TISSUE ENGINEERING
Volume 13, Number 11, 2007

DOI: 10.1089/ten.2006.0439

STRO-1 Selected Rat Dental Pulp Stem Cells Transfected with Adenoviral-Mediated Human Bone Morphogenetic Protein 2 Gene Show Enhanced Odontogenic Differentiation

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ABSTRACT

Dental pulp stem cells harbor great potential for tissue-engineering purposes. However, previous studies have shown variable results, and some have reported only limited osteogenic and odontogenic potential. Because bone morphogenetic proteins (BMPs) are well-established agents to induce bone and dentin formation, in this study STRO-1-selected rat dental pulp–derived stem cells were transfected with the adenoviral-mediated human BMP-2 gene. Subsequently, the cells were evaluated for their odontogenic differentiation ability in medium not containing dexamethasone or other stimuli. Cultures were investigated using light microscopy and scanning electron microscopy (SEM) and evaluated for cell proliferation, alkaline phosphatase (ALP) activity, and calcium content. Real-time polymerase chain reaction (PCR) was performed for gene expression of Alp, osteocalcin, collagen type I, bone sialoprotein, dentin sialophosphoprotein, and dentin matrix acidic phosphoprotein 1. Finally, an oligo-microarray was used to profile the expression of odontogenesis-related genes. Results of ALP activity, calcium content, and real-time PCR showed that only BMP2-transfected cells had the ability to differentiate into the odontoblast phenotype and to produce a calcified extracellular matrix. SEM and oligo-microarray confirmed these results. In contrast, the non-transfected cells represented a less differentiated cell phenotype. Based on our results, we concluded that the adenovirus can transfect STRO-1 selected cells with high efficacy. After BMP2 gene transfection, these cells had the ability to differentiate into odontoblast phenotype, even without the addition of odontogenic supplements to the medium.

INTRODUCTION

TISSUE ENGINEERING REQUIRES THREE KEY ELEMENTS: morphogenetic signals, including growth and differentiation factors; responding stem cells; and a biocompatible scaffold.[1] Stem cells have been isolated from many tissues, including bone marrow, neural tissue, skin, fat, and muscle. Currently, mesenchymal stem cells have also been found in dental pulp tissue.[2–4] Such dental pulp–derived adult stem cells (DPSCs) have exhibited multilineage potential and were

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able to differentiate toward odontoblast-like cells, adipocytes, myocytes, and neural cells.[2–6] These cells were relatively easy to expand *in vitro*, and could be passaged more than 25 times.[3] However, as a new stem cell source for tissue engineering, the *in vivo* transplantation of such cells in porous calcium phosphate, collagen, or titanium fiber mesh did not show adequate hard-tissue formation.[5,7] These results suggest a hierarchy of progenitors in adult dental pulp, including a minor population of self-renewing, highly proliferative, multipotent stem cells, in a larger compartment of probably more committed progenitors.[5]

For successful tissue-engineering strategies, stem cell technology needs to be optimized. Previous studies have already shown that dental pulp cells could be derived from human or even rat biopsies using STRO-1 cell selection.[4,6,8,9] STRO-1 is described as a marker of mesenchymal stem and precursor cells,[9,10] and selection with this marker increases the ability of osteo- and odontogenic differentiation, as well as development toward other differentiation pathways.[6,9–13]

Another strategy to enhance the efficacy of stem cells could be the addition of growth factors. Recombinant human bone morphogenetic protein (BMP)-2 can stimulate differentiation of dental pulp cells into odontoblasts.[14] The osteoinductive potential of recombinant human BMP-2 has also been confirmed in clinical studies.[15] However, there are some limitations for local delivery of BMP, including short half-life, large dose requirement, high cost, the need for repeated applications, and poor distribution.[16] Possibly, transfer of the *Bmp*2 gene into DPSCs could solve this problem. Recently, several studies have showed that adenoviral-mediated BMP-2 (AdBMP-2)-transfected cells in bone implants were capable of stable expression of BMP-2 and revealed exciting results in ectopic osteoinduction animal models.[17] Furthermore, adenoviruses are well known for efficient delivery of therapeutic genes and have already been applied to clinically relevant gene therapy.[18]

In this study, the differentiation characteristics of transfected STRO-1-selected rat DPSCs with adenoviral-mediated human *Bmp2* gene were evaluated. We hypothesized that *Bmp2*-transfected cells have greater odontogenic differentiation potential than non-transfected cells, even when cultured in medium without dexamethasone or other stimuli.

