Effects of core binding factor $\alpha 1$ or bone morphogenic protein-2 overexpression on osteoblast/cementoblast-related gene expressions in NIH3T3 mouse cells and dental follicle cells

K. Pan^{*†}, S. Yan^{*†}, S. Ge^{*†}, S. Li^{*†}, Y. Zhao[‡] and P. Yang^{*†}

^{*}Department of Periodontology and [†]Institute of Oral Biomedicine, School of Dentistry, Shandong University, Jinan, China, [‡]Department of Orthodontics, Stomatology Hospital, Tianjin Medical University, Tianjin, China

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Abstract

Objectives: Bone morphogenic protein-2 (BMP-2) has long been used to promote bone and periodontal regeneration, while core binding factor $\alpha 1$ (CBFA1) plays important roles in both osteogenic differentiation and tooth morphogenesis. The aim of this study was to evaluate the effects of CBFA1 or BMP-2 overexpression on osteoblast/cementoblast-related gene expressions in NIH3T3 cells and dental follicle cells (DFCs).

Materials and methods: CBFA1 or BMP-2 overexpression in NIH3T3 and DFCs was achieved by infection with retroviral vectors containing CBFA1 or BMP-2 cDNA. Cells stably integrated with CBFA1 or BMP-2 cDNA were selected with G418 for 14 days. Western blotting, real-time reverse transcriptase–polymerase chain reaction, and *in vitro* mineralization assay were performed to evaluate effects of CBFA1 or BMP-2 overexpression in cells undergoing osteoblast/cementoblast differentiation.

Results: Our results demonstrated that osteoblast/cementoblast-related gene expression levels in CBFA1-overexpressing NIH3T3 cells were higher than those in BMP-2-overexpressing cells. More mineral nodules were observed in CBFA1-overexpressing NIH3T3 cells than in BMP-2-overexpressing cells. CBFA1 overexpression in DFCs also increased osteoblast/cementoblast-related gene expression and promoted mineral nodule formation. However, no significant

changes in gene expression levels nor mineral nodule formation were found in BMP-2-overexpressing DFCs when compared with empty vector transduced DFCs.

Conclusions: CBFA1 overexpression up-regulated expression levels of osteoblast/cementoblast-related genes and enhanced *in vitro* osteogenic differentiation more efficiently than BMP-2 in both NIH3T3 cells and DFCs.

Introduction

Periodontitis is an inflammatory disease that leads to progressive destruction of tooth-supporting structures, including periodontal ligament, cementum, and alveolar bone. The ultimate goal of periodontal treatment is to restore architecture and function of periodontal tissues to predisease state by formation of new alveolar bone, cementum, and periodontal ligament (1,2). To achieve this, application of molecular factors in tissue engineering to promote periodontal regeneration has been an area of intensive interest. Putative factors reported to promote bone and periodontal regeneration include growth factors, such as bone morphogenetic/osteogenic proteins (BMP/OP) (3,4), transcription factors such as CBFA1 (5) and adhesion factors, such as bone sialoprotein and osteopontin (6,7).

In 1965, it was reported that certain substances in the extracellular bone matrix can induce new bone formation when implanted into intramuscular sites (8). This led to the discovery of BMPs/OPs, which were identified as members of the transforming growth factor- β superfamily (9,10). It has been reported that BMPs/OPs are pleiotropic morphogens governing the three key steps in the bone-induction cascade, including chemotaxis and mitosis, cartilage differentiation, and then replacement by bone (11). Since their discovery, BMPs/OPs have been applied to bone defects as alternative methods to promote bone

Correspondence: P. Yang, Department of Periodontology, School of Dentistry, Shandong University, 44-1 Wenhua Road, Jinan 250012, China. Tel.: +86 531 88382368; Fax: +86 531 82950194; E-mail: yangps@sdu.edu.cn

K. Pan and S. Yan contributed equally to this work.

regeneration, considering low therapeutic effects associated with bone grafts and metal implants (12–14). Recently, commercially available collagen-based products containing BMP-2 as osteo-inductive materials were approved by the US Food and Drug Administration (FDA) for human clinical use (15). BMP-2 Infuse™ bone graft (Medtronic, Minneapolis, MN, USA; Wyeth, Maidenhead, UK) was approved for certain interbody fusion procedures in 2002, for open tibial fractures in 2004, and for alveolar ridge and sinus augmentations in 2007 (16).

