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Modification of human BMSC with nanoparticles of polymeric biomaterials and plasmid DNA for BMP-2 secretion

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ABSTRACT

Background: Genetic modification of human bone marrow stem cells (hBMSCs) before administration to a patient is emerging as a viable approach to creating tailored cells that perform effectively in a clinical setting. To this end, safe delivery systems are needed that can package therapeutic genes into nanoparticles for cellular delivery.

Methods: We evaluated different plasmids on gene expression and compared the effective plasmids directly in hBMSCs. Then, we evaluated the transfection efficiencies of the polymeric carriers linoleic acid-substituted polyethylenimine (PEI-LA), polyethylenimine (PEI)-25, and PEI-2 using flow cytometry. We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to compare the toxicity of PEI-LA and PEI-25 on hBMSCs. We further assessed bone morphogenetic protein-2 (BMP-2) secretion and the osteogenic activity of hBMSCs transfected with the polymeric (PEI-LA and PEI-25) gWIZ-BMP-2 complex.

Results: Unlike the transformed cells that gave robust (>50%) transfection, only a few percent (<10%) of hBMSCs was transfected by the developed nanoparticles in culture. The plasmid DNA design was critical for expression of the transgene product, with the choice of the right promoter clearly enhancing the efficiency of transgene expression. Using the in-house designed PEI-LA, hBMSCs secreted BMP-2 in culture (~4 ng BMP-2/10⁶ cells/d), which indicates the feasibility of using PEI-LA as a delivery system. Furthermore, we demonstrated an increased osteogenic activity *in vitro* for hBMSCs transfected with the PEI-LA containing the BMP-2 expression system.

Conclusions: These results provide encouraging evidence for the potential use of a low toxic PEI-LA to genetically modify hBMSC.

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1. Introduction

Human bone marrow stem cells (hBMSCs) are currently under intense investigation as the basis of cell-based therapies for a variety of orthopedic deficiencies [1,2]. These cells have been the focus of interest in the field of regenerative medicine, not only for their differentiation and self-renewal potential, but also for their high capacity of directional migration and immunomodulatory effects [3,4]. Genetic modification of hBMSCs before administration to a patient (i.e., *ex vivo* gene therapy) has further emerged as an effective approach to create tailored cells that perform more effectively in a clinical setting [5,6]. By acting as a sustained delivery vehicle for desired therapeutic proteins, hBMSCs can direct specific cellular processing, ultimately stimulating the healing process at a bone site [7,8]. The success of gene modification depends largely on the development of a carrier that can efficiently deliver a target gene to hBMSCs with minimal toxicity [9,10]. To this end, nonviral gene carriers have received recent attention because of their safety, flexibility in chemical design, and large capacity for vector delivery [11,12]. Nonviral gene carriers enable condensation of long, string-like plasmid DNA (pDNA) into nanosize particles that allow their cellular internalization. Nonviral carriers are generally less effective for gene transfer to hBMSCs compared with viral systems, however, clinical application of nonviral carriers is safer and can be more widely practiced without concerns about immunogenicity and adverse reactions [13].

The hBMSCs are particularly difficult to transfect with exogenous pDNA, and viral transfections can give consistently high transfection efficiencies in the range of 50%–80% of the cell population [14,15]. Careful optimization of physical delivery methods, such as electroporation, in which electric pulses temporarily allow pDNA entry into cells, has been shown to give similar transfection efficiencies (40%–80%) [16,17]. The cationic lipid Lipofectamine 2000 and the hydrophobic peptide palmitic acid-arginine₁₅ have also been used on hBMSCs; lower transfection efficiencies in the range of 2%–35% [18] and ~10% [19], respectively, have been obtained with these carriers. Although generally considered safer than viral carriers, both electroporation and cationic liposomes have both displayed significant toxicities in cells; therefore, it is necessary to develop new nonviral carriers that are both highly efficient and nontoxic to hBMSCs. We previously reported an amphiphilic polymeric carrier, linoleic acid-substituted polyethylenimine (PEI-LA), which was derived from a nontoxic 2-kDa polyethylenimine (PEI) and the fusogenic lipid linoleic acid [20,21]. The PEI-LA gave superior transgene expression *in vitro* in rat BMSCs using the reporter gene, Green Fluorescent Protein (GFP) [22]. The pDNA/polymer nanoparticles were also effective in a rat subcutaneous implant model [23], in which a bone morphogenetic protein (BMP)-2 expression vector (BMP-2-IRES-AcGFP) was successfully expressed after implantation of the nanoparticles. The critical issue of the effectiveness of this nonviral delivery system for modification of hBMSCs with a therapeutically relevant transgene has not been explored to date.

In this study, we focused on this issue and explored the utility of PEI-LA to modify hBMSCs obtained from patients undergoing orthopedic (arthroplasty) surgery. We first

assessed the transfection efficiency of several expression vectors in immortal 293T cells and hBMSCs. We then investigated the feasibility of BMP-2 expression and osteogenic differentiation in hBMSCs *in vitro* using a custom-tailored expression vector (gWIZ-BMP-2) delivered by PEI-LA.

2. Materials and methods

2.1. Materials

We obtained Dulbecco's modified Eagle's medium (DMEM; high glucose with L-glutamine), Lipofectamine 2000, Opti-mem Hank's balanced salt solution (HBSS), and penicillin-streptomycin (10,000 U/mL to 10,000 µg/mL) from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologics (Lawrenceville, GA). We purchased the CyQUANT cell proliferation kit for DNA assay from Molecular Probes (Portland, OR). We also obtained 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), alkaline phosphatase (ALP) substrate *p*-nitrophenol phosphate, 8-hydroxyquinoline, *o*-cresolphthalein, 2-amino-2-methylpropan-1-ol, dexamethasone, glycerolphosphate, calcium standards kit and ascorbic acid from Sigma (St. Louis, MO). We reconstituted the BMP-2 stock solution (Wyath Pharmaceuticals, Inc.) in ddH₂O. Recombinant human basic fibroblast growth factor (bFGF) was obtained from the Biological Resource Branch of NCI-Frederickton (Bethesda, MD). The human BMP-2 enzyme-linked immunosorbent assay (ELISA) kit was from R&D Systems (Minneapolis, MN). We prepared the polymeric carrier PEI-LA (2.1 LA substitution per PEI) in house, as described previously [21].