MATERIAL AND METHODS

Cell culture

STRO-1-selected rat DPSCs (10th passage) obtained using fluorescence-activated cell sorting[9] were expanded in minimal essential medium alpha (α-MEM; GIBCO-BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μg/mL; Sigma, St. Louis, MO). The multilineage potential of this cell population was confirmed in a previous study.[19]

Adenovirus gene transfer

A replication-deficient adenoviral vector carrying the human *Bmp*2 gene AdBMP-2 was used in the current study. First, to acquire effective gene transfer, an optimum multiplicity of infection (MOI) was evaluated. Cells were seeded in a 24-well plate with a density of 2×10^4 cells/well. AdBMP-2 was added to the cells with different MOIs (0, 5, 10, and 100). Forty-eight h after transfection, the presence of BMP-2 in the culture supernatants was tested using a modified BMP-selective reporter bioassay inducing luciferase expression. Luciferase activity was quantified using the Bright-GloLuciferase Assay System (Promega, Madison, WI). For the standard curve, serial dilutions of recombinant human BMP-2 (rhBMP-2; R&D Systems, Abingdon, United Kingdom) were used. The lower detection limit of this assay was 1.6 ng/mL BMP-2.

Second, after achieving the optimum MOI, the cells were plated at the same density in 24-well plates. Twenty-four h after seeding, cells were infected with AdBMP-2 (dilution in α-MEM) at the optimum MOI. The transfected and non-transfected cells were cultured for 24 days in α-MEM-supplemented 10% FCS and 50 μg/mL gentamycin. Culture medium was changed at 3-day intervals.

Cell proliferation and alkaline phosphatase activity

One, 4, 8, 12, 16, and 24 days after gene transfer, the proliferation rates of the cells, as measured according to total deoxyribonucleic acid (DNA) content ($n=6$ for each time point), were assessed using a PicoGreen double strand DNA quantification kit (Molecular Probes, Leiden, The Netherlands).[4] One mL of Milli-Q water (Millipore, Billerica, MA) was added to each well to lyse the cells. One hundred μL of PicoGreen working solution was added to 100-μL supernatants of samples. After incubation, the fluorescence of each sample was measured using a fluorescence microplate reader (Bio-Tek Instruments Inc., Windoski, VT), with a 365-nm excitation filter and a 450-nm emission filter, and the DNA amounts were calculated from a standard curve.

The same supernatants as used for the PicoGreen assay were used to measure cellular ALP activity (Sigma). Twenty μL of 0.5-M 2-amino-2-methyl-1-propanol buffer was added to 80 μL of the samples or standards. Next, 100 μL of substrate solution (p-nitrophenyl phosphate) was added, and the mixtures were incubated at 37°C for 1 h. ALP activity was measured at 405 nm using an enzyme-linked immunosorbent assay microplate reader (Bio-Tek Instruments Inc.).

Calcium content

Four, 8, 12, 16, and 24 days after gene transfer, the rate of calcified extracellular matrix (ECM) deposition was assessed by measuring the calcium content of the samples ($n=6$) using an ortho-cresolphthalein complexone (OCPC, Sigma) method. One mL of 1-M acetic acid was added to each well overnight. Working solution was prepared; 5 mL of

OCPC solution was added to 5 mL of 14.8 M ethanolamine-boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline, and 88 mL of Milli-Q water. Ten μL of sample or standard was added to 300 μL of working solution. Serial dilutions of calcium chloride were used to generate a standard curve. The plate was incubated at room temperature for 10 min and then read at 575 nm (Bio-Tek Instruments Inc.).

Scanning electron microscopy

Cells were cultured on coverslips (Thermanox; Miles Laboratories Inc., Naperville, IL) ($n = 2$) and fixed in 2% glutaraldehyde (5 min) 1, 8, and 16 days after gene transfer. They were then dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Darmstadt, Germany). Gold was sputtered on the samples just before scanning electron microscopy (SEM) analysis. The specimens were examined using a Jeol 6310 scanning electron microscope (JEOL Europe BV, Nieuw Vennep, The Netherlands).

Real-time quantitative polymerase chain reaction

On days 1, 4, 8, and 16 after gene transfer, total ribonucleic acid (RNA) of every sample was isolated using the RNEasy kit (Qiagen, Venlo, The Netherlands). Quantity and quality of the RNA was measured using a spectrophotometer (SmartSpecs Plus, Biorad Laboratories, Veenendaal, The Netherlands). One μg of RNA was used to make complementary DNA (cDNA) using the reverse transcriptase polymerase chain reaction (PCR) method (Invitrogen, Breda, The Netherlands). To perform quantitative PCR, a SYBR Green PCR kit was used (Eurogentec, Liege, Belgium), and reactions were controlled in a spectrofluorometric thermal iCycler (Biorad, Richmond, CA). The odontogenic differentiation was monitored according to six markers: ALP, osteocalcin (OC), collagen type I (Col I), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize RNA expression (Table 1).

DNA was PCR-amplified under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. After the real-time PCR run, a Ct-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\ddot{A}Ct = Ct_{gene} - Ct_{GAPDH}$). Messenger RNA (mRNA) in each sample was calculated using a comparative ÄÄCt ($\ddot{A}Ct_{gene} - \ddot{A}Ct_{control}$) value method. The fold change in gene expression relative to the control was calculated using $2^{-\ddot{A}\ddot{A}CT}$.[20] Each sample was assessed in triplicate.

