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Osteoblast differentiation of rabbit adipose-derived stem cells by polyethylenimine-mediated *BMP-2* gene transfection *in vitro*

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ABSTRACT. The aim of this study was to examine the efficiency of polyethylenimine-mediated transfection of the human bone morphogenetic protein-2 (*BMP-2*) gene into rabbit adipose-derived stem cells (ADSCs), and its effect on osteoblast differentiation. Adipose tissue was isolated from the necks of adult Japanese white rabbits and cultured *in vitro* to obtain ADSCs. Gene delivery of *BMP-2* was mediated by polyethylenimine and stable transformants were selected by G-418. The expression of *BMP-2* mRNA was confirmed by reverse transcription-polymerase chain reaction, and of the BMP-2 protein by ELISA. Osteocalcin and collagen type I were detected by

western blot and by an alkaline phosphatase kit. Alizarin red S stain was also utilized to examine osteogenesis. The non-transfected group was considered as a control. In this study, we successfully derived ADSCs from rabbit adipose tissue. Through passages 3-6, the expression of CD29 and CD44 gradually increased, whereas the expression of CD34 and CD45 gradually decreased. Both mRNA and protein expression of BMP-2 were confirmed following polyethylenimine-mediated *BMP-2* gene delivery. In addition, the expression of alkaline phosphatase, osteocalcin, and collagen type I was found to be upregulated and alizarin red S staining was positive in transfected ADSCs, indicating BMP-2-induced osteogenesis. Therefore, this study determined that polyethylenimine was able to mediate *BMP-2* gene delivery and induce osteogenic differentiation of ADSCs.

Key words: BMP-2; Polyethylenimine; Adipose-derived stem cells; Gene delivery

INTRODUCTION

The use of adult stem cells has been an active research topic in regenerative medicine. By far the most studied adult stem cells have been bone marrow mesenchymal stem cells (BMSCs). However, several shortcomings limit the usage of BMSCs in patients, such as the difficulty of stem cell extractions, the limited availability of donors, and the issue of aging in stem cells. In seeking alternative sources of stem cells, studies have found that cells extracted from fatty tissues are also pluripotent, and therefore have called these cells adipose-derived stem cells (ADSCs) (Zuk et al., 2001). Compared to BMSCs, ADSCs are more abundant, and can be easily extracted from a variety of sources in a less invasive manner, therefore making ADSCs ideal candidates for tissue engineering.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β superfamily. Studies have shown that BMPs could induce osteogenesis in non-bone tissues, thus playing important roles in bone development and regeneration. The BMP family comprises at least 20 members. Among them, BMP-2 is considered to be the most active, capable of upregulating osteopontin expression and in itself sufficient to induce osteogenic differentiation (Riley et al., 1996).

The key to gene transfection is to choose the appropriate carrier for effective gene delivery into the designated tissues. Commercially available gene carriers (or vectors) include viral and non-viral vectors. Due to the concerns in cost and safety, viral vectors are of limited use in clinical applications. On the other hand, in recent years, non-viral vectors have been increasingly used in gene therapy with high degrees of efficiency and safety, low cost, and ease of large scale production. Of these, polyethylenimine (PEI), a new polymeric cationic gene vector, has gained increasing attention. However, there have been no reports demonstrating the effective mediation of *BMP-2* gene delivery into ADSCs using PEI. Thus, in the present study, we examined the transfection efficiency of PEI-mediated *BMP-2* into rabbit ADSCs, and assessed its effect on ADSC osteogenic differentiation. The results of this study might provide vital experimental data to further our understanding of bone regeneration.

