTCGTGCCTC-3'         | 100              |
|               | R: 5'-GCTGGCAGTGTGCACGTA-3'          |                  |
| OCN           | F: 5'-CTCACACTTCTCGCCCTATT-3'        | 107              |
|               | R: 5'-TTGGACACAAAGGGCTGAC-3'         |                  |
| DSP           | F: 5'-GCCCACCTTTACGCTTCAAAGAGA-3'    | 130              |
|               | R: 5'-GCCCAATGCAAATAATGTAA-3'        |                  |
| DMP1          | F: 5'-AAAAATTGTTGTGACTACGGAGG-3'     | 94               |
|               | R: 5'-GAGGAGACGATAATCCCATGCA-3'      |                  |
| GAPDH         | F: 5'-AAGGTTGAAGGTCGAGAGTCAA-3'      | 108              |
|               | R: 5'-AATGAAAGGCTTTATGTTGAG-3'       |                  |

bp, base pairs; F, forward; R, reverse; BMP2, bone morphogenetic protein 2; VEGF, vascular endothelial growth factor; ALP, alkaline phosphatase; OCN, osteocalcin; DSP, dentin sialophosphoprotein; DMP1, dentin matrix acidic phosphoprotein gene 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Western blotting. The protein extracts from the four groups of cells were separated on 12% sodium dodecyl sulfate-polyacrylamide gels (Beyotime Institute of Biotechnology, Haimen, China) and transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.) at 200 mA for 2 h. The membranes were blocked with 5% non-fat milk for 2 h, and were incubated overnight at 4˚C with the following primary antibodies: Rabbit polyclonal anti-VEGF (1:500; cat. no. ab46154; Abcam, Cambridge, MA, USA); rabbit polyclonal anti-BMP2 (1:500; cat. no. ab14933; Abcam) and rabbit polyclonal anti-dentin sialoprotein (DSP; 1:500; cat. no. sc-33586; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membranes were subsequently incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:20,000; cat. no. ab97051; Abcam) at 37˚C for 2 h. Rabbit polyclonal anti-GAPDH (1:2,500; cat. no. ab9485; Abcam) was selected as the internal control. The resultant bands were visualized by the Chemiluminescence Western Blotting Detection system (EMD Millipore) on X-ray films (Kodak, Rochester, NY, USA).

ALP staining. The transfected cells were seeded into 6-well plates at an initial density of 5x10^4 cells/well and were cultured to 70% confluence in 2 ml α-MEM containing 15% FBS. The ALP Staining kit (Jiancheng Biotech. Co., Ltd., Nanjing, China) was used to stain mineralized granules on day 16 subsequent to transfection (11). The number of mineralized nodules was counted three times using an Axio Observer Z1 microscope (Zeiss AG, Oberkochen, Germany) at a magnification of x200.

Statistical analysis. The data are presented as the mean ± standard deviation, and were evaluated by one-way analysis of variance to analyze the differences between the four groups of cells. SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Morphology, multipotent differentiation and phenotypes of SCAP. The kidney-shaped papillae, which had a dense and pink appearance, were separated from the third molars (Fig. 1A). The isolated primary SCAP presented with a short spindle-like appearance and formed classic cell colonies subsequent to eight days culture (Fig. 1B). Alizarin Red staining illustrated that SCAP formed abundant mineralized nodules 32 days subsequent to osteogenic induction (Fig. 1C), and Oil Red O staining indicated that SCAP formed lipid droplets 16 days following adipogenic induction (Fig. 1D). The freshly isolated SCAP exhibited typical forward scatter/side scatter characteristics (Fig. 1E), similar to those reported previously (4,7). The presence of the phenotypic markers, including STRO-1, CD146 and CD24, were observed to be 23.1%, 89.0% and 22.2%, respectively in the isolated cells (Fig. 1F-H).

Overexpression VEGF and BMP2 in SCAP. A total of four days subsequent to transfection, the mRNA expression levels of VEGF in SCAP-GFP, SCAP-BMP2, SCAP-VEGF and SCAP-BMP2-VEGF were 1.00±0.07, 1.32±0.09, 11.14±0.72 and 9.03±0.18, respectively. The relative mRNA expression levels of BMP2 in SCAP-GFP, SCAP-BMP2, SCAP-VEGF and SCAP-BMP2-VEGF were 1.00±0.12, 6.79±0.10, 3.11±0.05 and 5.94±0.12, respectively (Fig. 2A). The relative VEGF protein expression levels, which were quantified by measuring the grayscale of the western blotting bands in SCAP-GFP, SCAP-BMP2, SCAP-VEGF and SCAP-BMP2-VEGF, were 1.00±0.10, 1.09±0.09, 2.79±0.26 and 3.03±0.15, respectively. The relative BMP2
protein expression levels were 1.00±0.04, 3.32±0.09, 2.67±0.10 and 3.83±0.15, respectively (Fig. 2B and C). The mRNA and protein expression levels of VEGF and BMP2 were observed to be significantly enhanced in SCAP-BMP2-VEGF compared with SCAP-GFP. Notably, the current results demonstrated that VEGF gene transfection significantly enhanced the BMP2 expression of SCAP (P<0.05).