Oligo microarray

RNA samples from days 0 and 8 were analyzed using the Oligo GEArray Rat osteogenesis microarray (Cat. No. ORN-026; SuperArray, Bioscience Corporation, Frederick, MD). According to the instructions from the manufacturer, an annealing mix was made with 1 μg of RNA to synthesize cDNA. The Amplification Master mix was then added to the cDNA synthesis mix and incubated overnight at 37°C to produce cRNA. After prehybridizing the array membrane with the GEAhyb hybridization solution, 10 μg of cRNA in 750 μl of hybridization buffer was added to the membrane to allow hybridization overnight at 60°C. Afterward, the membrane was blocked for 40 min using GEAblocking solution. Subsequently, 2 mL alkaline phosphatase (AP)-streptavidin (1:7500) was added to the membrane and incubated for 10 min. Finally, the membrane was incubated with 1 mL of CDP-star chemiluminescence substrate at room temperature for 5 min. Then, the membrane was exposed to an X-ray film for detection. The images were analyzed using the GEArray Expression Analysis Suite.

Statistical analysis

Statistical significance in this study was evaluated using paired t testing. $p < 0.05$ was considered significant. Error bars represent means ± standard deviations (SD).

RESULTS

Gene transfer and cell proliferation

The test for MOI showed that cells could be transfected with BMP-2 adenovirus. After 48 h, the transfected cells secreted 4.3, 5.0, 34.5, and 101.9 ng/mL of rhBMP-2 at MOI

Table 1. The Rat-Specific Primer Sequences Used for Real-Time Polymerase Chain Reaction

	Sense primer	*Antisense primer*
Alp	5′-GCTTCACGGCATCCATGAG-3′	5′-GAGGCATACGCCATGACGT-3
OC	5′-GGCTTCCAGGACGCCTACA-3	5′-CATGCCCTAAACGGTGGTG-3′
Col I	5′-TGGAATCTTGGATGGTTTGGA-3′	5′-GCTGTAAACGTGGAAGCAAGG-3′
Dspp	5′-CGGTCCCTCAGTTAGTC-3′	5′-TACGTCCTCGCGTTCT-3′
Dmp1	5′-CGTTCCTCTGGGGGCTGTCC-3′	5′-CCGGGATCATCGCTCTGCATC-3′
Bsp	5′-ACTTCCCTTCGCAAGCTTAGG-3′	5′-AAACTTCCCGCGTATGTTGG-3′
Gapdh	5′-GAAGGGCTCATGACCACAGT-3′	5′-GGATGCAGGGATGATGTTCT-3