2.2. Expression vectors

We obtained the blank (control) gWIZ plasmid and its GFP-expressing derivative gWIZ-GFP from Aldevron (Fargo, ND). Commercially available BMP-2 expression vector pCMV6-XL4-BMP-2 was from Origene (Rockville, MD). Our first BMP-2 expression plasmid (BMP-2-IRES-AcGFP) was derived from the commercially available pIRES2-AcGFP (Clontech, Mountain View, CA), described previously [23]. The PCAG-emerald GFP (emGFP) plasmid, in which the emGFP was subcloned into a plasmid with chicken β-actin promoter and cytomegalovirus (CMV) enhancer, was a kind gift from Dr. Peter Kwan (University of Alberta, Canada). We constructed a new BMP-2 expression vector, gWIZ-BMP-2 (Fig. 1), by subcloning the BMP-2 fragment from pCMV6-XL4-BMP-2 into gWIZ. This is done by restriction digest of both plasmids with the NotI; digested gWIZ was treated with Antarctic phosphatase (New England Biolab, Ipswich, MA) to remove 5'-phosphate and minimize self-re-ligation. Both the vector and insert DNA fragments were purified with the QIAquick PCR Purification Kit (Qiagen, Ontario, Canada) from the enzymatic reactions before ligation with T4 DNA ligase (New England Biolab). We then transformed ligated plasmids into competent *Escherichia coli* cells (DH5α; Life Technologies, Ontario, Canada), according to the manufacturer's protocol, and then grew them onto Luria broth plates supplemented with 30 µg/mL kanamycin.

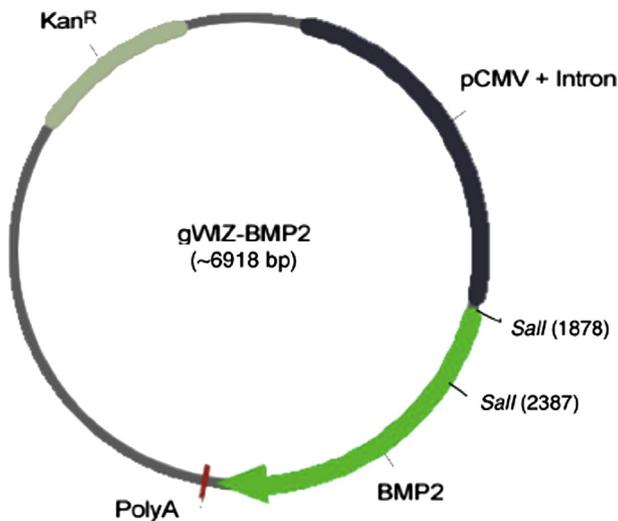


Fig. 1 – Plasmid map for the newly constructed gWIZ-BMP-2. Bone morphogenetic protein-2 from pCMV6-XL4-BMP-2 was sub-cloned into highly effective mammalian expression vector, gWIZ, which contains a modified promoter from the CMV immediate early gene promoter-enhancer. Directionality of the insert was verified by both colony polymerase chain reaction and restriction digest with *Sall*. (Color version of figure is available online.)

Transformants harboring recombinant constructs with the proper insert orientation (i.e., the 5'-end of BMP-2 lies directly downstream of the 3'-end of the pCMV plus Intron promoter in gWIZ) were screened by colony polymerase chain reaction; positive clones were further verified by restriction mapping with *Sall*, which generate asymmetric-size fragments that confirmed the directionality of the insert.

2.3. Cell culture

The hBMSCs were from bone marrow specimens obtained from femoral reaming during total hip arthroplasty procedures (15- to 48-y-old patients) after we obtained informed consent and approval from the institutional health research ethics board [24]. We cultured the cells in DMEM with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin supplemented with 5 ng/mL bFGF-2 for a total of 2 passages, according to Frank *et al* [25]. On confluence, the cells were split 1:3 using 0.08% trypsin/0.04% ethylenediaminetetraacetic acid. We incubated cultures at 37°C with 5/95% CO₂/air. As a control cell line, we cultured 293T human kidney fibroblast cells in the same way (but without bFGF) and used them in the indicated experiments.

2.4. Transfection of hBMSC

We performed a series of experiments to optimize hBMSC transfection protocols using the commercial carrier Lipofectamine 2000 and several polymeric carriers. To prepare carrier/pDNA complexes, we mixed the carriers with pDNAs at desired weight ratios and added them to cells at defined

concentrations (see Legends). With Lipofectamine 2000, we followed the manufacturer's suggestion for complex preparation. With polymeric carriers, we prepared the complexes in 150 mmol/L NaCl at indicated polymer:pDNA ratios and added them to culture medium at indicated concentrations (see Legends). Non-treated (NT) cells served as a control throughout the transfections. We seeded the hBMSC and 293T cells 24 h before transfection and changed culture medium to fresh medium (Opti-MEM I without serum for Lipofectamine 2000 transfections and basic medium for polymer transfections) before complex addition. We then added the complexes added to the cells, centrifuged them at 210 × *g* (5 min), and incubated them for 4–6 h, after which we changed the transfection medium to basic medium supplemented with serum and antibiotics. At indicated times (see Legends), we harvested the cells by trypsinization for flow cytometric assessment of GFP expression (see below). We also collected supernatants collected for BMP-2 analysis by an ELISA assay.