During the last decade, it has been reported that *CBFA1* (also known as *Runx2*, *OSF2*, *AML3*, and *PeBP2α4*), is a master gene for osteoblast differentiation and bone formation (17,18). Aetiological factors of cleidocranial dysplasia, an autosomal dominant disorder in human and mouse, characterized by defective bone formation, were reported to be various mutations at the *CBFA1* gene locus (19). In addition, CBFA1 protein was identified as essential for tooth formation (20,21) as patients suffering from cleidocranial dysplasia also show dental defects, including multiple supernumerary teeth, delayed eruption of permanent dentition and absence of cellular cementum formation (22,23).

The gene transfer approach presents an attractive alternative to conventional topical application of molecules to complex periodontal wound sites (24). Use of viral constructs containing one or more response modifier transgenes for long-term delivery may enhance periodontal regeneration. In the present study, CBFA1 or BMP-2 cDNA was stably integrated into NIH3T3 cells and DFCs using a retroviral system to evaluate effects of CBFA1 or BMP-2 overexpression on *in vitro* osteogenic differentiation. *In vitro* mineral nodule formation was then performed and changes in expression levels of osteoblast/cementoblast-related genes were determined.

Materials and methods

Cell culture

Retroviral packaging cell line PT67 (Clontech, Palo Alto, CA, USA) and murine fibroblast cell line NIH3T3 (American Type Culture Collection, Rockville, MD, USA) were maintained in α -minimum essential medium (α -MEM, HyClone, Logan, UT, USA) supplemented with 10% (v/v) foetal bovine serum (FBS, HyClone), 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 μ g/ml streptomycin (Invitrogen).

DFCs were isolated from 5- to 7-day-old BALB/c mice, as described previously (25). Briefly, bilateral first mandibular molar germs were dissected under a dissecting microscope and were treated with 1% trypsin at 4 °C for 1.5 h. After digestion, loose dental follicle tissues were

isolated and cultured in α -MEM supplemented with 20% (v/v) FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂ humidified incubator. Non-adherent tissues were removed 24 h later and culture medium was changed every 3 days. DFCs up to three passages were used for further experiments.

Construction of recombinant retroviral plasmids

CBFA1 cDNA was prepared by polymerase chain reaction (PCR) from plasmid pCMV5-CBFA1 (kindly provided by Dr Patricia Ducey, Department of Molecular Genetics, University of Texas M. D. Anderson Cancer Center) encoding full-length mouse CBFA1 cDNA with MASNSL as the N-terminal sequence (17,26). PCR primers were: 5'-CTCGAGATGGCGTCAAACAGCCTCTT-3' and 5'-ACGCGTTCAATATGGCCGCCAAACA-3'. PCR product was cloned into *XhoI*-*MluI* sites of the vector pIRES (Clontech) and the resulting construct was named as pIRES-CBFA1. A 2.2-kb CBFA1-IRES fragment was then released from pIRES-CBFA1 by digestion with *XhoI* and *SalI* (Takara, Tokyo, Japan), and was subcloned into retroviral vector pLEGFP-N1 (Clontech). The resulting construct was named pLEGFP-IRES-CBFA1. Similarly, BMP-2 cDNA was generated from plasmid pSP65-BMP-2 (provided by Dr Shaohua Liu, Department of Dentistry, Qilu Hospital of Shandong University, Jinan, China) and the primers were: 5'-ATACTCGAGATGGTGGCCGGGACCCG-3' and 5'-ATTACGCGTCTAGCGACACCCACAA CCCTCCA-3'. The PCR product was also ligated into the *XhoI*-*MluI* sites of pIRES. BMP-2-IRES fragment was released and subcloned into *XhoI*-*SalI* sites of pLEGFP-N1. The resulted construct was named pLEGFP-IRES-BMP-2.