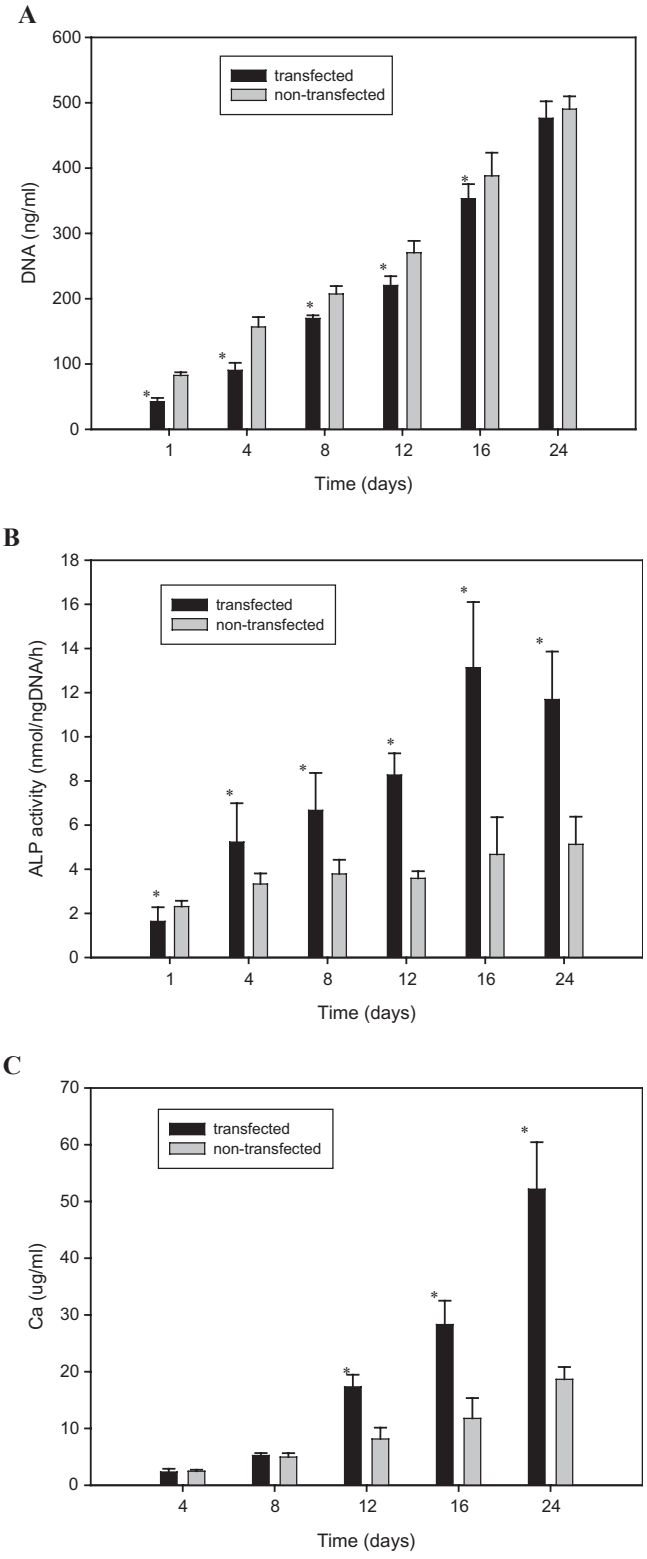

FIG. 1. Growth characteristics of transfected and non-transfected cells cultured in non-osteogenic medium. (**A**) The Proliferation characteristics, (**B**) ALP activity, and (**C**) Calcium content. The bars represent means ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$.

of 0, 10, 50, and 100, respectively. Therefore, in the main study, cells were infected with AdBMP-2 at a MOI of 100. After gene transfer, both populations showed exponential growth for 24 days. Cells grew more rapidly in the non-transfected group than in the transfected group ($p < 0.05$) (Fig. 1A).

ALP activity and calcium content

The transfected cells showed increasing ALP activity until day 16. At day 24, the ALP activity decreased. The transfected cells showed significantly higher levels of ALP activity than non-transfected cells ($p < 0.01$) (Fig. 1B). Finally, significantly higher levels of calcium were observed at days 12, 16, and 24 for the transfected cells ($p < 0.01$) (Fig. 1C).

SEM analysis

One day after transfection, SEM analyses showed that all cells had spread well on the surface of the coverslips. Most of the cells had multiple filopodia, and nuclei and nucleoli could be observed clearly. There were no differences between the 2 groups (Fig. 2A, B). Although no external source of phosphate (no β-glycerophosphate) was added to the cultures, a noticeable amount of mineralized ECM was observed at day 16 in the cultures of transfected cells (Fig. 2C). In contrast, the control cells only formed a confluent layer, without any obvious sign of mineralization (Fig. 2D).

Real-time quantitative PCR

Expression levels of mRNA for *Alp*, *Col I*, *OC*, *Bsp*, *Dspp*, and *Dmp1* were compared on days 1, 4, 8, and 16. mRNA expression levels of cells before gene transfer (day 0)

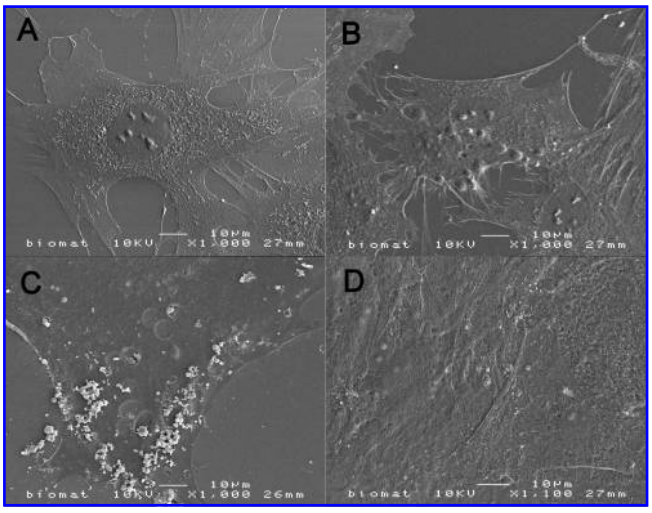

FIG. 2. Scanning electron micrographs of 2 cell populations. (**A**) Transfected cells on day 1, (**B**) non-transfected cells on day 1, (**C**) transfected cells on day 16, (**D**) non-transfected cells on day 16.