2.5. Bone morphogenetic protein-2 secretion by ELISA

We treated the manufacturer-supplied 96-well plates with BMP-2 capture antibody (1 µg/mL) for 8 h and blocked them with 1% bovine serum albumin overnight at 4°C. Then, we added 100 µL of the standards (in Opti-MEM plus 1% FBS) or samples to each well in duplicate and incubated them at room temperature for 2 h. Each well was aspirated and washed (four times) with 0.05% Tween-20 in phosphate-buffered saline. We added 100 µL of 0.5 µg/mL detection antibody to the wells and incubated it at room temperature for 2 h. After aspiration and washings, we added streptavidin–horseradish peroxidase to each well for 30 min. After a final wash, we added FAST OPD substrate solution (Sigma) was added to the wells and incubated them at room temperature for 30 min. We determined the absorbances by a microplate reader at 450 nm and converted them into a BMP-2 concentration (ng/mL) using the standard curve.

2.6. Flow cytometry for GFP analysis

We trypsinized cells and fixed them with 3.7% formaldehyde in clear (i.e., without phenol red) HBSS. We quantified the GFP expression by Beckman Coulter QUANTA SC flow cytometer (Brea, CA) using the FL1 channel, using the 488-nm blue laser. We calibrated instrument settings for each run to obtain a background level of GFP expression of 1%–2% for the untreated control cells. The mean fluorescence per cell for the total population or GFP-positive cell population was also determined [21].

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cytotoxicity

Briefly, we added 100 µL MTT solution (dissolved in clear HBSS at 5 mg/mL) into each well containing cells with 0.5 mL medium. After 2 h, we removed the medium and added 500 µL dimethyl sulfoxide to the wells to dissolve the formed MTT formazan crystals. We then measured the absorbance at 570 nm and used it as a relative measure of total cell activity [21]. We compared the MTT absorbance of transfected hBMSCs

with NT controls (100% viability) and expressed the viability of transfected cells as a percentage of untreated cells. We performed MTT assays at 24, 48, and 72 h after transfection.

2.8. Osteogenic activity of transfected hBMSCs

We determined the DNA content, ALP activity, and calcification in hBMSC cells as a measure of osteogenic activity. The hBMSC were grown in 24-well plates for this purpose and transfected with desired complexes. After complex removal, the cells were cultured in an osteogenic medium (DMEM supplemented with 10% FBS, 10 nmol/L dexamethasone, 10 mmol/L β -glycerolphosphate, and 50 mg/L ascorbic acid). At indicated times (see Results), we washed the cells with HBSS (two times) and lysed them with ALP buffer (0.5 mol/L 2-amino-2-methylpropan-1-ol and 0.1% Triton-X; pH 10.5) at 4°C. After 2 h, we transferred 150 μ L of lysed solutions into the 48-well plates and added 150 μ L 2 mg/mL ALP substrate (p-nitrophenol phosphate) to the cell lysate. We immediately determined the kinetics of absorbance change (at 405 nm) at 405 nm for up to 15 min.

We froze the remaining cell lysis solution at -20°C and thawed it at a suitable time for DNA analysis. We used the CyQUANT DNA kit for DNA analysis according to the manufacturer's instructions ($\lambda_{\text{abs}} = 480$ nm; $\lambda_{\text{em}} = 527$ nm). We used a DNA standard provided by the CyQUANT kit to estimate the DNA concentrations, which we also used to normalize the ALP activity obtained above.

For calcification, we rinsed the wells containing the hBMSCs (two times) by HBSS and then added 400 μ L 0.5 N HCl to dissolve the mineralized matrix overnight. We used 20 μ L of aliquots from each well to quantify the amount of dissolved calcium using the Sigma diagnostic kit (at 570 nm). We developed a standard curve using the obtained Ca^{2+} standards and used it to convert the obtained absorbance values into Ca^{2+} concentrations (in milligrams per deciliter).

2.9. Statistical analysis

Results are expressed as mean \pm standard deviation (SD). We performed data analysis using one-way analysis of variance with the SPSS software package (Chicago, IL). We used Tukey post hoc testing to determine intergroup variations, following the software directions. Statistical significance was determined at $P < 0.05$.

3. Results

3.1. Effect of plasmid on transgene expression in hBMSC and 293T cells

An initial study compared the transfection efficiency in hBMSCs with that of easy-to-transfect 293T cells, which are extensively used to evaluate gene delivery systems. Because our intent was to compare the efficiency of different pDNAs in these cell types, we used the commercial carrier Lipofectamine 2000 in this experiment. Figure 2 shows the results from flow cytometric analysis of GFP expression. We obtained a significantly higher percentage of GFP-positive cells for cells

treated with gWIZ-GFP and PCAG-emGFP plasmids, compared with all groups ($P < 0.05$) (Fig. 2A). The highest percentage of GFP-positive cells was $\sim 5\%$ in hBMSCs. The mean fluorescence of the hBMSC population was also higher for gWIZ-GFP and PCAG-emGFP-treated cells (Fig. 2B). Fluorescent microscopic analysis confirmed the results from flow cytometry (Fig. 1S), in which cells treated with gWIZ-GFP and PCAG-emGFP plasmids were readily observed to express GFP.

Figure 2C and D shows the results from 293T cells. We observed the highest percentages of transfected cells (80%–90%) for gWIZ-GFP and PCAG-emGFP plasmids ($P < 0.05$ versus all groups), followed by IRES-AcGFP-BMP-2 ($P < 0.05$ versus the rest of the groups) and then IRES-AcGFP ($P < 0.05$ versus NT, gWIZ blank, and pCMV6-XL4-BMP-2). We observed the highest mean fluorescence in cells transfected with PCAG-emGFP and gWIZ-GFP plasmids ($P < 0.05$ compared with all groups) (Fig. 2D), followed by BMP-2-IRES-AcGFP ($P < 0.05$ compared with the rest of the groups). Fluorescent microscopy results with 293T cells (Fig. 1S) were also consistent with the flow cytometry findings.