Production of viral stocks and viral infection

Packaging cells PT67 were plated in a 6-well plate (1×10^5 cells/well) 24–48 h before transfection with 4 μ g of pLEGFP-IRES-CBFA1, pLEGFP-IRES-BMP2, or empty vector, using Lipofectamine™ 2000 (Invitrogen). Forty-eight hours after transfection, stable virus-producing cell lines were selected with 400 μ g/ml G418 (Takara) for 2 weeks. Supernatant of these stable virus-producing PT67 cells was collected, filtered through a 0.45- μ m filter, and stored at –80 °C. The recombinant retrovirus was tested by PCR.

Viral titre was determined indirectly as previously described (27). In brief, NIH3T3 cells were plated in 6-well plates (2×10^5 cells/well). Twenty-four hours later, they were infected with diluted viral stocks (six 10-fold serial dilutions, $1-10^5$). Forty-eight hours after infection, the cells were selected by G418 until obvious cell colonies appeared. Viral titre corresponded to number of colonies

presented at the highest dilution that contained colonies, multiplied by the dilution factor.

Twenty-four hours before transduction, NIH3T3s and DFCs were plated in 6-well plates (1×10^5 cells/well). Cells were then incubated with viral stocks containing pLEGFP-IRES-CBFA1, pLEGFP-IRES-BMP2 or empty vector pLEGFP-N1, in the presence of polybrene (6 µg/ml, Sigma, St. Louis, MO, USA) for 8 h. Twenty-four hours after infection, NIH3T3 and DFCs were subjected to G418 (300 µg/ml) for 2 weeks. Stably transduced cells were used for the following experiments.

Real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted with Trizol reagent (Takara) from NIH3T3 cells and DFCs infected with pLEGFP-N1 (serving as negative control), pLEGFP-IRES-CBFA1 or pLEGFP-IRES-BMP-2. RNA isolated from non-infected cells served as empty control. One microgram of total RNA from each cell type was reverse transcribed to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania) according to the manufacturer's instructions.

For real-time PCR, LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany) was used and LightCycler PCR reaction mix was prepared according to manufacturer's instructions; primers used are listed in Table 1. In each reaction, 18 µl

of master mixture was placed into the glass capillaries and 2 µl of template cDNA was added. Capillaries were then placed into the rotor of the LightCycler (Roche). After a denaturation step at 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 59 °C for 5 s and 72 °C for 10 s were initiated. Amounts of mRNA were calculated for each sample based on the standard curve using the LightCycler Software 4.0 (Roche). Investigated genes included *CBFA1*, *BMP-2*, alkaline phosphatase (*ALP*), osteocalcin (*OC*), osteopontin (*OPN*), bone sialoprotein gene (*BSP*), collagen I gene (*Col I*), gene for cementum attachment protein (*CAP*) and cementum protein 23 gene (*CP23*). β-actin was used as an internal control.

Western blot analysis

Cells were harvested using 0.25% trypsin and washed twice with PBS. Proteins were extracted using lysis buffer (25 mM Tris-Cl, pH 7.2, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 0.1 M NaCl, 1 mM EDTA) containing a protease inhibitor cocktail (Roche). Proteins were separated using SDS–polyacrylamide gel electrophoresis and were transferred to a Protran nitrocellulose membrane, which was saturated overnight in Tris-buffered saline with Tween and 5% low-fat milk. The membrane was incubated with anti-BMP-2 (1 : 500) or anti-CBFA1 antibody (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with mouse or rabbit horseradish peroxidase-

Table 1 Specific primers used for real-time reverse transcriptase–polymerase chain reaction