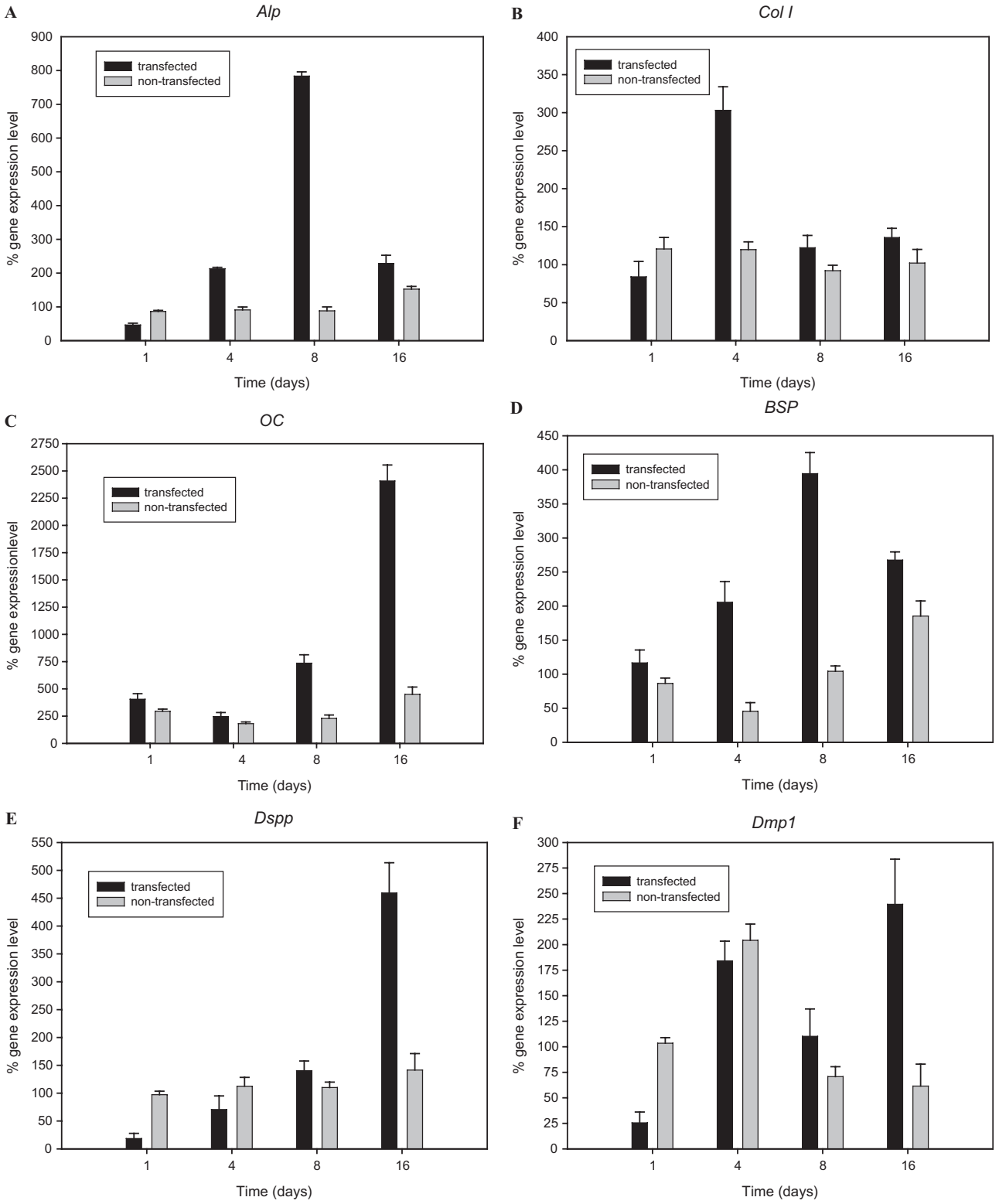

FIG. 3. Representative curves of the messenger ribonucleic acid levels of alkaline phosphatase (Alp) (**A**), collagen type I (Col 1) (**B**)=,; osteocalcin (OC) (**C**), bone sialoprotein (Bsp) (**D**), dentin sialophosphoprotein (Dspp) (**E**), and dentin matrix acidic phosphoprotein 1 (Dmp1) (**F**) quantified on day 16. Cells were from s cell fractions.

were set as baseline control (relative expression value, 100%). Results showed that *Alp* was gradually up-regulated in transfected cells approximately 0.46-, 2.1-, 7.8-, and 2.9-fold on days 1, 4, 8, and 16 respectively. In contrast, non-transfected cells showed no up- or down-regulation of *Alp* on these days (Fig. 3A). *Col I* showed up-regulation for the transfected cells only on day 4 (3-fold) (Fig. 3B). *OC* showed some up-regulation on days 8 (7.4-fold), and 16 (24-fold) in transfected cells and minimal increase in non-transfected cells (Fig. 3C). *Bsp* also revealed up-regulation of transfected cells on days 4 (2.1-fold), 8 (3.9-fold), and 16 (2.7-fold) (Fig. 3D). The expression of *Dspp* and *Dmp1*, two dentin-specific markers, showed similar profiles. They were down-regulated on day 1 (0.19- and 0.26-fold, respectively). On day 4, *Dmp1* showed upregulation in transfected cells, whereas *Dspp* showed no differences. On day 16, *Dspp* and *Dmp1*, were up-regulated approximately 4.6- and 2.4-fold, respectively (Fig. 3E, F).