3.2. Comparison of effective plasmids in hBMSCs

We compared the effective plasmids gWIZ-GFP and PCAG-emGFP directly in hBMSC (Fig. 3). An increase in transfection was evident with increasing plasmid concentration exposed to the cells. Based on the percentage of GFP-positive cells and mean fluorescence of cell population, higher transfection efficiency was evident with gWIZ-GFP plasmid compared with PCAG-emGFP. There was no difference in the mean fluorescence of transfected cells (Fig. 3C), which suggests that gWIZ-GFP was effective in transfecting a higher proportion of cell population rather than enhancing efficiency on a per-cell basis. At the highest concentration of gWIZ-GFP, up to 14% of hBMSCs were positive for GFP ($P < 0.05$ versus all groups). As expected, the control gWIZ plasmid gave no GFP expression ($< 1.5\%$ GFP-positive cells) for all concentrations used. Fluorescent microscopic analysis also confirmed the flow cytometric data (not shown).

3.3. Polymer-mediated transfection in hBMSCs

Using the most effective plasmid gWIZ-GFP, we evaluated transfection efficiencies of the polymeric carriers PEI-LA, PEI-25, and PEI-2 using flow cytometry (Fig. 4). The broadly effective polymeric gene carrier PEI-25 served a reference reagent. Both PEI-LA and PEI-25 at a polymer–pDNA weight ratio of 6:1 or 4:1 resulted in significantly increased transfection in hBMSC ($P < 0.05$ compared with the NT group). The transfection efficiency of PEI-LA was reduced significantly at the polymer–pDNA weight ratio of 2:1. Maximal transfection efficiency was achieved at the polymer–pDNA weight ratio of 6:1. Based on the mean fluorescence of GFP-positive cells, PEI-LA triggered a higher level of transgene expression compared with PEI-25 ($P < 0.05$).

Initial studies with BMP-2 expression were not successful when we attempted to use BMP-2-IRES-AcGFP for transfection; in other words, no BMP-2 secretion was evident (not shown). We therefore constructed gWIZ-BMP-2, because gWIZ-GFP gave superior expression compared with IRES-AcGFP in initial

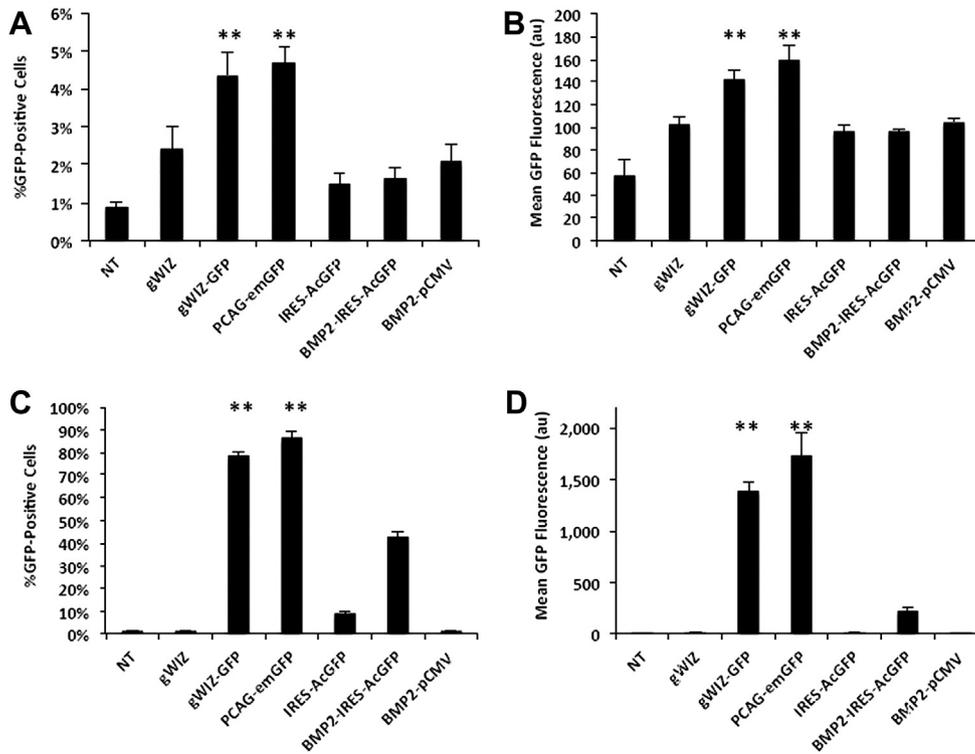


Fig. 2 – Comparison of transfection efficiency between hBMSC (A and B) cells and 293T cells (C and D). The cells were either non-treated (NT) or treated with Lipofectamine 2000 complexes of indicated plasmids (gWIZ, gWIZ-GFP, PCAG-emGFP, IRES-AcGFP, BMP-2-IRES-AcGFP, and BMP-2-pCMV). The complexes were prepared at a 1:1 ratio of Lipofectamine 2000:pDNA and added to the cells at 4 µg/mL. After 4 d, we assessed the transfection efficiency by flow cytometric analysis of GFP expression. Results are expressed as the mean percentage of GFP-positive cell population (A and C) and mean GFP expression per cell (B and D). ***P* < 0.05 versus all groups.

studies (Fig. 1). Figure 5 shows the extent of BMP-2 secretion from the polymer-mediated delivery of gWIZ-BMP-2. Compared with untreated hBMSCs, hBMSCs transfected with PEI-LA and PEI-25 at polymer-pDNA weight ratio of 6:1 resulted in

significant BMP-2 secretion after 2 d (*P* < 0.05). When the amount of BMP-2 secretion was normalized with the number of cells, hBMSCs transfected with the PEI-LA and PEI-25 displayed higher secretion than the no-treatment group (*P* < 0.05).