VEGF and BMP2 control the proliferation of SCAP. On days 4 and 8 subsequent to lentivirus-mediated gene transfection, SCAP-VEGF exhibited increased proliferation when compared with the other three groups of cells (P<0.05), while SCAP-BMP2-VEGF exhibited reduced proliferation (Fig. 2D). Mineralization nodule formation of gene-transfected SCAP. The mineralized nodules, which have a strong positive expression of ALP, were stained with a golden color (Fig. 4). The number of mineralized nodules in the SCAP-BMP2-VEGF group was significantly greater than in the remaining three groups (P<0.01). In addition, the number of mineralized nodules in the SCAP-BMP2 and SCAP-VEGF groups was observed to be significantly greater than that of SCAP-GFP (P<0.01).
SCAP are multipotent stem cells, which can differentiate into osteoblasts, odontoblasts and adipocytes (1-3). The current study confirmed the osteogenic and adipogenic differentiation capacities of SCAP. In agreement with previous studies (1-3,7,11,24), significant mineralized deposits and lipid droplets were formed by SCAP following osteogenic and adipogenic induction. Furthermore, the isolated cells from apical papilla exhibited the specific phenotypic characteristics of SCAP, including the presence of STRO-1, CD146 and CD24 (3). Previous studies have indicated that the positive percentages in the 1st passage of SCAP were approximately 20-30% (3,7), 47-84% (4) and 3-15% (7) for STRO-1, CD146 and CD24, respectively. In the present study, the representative expression of STRO-1, CD146 and CD24 of the 2nd passage SCAP was positive, and these results are consistent with previous studies (3,4,7).

Even though SCAP has the capacity to differentiate into osteoblasts and odontoblasts, and can form mineralized tissues...
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in vivo (9,11,25,26), the mechanisms of osteo-/odontogenic differentiation promotion remain to be fully elucidated. VEGF and BMP2, which exhibited specific expression in the proliferation and differentiation of osteoblasts, are regarded as important regulators of osteogenesis (27,28). In a normal bone healing model, VEGF was previously observed to exhibit maximal expression a number of days earlier than for BMP2 (29), indicating the sequence of vascular bed establishment and bone formation. In dentin development, BMP2 and VEGF were detected during the differentiation and maturation process of odontoblasts (30-33). Previous studies have reported that gene transfection of BMP2 or VEGF genes alone was effective in activating the osteo-/odontogenic potential of DPSCs by increasing the expression levels of osteo-/odontogenic genes, including ALP, OCN, COLI, bone sialoprotein, DSP and DMP1 (12,16). ALP, detected in the process of mineralization, was regarded as an early signal of osteo-/odontogenic differentiation of stem cells derived from dental tissues (24,34,35). OCN is typically recognized as a signal of osteo-/odontogenic differentiation at a later stage in the process (4). DMP1 serves an essential role in the maturation of ameloblasts, osteoblasts and odontoblasts, in addition to the progression of mineralization in certain stem cells (36). DSP, the amino-terminal part of DSPP, has been reported to be an important protein during the process of odontogenic differentiation (37). In the current study, the osteo-/odontogenic gene and protein expression levels were observed to be significantly upregulated in SCAP-BMP2 and SCAP-VEGF when compared with SCAP-GFP at the majority of the time points. This indicated that BMP2 or VEGF gene transfection alone aided in enhancing the osteo-/odontogenic differentiation of SCAP.

Previous studies have used combined delivery of BMP2 and VEGF in order to improve osteogenic differentiation of mesenchymal stem cells (20,22,38-43), osteoprogenitor cells (19,44) and osteoblasts (45). When pre-osteoblasts were induced by BMP2, the expression of VEGF was observed to increase (46). Similarly, VEGF gene transfection enhanced the expression of VEGF in the bone marrow stromal cells, in addition to marginally increasing the expression of BMP2 (41). However, a previous study contradicted this, stating that VEGF transfection inhibits the expression of BMP2 in mesenchymal stem cells (23). In the current study, VEGF gene transfection was observed to enhance the expression of VEGF, in addition to significantly increasing the expression of BMP2 in SCAP. These results indicated that VEGF may promote the osteo-/odontogenic differentiation of SCAP by acting cooperatively with BMP2. In addition, a previous study demonstrated that VEGF and BMP2 were able to promote bone regeneration by facilitating stem cell homing (39). A previous study indicated that BMP2- and VEGF-co-transfected BMMSCs resulted in the peak ALP expression on day 7 (22).
However, SCAP-BMP2-VEGF demonstrated an increased expression of ALP over time in the current study. The results of current study supported the hypothesis that BMP2 and VEGF gene co-transfection was more effective in improving osteo-/odontogenic differentiation of SCAP than single gene transfection.

A previous study indicated that VEGF increased the rate of proliferation in rat osteoblasts in a dose-dependent manner, while no significant alterations were observed with BMP2 (45). The results of the present study are consistent with this previous study (45), observing that the VEGF-transfected SCAP group exhibited marginally increased proliferation compared with the other groups on days 4 and 8 subsequent to transfection. The lowest levels of proliferation were observed in the BMP2 and VEGF co-transfection group on days 4 and 8, thus the BMP2 and VEGF had a synergistic effect on the proliferation of SCAP.

In summary, human dental papilla stem cells were successfully transfected with the BMP2 and VEGF genes. The SCAP-BMP2-VEGF group exhibited increased expression levels of osteo-/odontogenic differentiation-associated genes and protein, and increased mineralization deposits than the SCAP-BMP2, SCAP-VEGF and SCAP-GFP groups in vitro. These results suggest that the co-transfection of homologous BMP2 and VEGF genes is an effective strategy to improve the osteo-/odontogenic differentiation of SCAP in vitro.

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