Gene expression profiles in oligo-microarray

The Oligo GEArray Rat osteogenesis microarray profiled the expression of 113 key genes involved in osteogenic differentiation. This system was used to compare the multiple gene expression profiles representative of the transfected cells and non-transfected cells at day 8 with those of the control cells at day 0. Table 2 shows the up- or down-regulation of the different functional genes. A 2-fold change (or more) in signal density was defined as a relevant change in gene expression level. Thirty-six functional genes that were up-regulated in the transfected cells compared to non-transfected cells (Table 3). For the down-regulated genes, this number was 15. Several genes, belonging to the categories of transcription factors and regulators, growth factors, ECM, and cell proliferation (i.e. *Twist2*, *Csf3*, *Fgf3*, *Col5a1*, *Itga2*, *Smad4*, and *Vcam1)*, were down-regulated in the transfected cells. The results showed that genes involved in skeletal development, bone mineral metabolism, cell growth and differentiation, ECM molecules, cell adhesion molecules, and transcription factors and regulators were more up-regulated in transfected cells than in control and non-transfected cells (Table 2). In particular, bone gamma-carboxyglutamate protein 2 *(Bglap2)*; bone morphogenetic protein receptor, type 1A (*Bmpr1a*), *1b*, and *2*; *Biglycan*; *Anxa5*; *Bmp1* to *7*; *Col4a1*, *Matrix Gla protein*; Msh homeo box homolog 2 (*Msx2*); and *ameloblastin* showed higher up-regulation in transfected cells than in non-transfected cells (Table 3).

DISCUSSION

Gene therapy has shown potential for dental tissue engineering. In previous studies, *Bmp7* and *Bmp11* have been applied in *in vitro* gene therapy and resulted in reparative

Table 2. Functional Gene Expression in Transfected and Non-Transfected Cells on Day 8, Compared With Control Cells on Day 0

		Transfected		*Non-transfected*	
Major category	*Subcategory*	*Up*	*Down*	*Up*	*Down*
Skeletal development	Bone mineralization	7			1
	Cartilage condensation	4		2	1
	Ossification	9		2	
	Osteoclast differentiation	1		1	1
	Other genes involved	7		2	1
Bone mineral metabolism	Calcium ion binding and homeostasis	9		3	3
	Phosphate transport	5	1		3
Cell growth and differentiation	Regulation of the cell cycle	5	1	5	
	Cell proliferation	8	1	5	
	Growth factor and receptors	21	2	7	4
	Cell differentiation	7	1	3	
Extracellular matrix (ECM) molecules	Basement membrane constituents	2			1
	Collagens	1	1		1
	ECM protease inhibitors	2			
	ECM proteases	5	1	3	1
	Structural constituents of tooth enamel	2		1	
	Other ECM molecules	24	3	7	4
Cell adhesion molecules	Cell-cell adhesion			1	
	Cell-matrix adhesion	1	1		
	Other cell adhesion molecules	5	1		2
Transcription factors and regulators	Transcription factors and regulators	7	2	4	