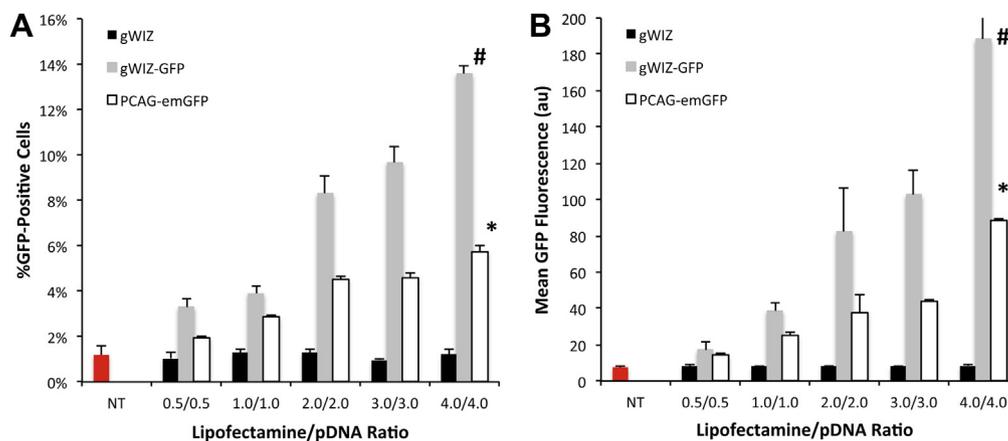


Fig. 3 – Detailed comparison of gWIZ-GFP and PCAG-emGFP plasmids for modification of hBMSCs. The plasmids were delivered by using Lipofectamine 2000 at the indicated ratios (the numbers on the horizontal axis correspond to concentrations of pDNA/Lipofectamine 2000 in micrograms per milliliter/micrograms per milliliter). We investigated GFP expression by flow cytometry 24 h after transfection. Non-treated cells (NT) served as the control. Results are expressed as the percentage of GFP-positive population (A) and mean fluorescence of cell population (B). #*P* < 0.05 for gWIZ-GFP versus PCAG-emGFP; **P* < 0.05 for PCAG-emGFP versus gWIZ. (Color version of figure is available online.)

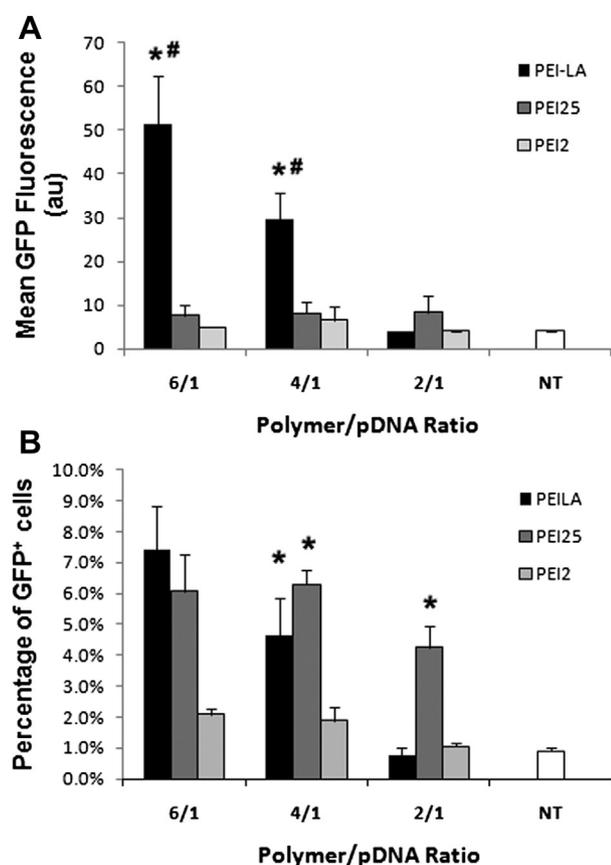


Fig. 4 – Transfection efficiency in hBMSCs mediated with polymeric carriers. We transfected the cells with gWIZ-GFP complexes of PEI-LA, PEI-25, and PEI-2 and analyzed GFP expression by flow cytometry 24 h after transfection. Polymer-gWIZ-GFP ratios used for transfection are indicated in the horizontal axis (as micrograms per milliliter/micrograms per milliliter). (A) Mean GFP fluorescence induced in the cell population. (B) Percentage of GFP-positive cell population. * $P < 0.05$ versus NT (not shown); # $P < 0.05$ versus PEI-25 at the same ratio.

3.4. Toxicity of PEI-LA and PEI-25 in hBMSCs

To evaluate the toxicity of PEI-LA and PEI-25 in hBMSCs, we examined cell viability using the MTT assay at 24, 48, and 72 h after BMP-2 gene transfection. As shown in Figure 6, cells treated with PEI-25 complexes displayed dramatically decreased growth. However, PEI-LA was not obviously toxic in hBMSCs during the study period. Increasing the PEI-LA-pDNA weight ratio from 2:1 to 6:1 did not significantly affect cell viability. The PEI-LA did not change the gross morphology of hBMSCs (Fig. 6B), even at a higher polymer-DNA weight ratio. However, all PEI-25 complexes tested generated pronounced cell debris and morphological changes in hBMSCs.