Gene	Primer sequence	Melting temperature (°C)	Size (bp)	Accession number
BMP-2	5'-CCTCAGCAGAGCTTCAGGTT-3'	59.75	153	NM_001200
	5'-CCAACCTGGTGTCCAAAAGT-3'	59.86		
CBFA1	5'-CCCAGCCACCTTACCTACA-3'	59.99	150	NM_009820
	5'-TATGGAGTGCTGCTGGTCTG-3'	60.01		
Col I	5'-TGACTGGAAGAGCGGAGAGT-3'	60.14	151	NM_007742
	5'-GTTTCGGGCTGATGTACCAGT-3'	60.00		
OC	5'-AAGCAGGAGGGCAATAAGGT-3'	60.10	152	NM_001032298
	5'-TAGGCGGTCTTCAAGCCATA-3'	60.73		
OPN	5'-TCTGATGAGACCGTCACTGC-3'	59.99	170	AF515708
	5'-AGGTCCTCATCTGTGGCATC-3'	60.08		
BSP	5'-GAAGCAGGTGCAGAAGGAAC-3'	60.00	154	NM_008318
	5'-ACTCAACGGTGCTGCTTTT-3'	59.92		
ALP	5'-AAGCCAGACACAAGCATTCC-3'	59.97	151	X13409
	5'-GAGAGCGAAGGGTCAGTCAG-3'	60.14		
CAP	5'-CCTGGCTCACCTTCTACGAC-3'	59.87	156	AY455942
	5'-CCTCAAGCAAGGCAAATGTC-3'	60.78		
CP23	5'-GGCGATGCTCAACCTCTAAC-3'	59.84	158	NM_001048212
	5'-GATACCCACCTCTGCCTTGA-3'	60.07		
β-actin	5'-CCCTGAAGTACCCCATTTGAA-3'	59.78	158	NM_007393
	5'-CTTTTCACGGTTGGCCTTAG-3'	59.74		

conjugated immunoglobulin G (1 : 10 000, Sigma). Blots were visualized using diaminobenzidine, and β -actin was used as internal control. Images of the blots were analysed using the JD801 IMAGE 4.11 program (Dahui Biotechnology, Guangzhou, China).

Mineralization assay

Infected and non-infected NIH3T3 cells and DFCs were plated in 24-well plates (1×10^4 cells/well) in α -MEM containing 10% FBS. After 24 h, cells were incubated with mineralizing media (α -MEM containing 10% FBS, 50 μ g/ml ascorbic acid, 10 nM dexamethasone, and 10 mM β -glycerophosphate). Biomineralization assay (*in vitro* mineralization assay) was performed at weeks 2, 3 and 4 with von Kossa staining (28). Nodules were photographed using an Olympus CX71 camera (Tokyo, Japan) and areas covered with mineral nodules in each well were determined using image analysis system DT2000 software V1.0 (Tansi Technology Co. Ltd, Nanjing, China).

Statistical analysis

Data are presented as mean $\pm$ standard error from at least three experiments, and *t*-testing was used to determine significance using SPSS 12.0 software. $P < 0.05$ was considered statistically significant.

Results

Construction and identification of recombinant plasmids

Recombinant retroviral plasmids pLEGFP-IRES-CBFA1 and pLEGFP-IRES-BMP-2 were identified by restriction endonucleases. As shown in Fig. 1, sizes of CBFA1 and BMP-2 fragments were 1587 and 1191 bp, respectively. Sequence of CBFA1 and BMP-2 were consistent with their sequences published in GeneBank (BMP-2: NM_001200; CBFA1: NM_009820).

Retroviral infection of NIH3T3s and DFCs

pLEGFP-IRES-CBFA1 and pLEGFP-IRES-BMP-2 were transfected into packaging cell line PT67 and stable virus-producing PT67 cells were obtained by G418 selection. NIH3T3s and DFCs were then infected with virus-containing supernatants collected from PT67 cells. After G418 selection for 2 weeks, stable CBFA1 or BMP-2 overexpressing NIH3T3s and DFCs were obtained. Green fluorescence could be observed using an inverted microscope and cells were without significant morphological changes (Fig. 2 A1, A2). Real-time RT-PCR confirmed that expression level of CBFA1 or BMP-2 was increased in