MATERIAL AND METHODS

Material

PEI (13 kDa) was obtained from Sigma Aldrich (St. Louis, MO, USA). A green fluorescent protein expression plasmid (pEGFP-N1) was purchased from Clontech (Palo Alto, CA, USA). The Lipofectamine™ 2000 transfection kit was from Invitrogen (Carlsbad, CA, USA). The plasmid extraction kit was from Qiagen (Valencia, CA, USA). *Escherichia coli* strain DH5α was purchased from Shanghai ShiDai Biological Technology Co. (Shanghai, China). The cell culture medium Dulbecco's modified Eagle medium (DMEM) was from Hyclone (Logan, UT, USA), and fetal bovine serum (FBS) was from Gibco (Gaithersburg, MD, USA). CD29, CD34, CD44, and CD45 fluorescent antibodies were purchased from Cahag Laboratories (San Francisco, CA, USA). The alkaline phosphatase (ALP) quantitative detection kit was from Sigma Aldrich, and the RNA extraction kit and one-step reverse transcription-polymerase chain reaction (RT-PCR) kit were from Qiagen. The western blotting detection kit was purchased from Beijing Zhongshan Biotechnology Company (Beijing, China). Finally, the identification of eukaryotic expression plasmid pEGFP-N1-BMP-2 and PCR primers was synthesized by Guangzhou SaiYa Biotechnology Co., Ltd. (Guangzhou, China).

ADSC isolation, culture, and characterization

Adult Japanese white rabbits were anesthetized, followed by routine skin preparation and disinfection. A 5-cm cut was made in their side, and an approximately 10 g fat specimen was extracted. The fat pad was immediately placed on a Petri dish and rinsed several times with phosphate-buffered saline (PBS) to remove visible small blood vessels, capsules, and red blood cells. The adipose tissue was then minced into pieces, and added to 2 volumes of 0.1% type I collagenase for digestion and placed on an oscillation shaker at 37°C for 60 min. An equal volume of culture medium containing 10% FBS was then added to stop digestion. The sample was then passed through a 200-M filter, and centrifuged for 5 min at 1000 rpm. The supernatant was removed and the tissue was resuspended and subsequently cultured in an incubator with 5% CO_2 at 37°C. The culture medium was initially changed after the first 2 days, then once every 3 days. Cell morphology and growth were observed under an inverted microscope.

Flow cytometry was used to detect the ADSC surface markers. ADSCs were collected at passages 3 and 6, washed once by PBS, then put into five Eppendorf tubes at a concentration of 1 x 1010 cells /L. Four tubes were incubated with 20 μL fluorescence-labeled monoclonal anti-rabbit antibody (CD29, CD34, CD44, or CD45 with dilution of 1:200 for all) for 30 min, and then cells were fixed with 10% paraformaldehyde (PFA).

BMP-2 transfection

Grouping

Cells were grouped into study cohorts as follows: group A was transfected with pEGFP-N1-BMP-2; group B was transfected with pEGFP-N1; and group C was not transfected. Each group contained 10 samples.

Transfection and selection

After three passages, ADSCs were seeded onto 24-well plates at a density of 2 x 10^4 cells per well for 24 h. Cells were washed briefly with PBS before transfection. pEGFP-N1-BMP-2 and pEGFP-N1 plasmids (1 μg) were diluted in 50 μL serum-free antibiotics-free medium, and mixed with equal volumes of PEI prepared in PEI/DNA nano-complex with a preset N/P value according to manufacturer instruction. The complex was then added into each well and incubated for 4 h. The medium was aspirated and ADSCs were washed briefly by PBS, followed by addition of new medium containing 10% FBS. The ADSCs were cultured for another 48 h, followed by immunohistochemistry and fluorescent imaging. For cell selection, DMEM containing G-418 (400 μg/mL) and 15% FBS was introduced onto the 24-well plates and cells were cultured and passaged for 2 weeks. Surviving cells were further selected with DMEM containing 200 μg/mL G-418 and 5% FBS for another 4 weeks.