3.5. Osteogenic activity of hBMSCs transfected with polymeric gWIZ-BMP-2 complex

To assess osteogenesis in hBMSCs, we evaluated the DNA content and ALP activity of the cultures on days 7 or 14, and

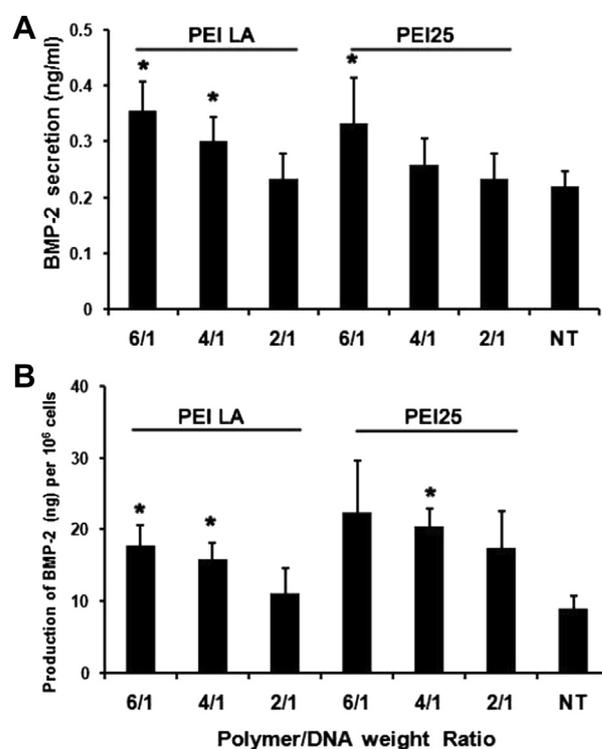


Fig. 5 – Bone morphogenetic protein-2 secretion from hBMSCs modified with PEI-LA and PEI-25 complexes of gWIZ-BMP-2. The polymer-gWIZ-BMP-2 ratios used for transfection are indicated in the horizontal axis (as micrograms per milliliter/micrograms per milliliter). Bone morphogenetic protein-2 accumulation in the tissue culture supernatants was allowed for 2 d after transfection; we determine BMP-2 concentration in the supernatants using an ELISA assay. Secretion is expressed as nanograms per milliliter of medium (A) or nanograms per 10⁶ cells (B) over 2 d. * $P < 0.05$ versus NT.

assessed calcification on days 28 and 35. There was no significant difference in DNA content between the PEI-LA transfected hBMSCs and the untreated hBMSCs (Fig. 7A). The hBMSCs transfected with gWIZ-BMP-2/PEI-LA at polymer-pDNA weight ratios of 6:1 or 4:1 expressed significant higher levels of ALP activity than the untreated hBMSC control (Fig. 7B). Calcium deposition by the PEI-LA transfected hBMSCs at a higher weight ratio (6:1) was significant on day 28 after gWIZ-BMP-2 transduction (Fig. 8). The hBMSCs transfected with gWIZ-BMP-2 using PEI-LA at a polymer-DNA weight ratio of 6:1 or 4:1 induced a marked elevation on day 35 after transfection ($P < 0.05$ compared with the NT group). In addition, alizarin red-S staining on day 35 revealed a significant increase in calcium nodules in transfected hBMSCs with PEI-LA.

4. Discussion

Nonviral carriers have been used for pDNA delivery into hBMSCs; this study pursued PEI-based polymers toward this goal. Polyethylenimine-25 is known to be highly efficient in

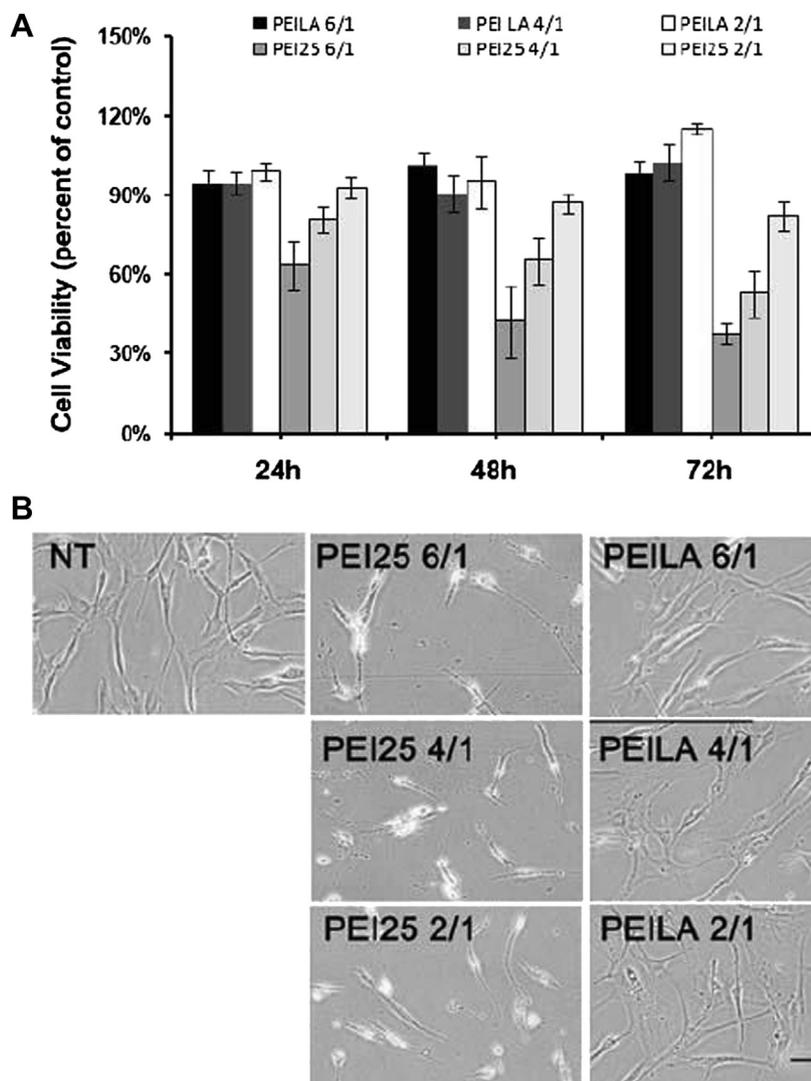


Fig. 6 – Viability of hBMSCs treated with PEI-LA and PEI-25 complexes of gWIZ-BMP-2. Polymer–gWIZ-BMP-2 ratios used for transfections are indicated as micrograms per milliliter/micrograms per milliliter. We performed MTT assays 24, 48, and 72 h post-transfection; cell viabilities obtained are expressed as a percentage of untreated cells (taken as 100% viability [A]). (B) Light microscopy images of treated hBMSCs. Note the significantly reduced cell density for cells treated with PEI-25 complexes.