Figure 1. Results of agarose gel electrophoresis of PCR products and restriction endonuclease digestion analysis. Lane M, wide range DNA marker (500–12000 bp, Takara); lane 1, BMP-2 gene amplified from plasmid pSP65-BMP-2; lane 2, plasmid pLEGFP-IRES-BMP-2 digested with Xho I and Mlu I; lane 3, plasmid pLEGFP-IRES-BMP-2 digested with Xho I and Sal I; lane 4, CBFA1 gene amplified from plasmid pCMV5-CBFA1; lane 5, plasmid pLEGFP-IRES-CBFA1 digested with Xho I and Mlu I; lane 6, plasmid pLEGFP-IRES-CBFA1 digested with Xho I and Sal I.

pLEGFP-IRES-CBFA1 or pLEGFP-IRES-BMP-2 infected cells compared to control cells ($P < 0.01$) (Fig. 2 B1, B2). Presence of CBFA1 or BMP-2 protein in cell extract also was detected by Western blotting with CBFA1 or BMP-2 antibody (Fig. 2 C1, C2). Our results demonstrated that CBFA1 and BMP-2 protein were up-regulated in pLEGFP-IRES-CBFA1 and pLEGFP-IRES-BMP-2 infected cells, respectively ($P < 0.05$) (Fig. 2 D1, D2).

Effects of CBFA1 or BMP-2 overexpression on changes in osteoblast/cementoblast-related gene expressions

Expression levels of osteoblast/cementoblast-related genes including *Col I*, *ALP*, *OPN*, *BSP*, *OC*, *CAP* and *CP23* were determined using real-time RT-PCR. In NIH3T3 cells (Fig. 3A), overexpression of CBFA1 up-regulated expression levels of *ALP*, *OC*, *Col I*, *BSP* and *OPN* ($P < 0.05$), while BMP-2 overexpression increased expression levels of *ALP*, *OC*, *Col I* and *BSP* but decreased *OPN* expression level ($P < 0.01$). Furthermore, expression level of *Col I* in CBFA1 overexpressing NIH3T3 cells was higher than that in BMP-2 overexpressing NIH3T3 cells ($P < 0.05$), while expression levels of *ALP* and *BSP* in CBFA1 overexpressing NIH3T3 cells were lower than those in BMP-2 overexpressing NIH3T3 cells ($P < 0.01$). In DFCs (Fig. 3B),

Figure 2. NIH3T3 and DFCs stably overexpressing CBFA1 or BMP-2. NIH3T3 and DFCs were infected by retrovirus and selected by G418. (A1, A2) Green fluorescence could be detected in the stably transduced cells but without significant morphological changes (at $\times 200$ magnification). (B1, B2) Real-time RT-PCR confirmed that the transduced cells overexpressed CBFA1 or BMP-2 gene. (C1, C2) Representative images demonstrated that the presence of CBFA1 or BMP-2 protein in cell extract could be detected by Western blot with CBFA1 or BMP-2 antibody. (D1, D2) Protein levels were normalized with that of β -actin. CBFA1 and BMP-2 were up-regulated in pLEGFP-IRES-CBFA1 and pLEGFP-IRES-BMP-2 infected cells, respectively. ** $P < 0.01$ vs. NIH3T3 and NIH3T3-EGFP, † $P < 0.05$ vs. DFCs and DFCs-EGFP, †† $P < 0.01$ vs. DFCs and DFCs-EGFP. Statistical significance was determined using t test.

expression levels of all the above-mentioned osteoblast/cementoblast-related genes were detected. Furthermore, overexpression of CBFA1 enhanced the expression levels of *BSP*, *OPN*, *Col I*, *CAP* and *CP23* ($P < 0.05$), while

overexpression of BMP-2 only enhanced the expression level of *CP23* ($P < 0.01$). In contrast, both CBFA1 and BMP-2 overexpression inhibited expression of *ALP* ($P < 0.01$), with the inhibitory effect more prominent in