Detection of BMP-2 after transfection

RT-PCR for detection of BMP-2 mRNA

After G-418 selection, total mRNA was extracted from cultured ADSCs using TRIzol (Invitrogen) according to instructions. RNA reverse transcription reaction was performed sequentially adding the following reagents: RNA solution 8 μL, Oligo (dT), ribonuclease inhibitor 0.5 μL, 5X first-strand buffer 4 μL, ribonuclease inhibitor 0.5 μL, dNTP mix 2 μL, 0.1% DEPC-treated water 2 μL, MMLV Reverse Transcriptase 1 μL. The reaction conditions were 37°C for 60 min; 90°C for 5 min; cooled immediately on ice for 5 min. The product was set at -20°C. For PCR analysis the following reagents were added sequentially: template cDNA 10 μL, 10X buffer 4 μL, $MgCl_2$ 3 μL, dNTP 0.6 μL, BMP-2 (Primer1 upstream) 1.2 μL, BMP-2 (Primer2 downstream) 1.2 μL, β-actin1 (upstream) 0.4 μL, β-actin2 (downstream) 0.4 μL, Taq DNA polymerase 1 μL, DEPC water (0.1%) 28.7 μL. The reaction conditions were pre-denaturation at 94°C for 3 min; denaturation at 94°C for 40 s, annealing at 57°C for 45 s, extension at 72°C for 1 min, in a total of 30 cycling; and a last cycle extended at 72°C for 7 min. After the end of the reaction, the product was saved at 4°C and analyzed by 1% agarose gel electrophoresis. *BMP-2* primers were: 5'-GCC CTT TTC CTC TGG CTG AT-3' and 5'-TTG ACC AAC GTC TGA ACA ATG G-3'.

ELISA for detection of BMP-2 protein

The cells in groups A, B, and C were digested and seeded onto 24-well plates. Five wells were used for each group and medium was changed every 3 days. The samples were collected at 3, 7, 14, and 21 days, and then analyzed by ELISA kits according to manufacturer protocol to determine the expression BMP-2.

Osteogenic differentiation of ADSCs after transfection

ALP detection

For transfected and non-transfected ADSCs, cells were seeded onto 24-well plates at

the concentration of 1 x 10^6 cells per well. Cells were collected and prepared with sonication at 3, 5, 7, 10, 14, and 21 days after transfection. ALP detection was performed according to manufacturer instruction.

Osteocalcin (OC) and collagen (type I) detection

Western blot analysis was used to detect OC and type I collagen. ADSCs were collected 7 or 14 days after culture from stable transfections of plasmid pEGFP-N1-BMP-2 (group A), or without transfection (group C). The protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Hybond™-P; Amersham Biosciences, Munich, Germany). Membranes were blocked with 0.2% Tween 20 and 5% non-fat dry milk in PBS. The lysates were then incubated with primary antibodies (1:200) for 2 h at room temperature followed by 3 washes with PBS. A horseradish peroxidase-labeled secondary antibody was then added and incubated for 1 h at room temperature followed by 3 washes with PBS and detected by X-ray film. The results were repeated three times and β-actin was used as internal control.

Alizarin red S stain

ADSCs were analyzed for calcified calcium nodule alizarin red S staining after 21 days in culture. Briefly, cells were washed with 3X PBS and fixed with 10% PFA, followed by two washes of phosphate buffer. Subsequently, 0.1% alizarin Red-Tris-HCl, pH 8.3, dye was added to the sample for 30 min at 37°C, followed by examination by light microscopy.

Statistical analysis

Data were calculated from experiments performed in triplicate and reported as means ± SD. Comparisons were made using either the Student *t*-test or one-way ANOVA *post hoc* tests. Statistical significance was determined at $P < 0.05$.

RESULTS

Characteristics of ADSCs

Flow cytometric analysis showed that after 3 to 6 passages, CD29 and CD44 were upregulated in ADSCs (Table 1). After passage 3 or 6, the percentages of CD29-positive cells were 92.15 or 95.47%, and the percentages of CD44-positive cells were 91.78 or 96.03%, respectively. Conversely, CD34 and CD45 were downregulated in ACSCs (Table 2). The percentages of CD34-positive cells were 4.46% after 3 passages and 1.03% after 6 passages. The percentages of CD45-positive cells were 5.67% after 3 passages and 0.89% after 6 passages. Upregulation of CD29 and CD44, and downregulation of CD34 and CD45 were more marked in late passages (Figure 1).