condensing pDNA and mediating efficient gene transfer without the use of an endosome-disruption component [26]. However, the toxicity of these polymers has been always considered to limit their clinical application [27]. This was shown to be the case here in our head-to-head comparison with the in house–developed PEI-LA; detrimental changes in cell morphology and cell growth were evident at effective doses of PEI-25, but not with the PEI-LA. Based on an analysis of total DNA content in cultures, this study showed that PEI-LA complexes did not significantly affect hBMSC proliferation in long-term culture (Fig. 7A). The present study demonstrated that PEI-LA–mediated transfection was effective in hBMSCs, with an optimized transfection efficiency of ~8%. The transfection efficiency of PEI-LA was equivalent and/or superior to PEI-25, depending on the polymer–DNA weight ratio (Fig. 4). Cellular morphology was similar 24 h after

gene transfection with PEI-LA, compared with unmodified control cells. In addition, the reduction in cell viability in response to PEI-25–pDNA complexes clearly showed that hBMSCs is sensitive to treatment conditions. From a clinical perspective, the cytotoxicity of PEI-25 may induce more necrotic cell debris, which can elicit local inflammation. This concern should lessen with the use of PEI-LA.

Previously, PEI-LA was shown to mediate *in situ* BMP-2 expression at a rat subcutaneous site when it was used to deliver BMP-2-IRES-AcGFP plasmid [23]. This plasmid and its originating plasmid (i.e., IRES-AcGFP) were ineffective when used to modify hBMSC *in vitro*, where the nature of the plasmid was critical to derive a detectable transgene expression. Although a major difference between the two cell types used in this study was evident in the efficiency of transfection, both cell types gave the best transfection with gWIZ-GFP. We

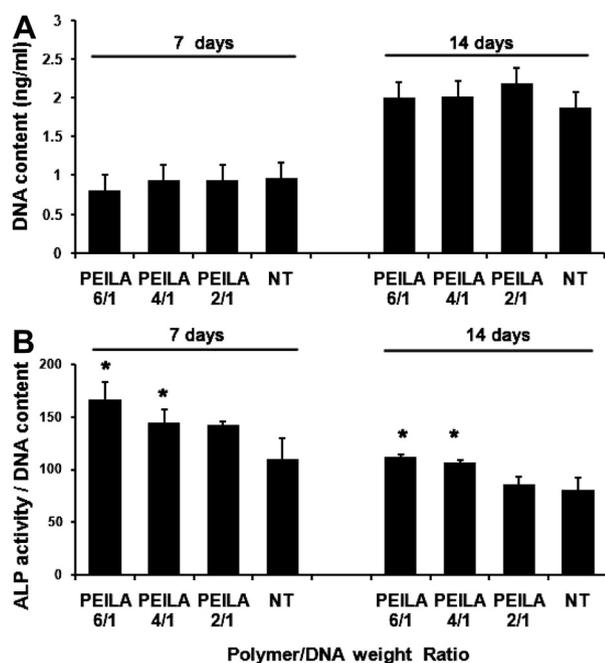


Fig. 7 – DNA content (A) and specific ALP activity (B) in hBMSC cultures treated with PEI-LA complexes of gWIZ-BMP-2. The polymer/gWIZ-BMP-2 ratios used for transfections are indicated as $\mu\text{g}/\text{mL}/\mu\text{g}/\text{mL}$. Transfections with complexes were carried out for 24 hours, after which the medium was changed to OM. The DNA content and ALP activity was determined after 7 and 14 days of post-transfection. * $P < 0.05$ vs. NT.

observed maximal GFP expression 1 d post-transfection, which gradually decreased over the study period. This was similar to the transfection observed in rat BMSC, as reported in a previous study from our group [22]. The most robust expression of the reporter (GFP) gene obtained from gWIZ-GFP led us to prepare a new BMP-2 expression construct: namely, gWIZ-BMP-2. The gWIZ mammalian expression vectors contain a modified promoter from the CMV immediate early gene promoter-enhancer, which had been systemically modified to remove sequences that are deleterious to high levels of expression, followed by intron A from the CMV immediate early gene, coupled with a high-efficiency artificial transcription terminator (information provided by Aldevron, Fargo, ND). Although the details of this modification are not fully known, it seems to be clearly advantageous in deriving transgene expression in hBMSCs.

It is well known that BMP-2 protein has strong osteoinductive activity that has been successfully applied in clinics [28]. However, direct delivery of BMP-2 protein has certain limitations, such as poor distribution, a short half-life, large dose requirements, and high cost, which make regional gene therapy an attractive alternative approach for BMP-2 delivery [29,30]. Transfection of BMP-2 gene into mesenchymal stem cells can stimulate the osteogenic pathway via autocrine and paracrine mechanisms [31–33]. Two feasible strategies for delivering BMP-2 gene into the transplant sites are transplantation of BMP-2-producing hBMSCs and direct