Figure 3. Effects of CBFA1 or BMP-2 overexpression on osteoblast/ cementoblast-related gene expressions. Total RNA was isolated from NIH3T3, DFCs and the cells infected with pLEGFP-N1, pLEGFP-IRES-CBFA1 and pLEGFP-IRES-BMP-2. Real-time RT-PCR was performed to compare the effects of CBFA1 or BMP-2 overexpression on osteoblast/ cementoblast-related gene expressions, including Col I, ALP, OPN, BSP, OC, CAP and CP23. All values were normalized with β -actin levels and the data were presented as the mean $\pm$ SE for three experiments. * P < 0.05 vs. NIH3T3 and NIH3T3-EGFP, ** P < 0.01 vs. NIH3T3 and NIH3T3-EGFP, † P < 0.05 vs. NIH3T3-BMP-2, †† P < 0.01 vs. NIH3T3-BMP-2, § P < 0.05 vs. DFCs and DFCs-EGFP, §§ P < 0.01 vs. DFCs and DFCs-EGFP, ‡† P < 0.01 vs. DFCs-BMP-2. Statistical significance was determined using t test.

CBFA1 overexpressing DFCs than in BMP-2 overexpressing DFCs (P < 0.01).

Effects of CBFA1 or BMP-2 overexpression on mineralization

In vitro mineral nodule formation assay was performed to determine whether CBFA1 or BMP-2 overexpression would affect *in vitro* mineralization. In NIH3T3 cells (Fig. 4B), both CBFA1 and BMP-2 overexpression induced formation of mineral nodules, but more mineral nodules were detected in CBFA1 overexpressing cells than in BMP-2 overexpressing cells (P < 0.01). In DFCs (Fig. 4C), overexpression of CBFA1 enhanced *in vitro* mineralization, as indicated by the larger size of mineral nodules formed in CBFA1 overexpressing DFCs than in control DFCs (P < 0.01). In contrast, there was no significant difference in mineral nodule formation between BMP-2 overexpressing DFCs and control DFCs (P > 0.05).

Figure 4. Effects of CBFA1 or BMP-2 overexpression on mineral nodule formation. Cells were cultured in mineralizing media and von Kossa staining was performed to evaluate the mineral nodule formation on week 2, 3, 4. The nodules were microphotographed with Olympus camera ($\times 100$ magnification) (A1, A2) and the area covered by mineral nodules in each well was determined. The data were presented as the mean $\pm$ SE for three experiments. ** P < 0.01 vs. NIH3T3 and NIH3T3-EGFP, †† P < 0.01 vs. NIH3T3-BMP-2, §§ P < 0.01 vs. DFCs, DFCs-EGFP and DFCs-BMP-2. Statistical significance was determined using t test.

Discussion

The attraction of using local gene therapy to induce repair and formation of bone and periodontium is that genes can be delivered to the appropriate anatomical site, and duration of protein expression can be determined by selecting proper vector and/or promoter (29). Retroviruses can integrate their genes efficiently into the genome of target cells and provide long-term expression of the transgenes (30). In this study, retroviral strategies were adopted to transfer CBFA1 or BMP-2 into NIH3T3 cells and DFCs cells to evaluate their effects on osteoblast/ cementoblast-related gene expression profiles. Our data indicated that overexpression of CBFA1

enhanced osteoblast/cementoblast-related gene expressions and promoted *in vitro* mineral nodule formation in both NIH3T3s and DFCs. Overexpression of BMP-2 also enhanced gene expression levels and promoted *in vitro* mineralization in NIH3T3 cells, but failed to show such effects in DFCs.

CBFA1 is the principal osteogenic master gene during bone formation. Studies have indicated that *CBFA1* is a positive regulator that up-regulates expression levels of bone matrix genes such as *Col I*, *OPN*, *BSP* and *OC* in mesenchymal cells and non-osteoblast cells (17,31–34). Consistent with these previous findings, our results demonstrated that *CBFA1* up-regulated expression levels of *Col I*, *OPN*, *BSP* and *OC*. *CBFA1* also increased expression levels of *CAP* and *CP23*, which are both putative markers for cementoblasts. We speculated that overexpression of *CBFA1* might induce NIH3T3/DFCs to differentiate towards osteoblast/cementoblasts by up-regulating osteoblast/cementoblast-related genes.