Expression of *BMP-2* mRNA after transfection

After G-418 selection, transfected ADSCs were analyzed by RT-PCR to determine

the expression of exogenous *BMP-2*. The results showed that *BMP-2* mRNA was expressed in the cells transfected with pEGFP-N1-BMP-2 (group A), but not in the cells transfected with pEGFP-N1 (group B) or in the cells without transfection (group C) (Figure 2).

Table 1. Characteristics of adipose-derived stem cells after three or six passages (%) (means ± SD).

Passage #	Antibodies			
	CD29	CD44	CD34	CD45
3	92.15 ± 3.07	91.78 ± 3.14	4.46 ± 1.01	5.67 ± 1.09
6	95.23 ± 4.12	96.03 ± 4.06	1.03 ± 0.23	0.89 ± 0.04

Table 2. The adipose-derived stem cells activities of adipose-derived stem cells in each group measured at different times (U/L, N = 10, means ± SD).

Groups	7 days	10 days	14 days	21 days
A	39.60 ± 1.80	52.71 ± 3.44	95.14 ± 2.86	51.65 ± 3.21
B	20.23 ± 2.34	29.53 ± 2.62	50.11 ± 2.59	48.47 ± 3.03
C	19.35 ± 1.62	26.69 ± 3.26	48.43 ± 2.60	47.73 ± 2.59

Group **A:** transfected with pEGFP-N1-BMP-2; Group **B:** transfected with pEGFP-N1; and Group **C:** not transfected.

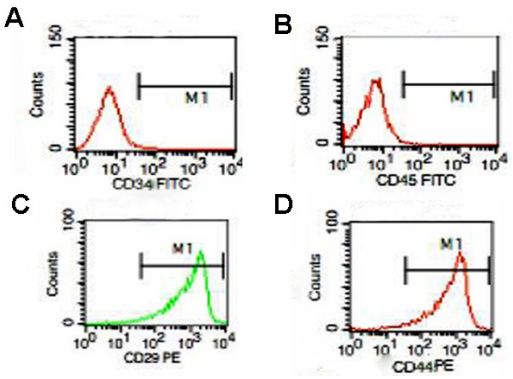

Figure 1. Flow cytometric analysis of adipose-derived stem cells after three passages. **A.** CD34; **B.** CD45; **C.** CD29; **D.** CD44.

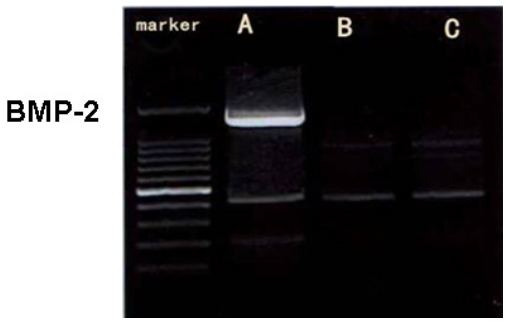

Figure 2. Electropherogram of reverse transcription-polymerase chain reaction showing the expression of *BMP-2* mRNA. *Lane A*, transfected with pEGFP-N1-BMP-2; *lane B*, transfected with pEGFP-N1; *lane C*, not transfected.

Expression of BMP-2 protein after transfection

The levels of secreted BMP-2 proteins in the different groups after 3, 7, 14, and 21 days following transfection are shown in Figure 3. At each time point, the amount of BMP-2 protein in group A was significantly higher than the amounts in groups B or C ($P < 0.05$), and the difference of BMP-2 protein levels was not statistically significant between groups B and C ($P > 0.05$). In group A, the BMP-2 protein expression level at one specific time point was not different than the expression levels at other times ($P > 0.05$). The results suggested that BMP-2 transfection by the plasmid pEGFP-N1-BMP-2 was very stable.