injection of BMP-2 expression systems [34,35]. To this end, delivery of gWIZ-BMP-2 with PEI-LA enabled significant BMP-2 secretion at $\sim 4 \text{ ng}/10^6 \text{ cells/d}$. Although we obtained equivalent secretion with PEI-25, the main reason for this was the reduced cell numbers that artificially enhanced the secretion rate per cell basis. The BMP-2 secretion rate achieved with PEI-LA was comparable to some of the nonviral and viral gene delivery rates reported. Electroporation with neurotrophin-3 gene [36], as well as an adenoviral transfection for BMP-2 and vascular endothelial growth factor [37], gave protein secretion rates of $\sim 3 \text{ ng}/10^6 \text{ cells/d}$ in hBMSCs. A higher rate of $\sim 50 \text{ ng BMP to } 2/10^6 \text{ cells/d}$ was reported after transfection with another adenoviral system [15], which indicates the possibility of further improving BMP-2 secretion rates. Even higher protein secretion rates were achieved in some cases; for example, $\sim 160 \text{ ng EGF}/10^6 \text{ cells/d}$ (with DOPE/DOTAP mixture Escort) [38] and $\sim 375 \text{ ng secreted ALP}/10^6 \text{ cells/d}$ (with electroporation) [16]. It is likely that further improvement in transfection procedures and/or design of the expression vector will be needed to achieve this level of recombinant protein secretion. The absolute level of BMP-2 secretion needed for tissue repair is not known at this stage, but other authors have reported successful bone repair with cells that expressed $\sim 3 \text{ ng}/10^6 \text{ cells/d}$ [37], so our approach appears to satisfy the secretion criteria for *in vivo* bone repair.

To evaluate the influence of BMP-2 transfection on the osteogenic differentiation of hBMSCs, we first examined ALP activity levels in hBMSCs. Alkaline phosphatase is considered to be an early marker for osteoblastic differentiation that becomes up-regulated *in vitro* within 2 wk of osteogenesis [39]. It is not surprising that the higher polymer-pDNA weight ratio led to significantly increased ALP activity, because transfection efficiencies with PEI-LA were observed to increase with increasing weight ratio [21]. We expected BMP-2 transfection to induce *in vitro* calcification in hBMSCs; there was a lack of calcification 21 d after osteogenic induction, but calcification was evident after 28 and 35 d. The induction of calcification in hBMSCs was expected to be closely related to up-regulation of several other markers. For example, bone-sialoprotein and runx2 expression were up-regulated with BMP-2 cultured hBMSCs in our research [40]. However, it is possible for adipogenic markers to be up-regulated as well with BMP-2 transfected hBMSCs [40]. Although there was no visual evidence of adipocyte formation in our cultures in this study, future studies will focus on this issue and will better characterize the molecular changes associated with forced BMP-2 expression in hBMSCs.

Collectively, we have shown that gWIZ-GFP and gWIZ-BMP-2 plasmids are effective expression vectors for hBMSCs and that PEI-LA is an efficient nonviral carrier for pDNA delivery into hBMSCs. Unlike the lipid-based systems, this polymeric carrier has potential for further improvement and can be particularly useful in *in vivo* delivery of osteogenic genes. Bone morphogenetic protein-2 secretion was successfully demonstrated and osteoinduction was induced *in vitro* upon modification of hBMSCs with PEI-LA/gWIZ-BMP-2 plasmid complexes. These studies lay the foundation for further investigation into polymer-mediated gene delivery for bone tissue engineering and bone repair.

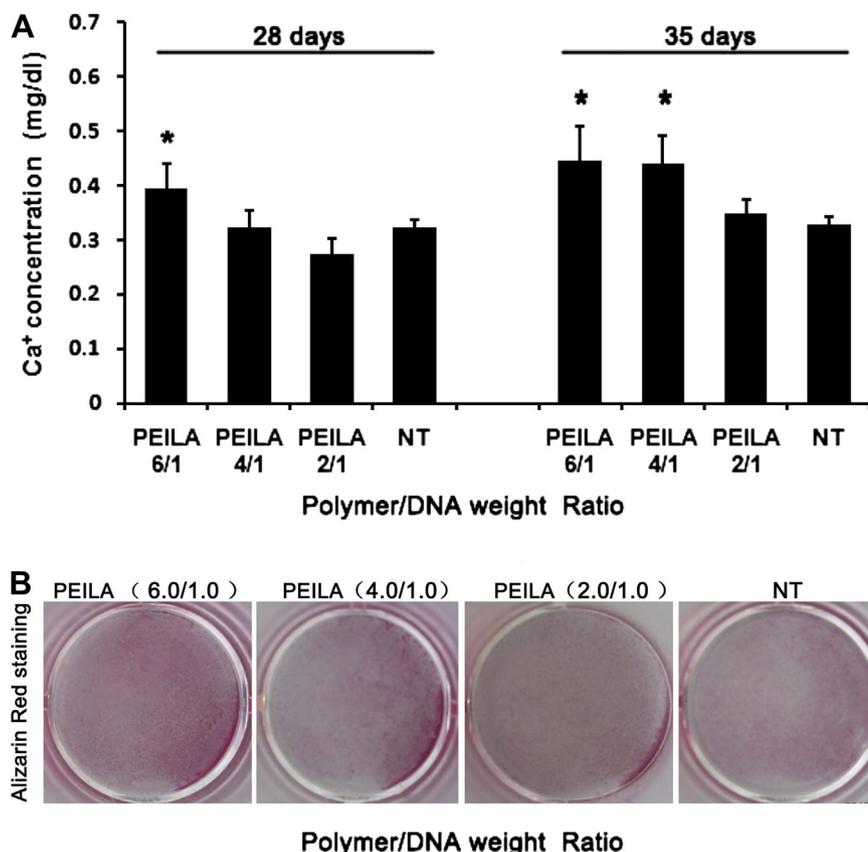


Fig. 8 – Calcification in hBMSC cultures treated with PEI-LA complexes of gWIZ-BMP-2. Transfections with complexes were carried out for 24 hours, after which the medium was changed to OM. The extent of calcification was quantitated spectroscopically after 28 and 35 days of post-transfection (A). *P < 0.05 vs. NT. (B) Representative Alizarin Red-stained cultures after 35 days of post-transfection. (Color version of figure is available online.)

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