Table 3. Upregulation-Expression Genes in Transfected Cells

Ratio	*Symbol*	*GenBank*	*Unigene*	*Description*	*Functional Category*
23.2	*Bglap2*	NM_013414	9722	Bone gamma-carboxyglutamate protein 2	Bone mineralization, Calcium ion binding and homeostasis, Osteoclast differentiation, Cell adhesion
18.19	*Bmpr1a*	NM_030849	88925	Bone morphogenetic protein receptor, type 1A	Receptor, Extracellular matrix
10.94	*Bmpr1b*	XM_227759	124805	Bone morphogenetic protein receptor, type 1B	Cartilage condensation, Receptor
10.47	*Bgn*	NM_017087	783	Biglycan	Extracellular matrix
9.35	*Anxa5*	NM_013132	3318	Annexin A5	Calcium ion binding and homeostasis
7.88	*Bmp1*	XM_573814	9305	Bone morphogenetic protein 1	Calcium ion binding andhomeostasis, Growth factor, ECM protease
7.4	*Bmp7*	XM_342591	18030	Bone morphogenetic protein 7	Skeletal development, Phosphate transport, Growth factor, Extracellular matrix
5.9	*Col4a1*	XM_214400	53801	Procollagen, type IV, alpha 1	Phosphate transport, Collagen, Basement membrane constituent
4.14	*Map*	NM_012862	2379	Matrix Gla protein	Bone mineralization, Ossification, Calcium ion binding and homeostasis
3.89	*Msx2*	NM_012982	10414	Msh homeo box homolog 2 (Drosophila)	Transcription factors and regulator
3.44	*Ambn*	NM_012900	11370	Ameloblastin	Bone mineralization, Ossification, Structural constituent of tooth enamel
3.4	*Bmp3*	NM_017105	53974	Bone morphogenetic protein 3	Growth factor
3.3	*Bmp2*	NM_017178	90931	Bone morphogenetic protein 2	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
3.29	*Madh7*	NM_030858	29980	MAD homolog 7 (Drosophila)	Transcription factors and regulator
3.02	*Cd36*	XM_216076	102418	Cd36 antigen	Cell adhesion
2.88	*Bmpr2*	XM_217409	59276	Bone morphogenic protein receptor, type II	Receptor, Extracellular matrix
2.83	*Spp1*	NM_012881	8871	Secreted phosphoprotein 1	Bone mineralization, Ossification, Growth factor, Cell proliferation and differentiation, Extracellular matrix, Cell-matrix adhesion
2.81	*Ahsg*	NM_012898	32083	Alpha-2-HS-glycoprotein	Bone mineralization, ECM protease inhibitor
2.72	*Bmp6*	NM_013107	40476	Bone morphogenetic protein 6	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
2.7	*Col12a1*	XM_243912	11218	Procollagen, type XII, alpha 1	Phosphate transport, Extracellular matrix, Cell adhesion
2.7	*Bmp4*	NM_012827	10318	Bone morphogenetic protein 4	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
2.66	*Sparc*	NM_012656	98989	Secreted acidic cysteine rich glycoprotein	Calcium ion binding and Homeostasis, Basement membrane constituent
2.63	*Igf2*	NM_031511	118681	Insulin-like growth factor 2	Growth factor
2.6	*Fgfr2*	XM_341940	12732	Fibroblast growth factor receptor 2	Bone mineralization, Cell proliferation, Extracellular matrix
2.6	*Smad2*	NM_019191	2755	MAD homolog 2 (Drosophila)	Cell differentiation, Transcription factors and regulator
2.58	*Smad3*	NM_013095	10636	MAD homolog 3 (Drosophila)	Cell proliferation, Transcription factors and regulator
2.49	*Calcr*	NM_053816	10062	Calcitonin receptor	Calcium ion binding and Homeostasis, Extracellular matrix
2.46	*Smad1*	NM_013130	10635	MAD homolog 1 (Drosophila)	Bone mineralization, Transcription factors and regulator
2.45	*Col9a1*	XM_223124	90726	Procollagen, type IX, alpha 1	Phosphate transport, Extracellular matrix, Cell adhesion
2.43	*Alpl*	NM_013059	82764	Alkaline phosphatase, tissue-nonspecific	Ossification
2.31	*Ibsp*	NM_012587	9721	Integrin binding sialoprotein	Ossification, Extracellular matrix, Cell adhesion
2.31	*Bmp5*	XM_236415	7994	Bone morphogenetic protein 5	Skeletal development, Phosphate transport, Growth factor, Extracellular matrix
2.21	*Dspp*	NM_012790	21392	Dentin sialophosphoprotein	Ossification, Extracellular matrix
2.16	*Mmp10*	NM_133514	9946	Matrix metallopeptidase 10	ECM protease
2.1	*Dmp1*	NM_203493	19340	Dentin matrix protein 1	Ossification, Extracellular matrix
2.04	*Fgfr3*	NM_053429	23671	Fibroblast growth factor receptor 3	Cell differentiation, Extracellular matrix

dentin formation *in vivo*.[21,22] However, no study has investigated the differentiation efficacy of *Bmp2* gene transfected dental pulp stem cells. The current study showed that STRO-1-selected rat DPSCs could be transfected with AdBMP-2 and that the transfected cells secreted human BMP-2 in high levels ($\sim$102 ng/mL) after 48 h. Evidently, BMP-2 accumulated in this study to a functional concentration to induce cell differentiation *in vitro*.[14,23,24] Although only the BMP-2 protein expression at 48 h was tested, other studies have showed that the adenovirus also has the ability to mediate high levels of gene expression in cells for incubation times longer than 3 weeks.[25]

In our study set-up, we specifically chose cell-culturing conditions without external addition of dexamethasone. Stem cells cultured with dexamethasone before implantation have demonstrated greater bone formation but over a shorter length of time. The result of culturing cells with osteogenic supplements may mask part of the benefit of BMP-2 produced from our genetically modified cells.[26]

In our results, we first addressed proliferation characteristics. Previous studies have indicated that an adenovirus dose of 10^7 particle units or an MOI of more than 50 can provoke a cytotoxic response to the infected cells.[27,28] In our study, the transfected cells showed exponential growth, although there was a small delay for cell proliferation in the early phase. This delay can be due to a hampering effect of the transfection or the inverse relationship between proliferation and differentiation. When cells are more proliferative, then the differentiation of the cells is slowed, and vice versa.[29]

ALP is a prerequisite for the differentiation of dental pulp *in vivo*. In our study, transfected DPSCs showed significantly higher ALP activity rates than non-transfected cells, which was analogous to that of cells under the influence of a standard osteogenic medium.[4] The real-time PCR results confirmed the up-regulation of ALP expression. However, the expression of mRNA always occurs before protein expression, and thus it is understandable that mRNA expression of ALP reached a peak on day 8, whereas ALP activity in the biochemical assay showed a peak value on day 16. Calcium content and SEM observation demonstrated more calcified matrix deposition for transfected cells. The transfected cells showed the ability to form multilayers and mineralized nodules on a smooth surface *in vitro*, even without the addition of an external source of phosphate.