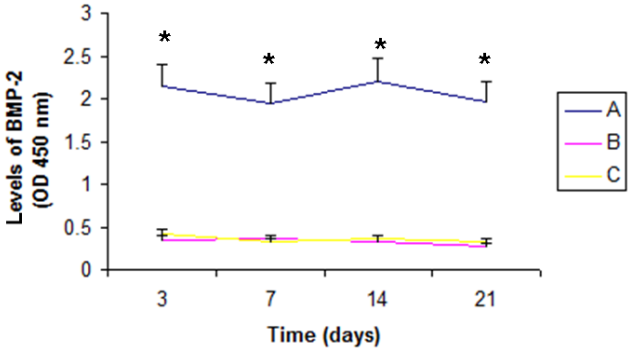

Figure 3. Levels of BMP-2 protein expressed by adipose-derived stem cells in each group measured at different times by ELISA. **A.** Transfected with pEGFP-N1-BMP-2; **B.** transfected with pEGFP-N1; **C.** not transfected. *P < 0.05.

Osteogenic differentiation in ADSCs after BMP-2 transfection

ALP detection

After 3, 5, 7, 10, 14 or 21 days following transfection, ALP tests were performed. The results showed that ALP levels started to increase after 7 days following transfection. The ALP content peaked at approximately 14 days after transfection, then decreased gradually. In contrast, ALP activity remained high in the non-transfected group (Figure 4). The ALP activity in group A was significantly higher than that in groups B or C ($P < 0.05$). The results suggested that transfected cells secreted BMP-2, which induced differentiation of ADSCs towards osteoblasts.

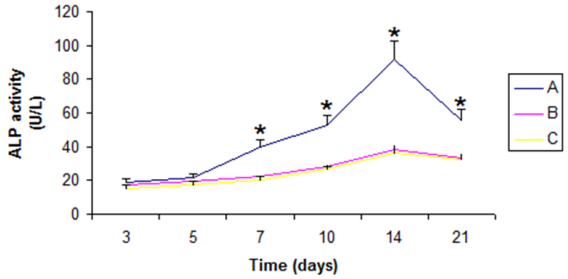

Figure 4. Adipose-derived stem cells (ALP) activities of adipose-derived stem cells in each group measured at different times (U/L). **A.** Transfected with pEGFP-N1-BMP-2; **B.** transfected with pEGFP-N1; **C.** not transfected. *P < 0.05.

OC and type I collagen expression

After 7 and 14 days following transfection, western blot analysis showed that group A ADSCs secreted marked amounts of OC and type I collagen than did the non-transfected group C cells (Figure 5). The results suggested that transfected ADSCs differentiated into osteoblasts.

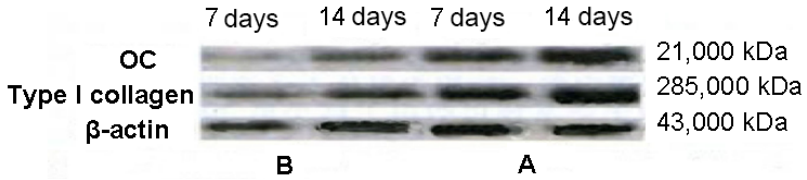

Figure 5. Western blot of osteocalcin (OC) and type I collagen. **A.** Transfected with pEGFP-N1-BMP-2; **B.** not transfected.

Alizarin red S staining

After 21 days following transfections, ADSCs in group A were positively stained by alizarin red S staining, whereas ADSCs in groups B and C were negative for alizarin red S staining (Figure 6). The results indicated that BMP-2 transfection induced osteogenesis in ADSCs.

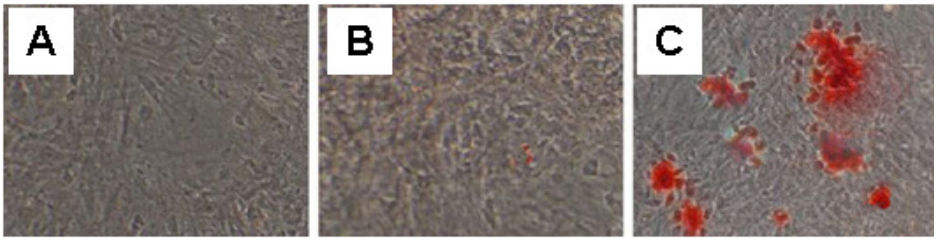

Figure 6. Alizarin red S staining 21 days after transfection. **A.** Not transfected; **B.** transfected with pEGFP-N1; **C.** transfected with pEGFP-N1-BMP-2.