BMP-2 induces expression of numerous transcription factors, which constitute a network of activities and molecular switches for bone development and osteoblast differentiation (35–37); *CBFA1* is among these transcriptional regulators. BMP-2 has been reported to increase *CBFA1* mRNA level in several immortalized cell lines, such as human bone marrow stromal cells (38), C2C12 cells (39) and 2T3 cells (40), and, therefore, promote these cells to differentiate along the osteoblastic pathway. Our results also demonstrated that in NIH3T3 cells, BMP-2 enhanced *CBFA1* and other osteoblast-related gene expression levels. However, in our study no changes in expression levels of these osteoblast-related genes were detected in BMP-2 overexpressing DFCs. This result was at variance from some previous findings that have shown that BMP-2 induced osteoblast differentiation of DFCs, as reflected by enhanced *Cbfa1*, *BSP* and *OC* mRNA expressions and increasing mineralization formation (41,42). BMP-2 exerts its effects through specific receptors on the cell surface by binding to them receptors and interacting with cytoplasmic effector molecules to initiate a complex cascade of intracellular events leading to alteration in gene function (35,43). In these previous reports, researchers achieved their results by treating DFCs with BMP-2 protein (41,42), while in our study the DFCs were infected with retroviral constructs containing BMP-2 cDNA instead of BMP-2 protein treatment. Although our G418 selection results demonstrated that the *BMP-2* gene was stably integrated into the genome of DFCs, concentration of BMP-2 protein secreted by infected DFCs may not be high enough to induce significant changes in gene expression levels. Our results indicated that using retroviral gene transfer strategy *CBFA1*-induced changes in osteoblast-related gene expression levels and *in vitro*

mineralization were more prominent than those induced by BMP-2.

In addition to these osteoblast-related genes, *CBFA1* overexpression in DFCs also enhanced expression levels of cementoblast-related genes including *CAP* and *CP23*, while overexpression of BMP-2 only enhanced expression level of *CP23*. Although BMP-2 has been used to promote bone and periodontal regeneration (44,45), it has been reported that BMP-2 does not have a significant effect on cementum regeneration (46). It has also been reported that BMP-2 inhibits differentiation and mineralization of cementoblasts *in vitro* (47). Among the BMPs/OPs family, BMP-7/OP-1 have also been approved by the FDA as osteo-inductive materials for human clinical use (15). BMP-7/OP-1 Osigraft™ (Stryker Biotech, Kalamazoo, MI, USA) was approved for long bone fractures and as an alternative to autografts in patients requiring posterolateral lumbar spinal fusion (15). It has been reported that single application of rhOP-1 preferentially induces cementogenesis on denuded root surfaces in primates (48,49) and rhOP-1 showed higher ability to induce cementogenesis when compared with rhBMP-2 (50). Our results, which demonstrated that cementoblast-related gene expression changes induced by BMP-2 overexpression were less prominent than those induced by *CBFA1* overexpression, also indicated that effects of BMP-2 on cementogenesis were relatively mild. Considering that BMP-7/OP-1 plays more important roles in cementogenesis than BMP-2, further studies regarding this growth factor are necessary.

In vitro mineralization assay demonstrated that in NIH3T3 cells, both *CBFA1* and BMP-2 overexpression induced mineral nodule formation. Moreover, more mineral nodules were detected in *CBFA1* overexpressing NIH3T3 cells than in BMP-2 overexpressing NIH3T3 cells. *CBFA1* overexpression in DFCs also enhanced mineral nodule formation, but no changes in mineral nodule formation were detected in BMP-2 overexpressing DFCs. Based on our findings that *CBFA1* overexpression increased expression levels of *OPN* and *Col I* and promoted mineral nodule formation more efficiently than BMP-2 overexpression in both NIH3T3s and DFCs, we assume that *OPN* and *Col I* might be more important to the mineralization process.

In conclusion, our investigation has indicated that *CBFA1* enhanced expression levels of osteoblast/cementoblast-related genes and promoted mineral nodule formation more efficiently than BMP-2 in both NIH3T3 cells and DFCs. The results suggested that *CBFA1* might be more important than BMP-2 on up-regulation of osteoblast/cementoblast-related gene expressions using the gene transfer method. Based on the fact that BMP-7/OP-1 is more important in cementogenesis than BMP-2, we will include this growth factor in our future studies on cementogenesis.

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