Collagen type I is the predominant protein and the basis for dentin repair. Some noncollagenous proteins in dentin and bone include OC and BSP. In addition, DSPP is the major part of noncollagenous proteins in dentin and plays a crucial role during dentin mineralization.[30] DMP-1 is another specific protein in dentin and is a candidate gene for dentinogenesis imperfecta.[31] Although the different genes showed different mRNA expression profiles, gene expression was enhanced after AdBMP-2 transfection. This change was comparable with that of DPSCs under osteogenic culture or treated with rhBMP-2 protein.[2,4,14] The expression of these markers offers strong evidence that DPSCs differentiated into odontoblasts, because of the presence of BMP-2 protein originating from the adenoviral-mediated *Bmp-2* transfer.

In two previous studies, representative gene expression profiles and functional classifications of human DPSCs and mesenchymal stem cells (MSCs) from bone marrow were compared using a cDNA microarray system containing more than 4,000 or 12,814 human genes.[32,33] Human DPSCs and MSCs were found to have similar levels of gene expression for more than 4,000 known genes. A few genes, including Collagen type XVIII α1, insulin-like growth factor-2, discordin domain tyrosine kinase 2, and cyclin-dependent kinase 6, were more highly expressed in DPSCs.[32] Furthermore, the studies revealed that, upon prolonged culturing, each cell type regulates the expression levels for several genes belonging to cell division, cell signalling, cell structure, and metabolism, differently.[33] In the present study, the results of the functional oligo-microarray for rat osteogenesis showed that the transfected cells had higher expression levels of functional genes. These genes were divided into several functional categories, including skeletal development, bone mineral metabolism, cell growth and differentiation, ECM molecules, cell adhesion molecules, and transcription factors and regulators. The high expression levels of *Alp*, *Dspp,* and *Dmp1* in the transfected cells confirmed the PCR and ALP activity results of our study. Furthermore, BMP-2, -4, -6, and -7, which are strong promoters of osteogenesis, mineralization formation, and tooth morphogenesis,[17,34] showed markedly greater expression levels than the non-transfected cells. The higher expression levels for the BMP receptors (*Bmpr1a*, *Bmpr1b,* and *Bmpr2*) in transfected cells are probably a logical result of the higher levels of secreted BMP-2. Furthermore, the increase in protein expression of BMP-2 results in signals from the plasma membrane to the nucleus requiring several SMAD proteins.[35] This could explain the upregulation for *Smad 1*, *2*, *3*, and *7* in the transfected cells. In the present study, *Msx2* was also up-regulated after transfection. This is noticeable because Shi *et al.* described that DPSCs and BMSCs expressed *Msx2* was at a low levels.[36] The BMP–Msx pathway mediates reciprocal interactions between the epithelium and mesenchyme during early bone and tooth development. BMP-4 is known to induce *Msx2* expression in cultured dental mesenchyme.[37]

Two final genes that were interestingly up-regulated were *Bglap2* and *Ambn*. *Bglap2*, encoding a highly conserved protein associated with mineralized bone matrix and secreted by calcified tissues, was highly expressed in transfected cells, and *Ambn* encodes ameloblastin, which acts as a structural component of tooth enamel matrix.[35] This gene manifested up-regulation only in transfected cells. Generally, more active odontogenesis-related genes were undoubtedly found in the transfected cells, meaning that these cells were more

directed toward the odontogenic pathway.

In summary, rat STRO-1-selected dental pulp stem cells were successfully transfected with AdBMP-2. Transfected cells underwent more effective induction toward the odontoblast phenotype than non-transfected cells, even in medium without externally added substitutes (dexamethasone, β-glycerophosphate). The results of this study were in accordance with our hypothesis, and the combination of a more homogeneous cell population with gene transfection could be a strategy to improve the clinical efficacy of DPSCs for hard tissue engineering. To prove that presence of BMP is a denominator for odontogenic differentiation, and thus to reconcile previous discordant reports, animal studies are necessary. Therefore, further evaluation should be performed in 3-dimensional scaffolds *in vitro* and *in vivo*.

ACKNOWLEDGMENTS

The authors thank W.J.E.M. Habraken and E.L. Vitters for technical assistance. SEM was performed at the Microscopic Imaging Centre of the Nijmegen Centre for Molecular Life Sciences, The Netherlands. This work was supported by the Royal Netherlands Academy of Arts and Sciences (KNAW; No.06CDP030).

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