DISCUSSION

Current clinical methods for orthopedic therapies include the use of autologous bone, allografts with free vascularized bone, heterologous bone, and bone grafts. However, none of these therapies is perfect. The dramatic developments in tissue engineering have brought new hope to patients with bone defects, as the implantation of bio-complexes combining stem cells capable of osteogenesis with biologic scaffoldings has the potential to induce bone regrowth and regeneration.

Tissue engineering has three elements (Xu et al., 2002; Zhang et al., 2009): seed cells, transcription factors, and scaffolding. The selection of seed cells is critical for the success of bone transplantation, and the primary choice has been BMSCs. However, there are drawbacks associated with the clinical usage of BMSCs, such as insufficient sources, difficulties in cell extraction, low survival rates, and immune-rejection in the patients. Since 2001, ADSCs have begun to be used as seed cells in tissue-engineering protocols (Zuk et al., 2001). ADSCs are readily available and pluripotent, and can be easily and stably transfected with genes of interest

with little subsequent immune-rejection (Fischer et al., 2009). Our previous studies examined the mechanisms of ADSC differentiation, and successfully demonstrated the isolation of pluripotent ADSCs from fatty tissues (Li et al., 2007; Wang et al., 2012).

Transcription factors are important for inducing differentiation in stem cells. BMPs are members of the transforming growth β superfamily and consist of over 20 isoforms (Wozney et al., 1988). Among these, BMP-2 is especially potent, and is known to induce complete osteogenic differentiation of stem cells. The recombinant product, rBMP-2, is to date the only compound approved by the U.S. Food and Drug Administration for clinical trials for bone defects. However, there are many disadvantages associated with the application of inducible rBMP-2, such as non-specificity, rapid degradation, availability, and costs (Tsuchida et al., 2003), limiting its full potential for clinical application. One method proposed to overcome those drawbacks is to introduce endogenous *BMP-2* through gene transfection (Li et al., 2007).

There are several common gene transfection methods: physical (calcium phosphate co-precipitation, electroporation transfection), chemical (non-viral vector-mediated transfection), and biological (virus-mediated transfection). A commonly used gene transfection vector is adenovirus, which has a high transfection efficiency. However, it is non-specific and leads to high immune reaction in patient tissues (Levy et al., 1998; Winn et al., 2000). Recently, safe and easy-to-produce cationic polymer nanoparticles have been developed as non-viral vectors. Of these, PEI has substantial potential to be used clinically. Previous studies on PEI-mediated cytotoxicity and gene transfection efficiency showed that the application of PEI (13 kDa, high branching) on ADSCs induced minimal cytotoxicity but achieved considerable transfection efficiency (Wang et al., 2013).

There are several methods to evaluate the osteogenic differentiation of stem cells, including cell ALP activity, type I collagen and OC expression, and calcium nodule formation (Owen et al., 1990; Komori et al., 1997). In the present study, we investigated whether PEI could mediate delivery of the *BMP-2* gene into ADSCs and assessed the osteogenic differentiation of transfected cells. The results demonstrated that PEI successfully induced BMP-2 expression in ADSCs, and that the expression of BMP-2 could last as long as 3 weeks. After 7 to 14 days, ALP activity, type I collagen synthesis, and OC secretion were enhanced, indicating a high efficiency of PEI-mediated *BMP-2* transfection and subsequent osteogenic differentiation in ADSCs.

Taken together, PEI-mediated *BMP-2* gene transfection was shown to be highly efficient and induced osteo-differentiation in ADSCs. Further studies will be required to further our understanding of the exact mechanisms of PEI-mediated gene transfection into ADSCs.

Conflicts of interest

The authors declare no conflict of interest.

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