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Nanoplex-Mediated Co-delivery of Fibroblast Growth Factor and Bone Morphogenetic Protein Genes Promotes Osteogenesis in Human Adipocyte-Derived Mesenchymal Stem Cells

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Abstract

This study highlights the importance of transfection mediated coordinated bone morphogenetic protein 2 (BMP-2) and fibroblast growth factor 2 (FGF-2) signaling in promoting osteogenesis. We employed plasmids independently encoding BMP-2 and FGF-2 complexed with polyethylenimine (PEI) to transfect human adipose derived mesenchymal stem cells (hADMSCs) *in vitro*. The nanoplexes were characterized for size, surface charge, *in vitro* cytotoxicity and transfection ability in hADMSCs. A significant enhancement in BMP-2 protein secretion was observed on day 7 post-transfection of hADMSCs with PEI nanoplexes loaded with both *pFGF-2* and *pBMP-2* (PEI/(*pFGF-2* + *pBMP-2*)) versus transfection with PEI nanoplexes of either *pFGF-2* alone or *pBMP-2* alone. Osteogenic differentiation of transfected hADMSCs was determined by measuring *osteocalcin* and *Runx-2* gene expression using real time polymerase chain reactions. A significant increase in the expression of *Runx-2* and *osteocalcin* was observed on day 3 and day 7 post-transfection, respectively, by cells transfected with PEI/(*pFGF-2* + *pBMP-2*) compared to cells transfected with nanoplexes containing *pFGF-2* or *pBMP-2* alone. Alizarin Red staining and atomic absorption spectroscopy revealed elevated levels of calcium deposition in hADMSC cultures on day 14 and day 30 post-transfection with PEI/(*pFGF-2* + *pBMP-2*) compared to other treatments. We have shown that co-delivery of *pFGF-2* and *pBMP-2* results in a significant enhancement in osteogenic protein synthesis, osteogenic marker expression and subsequent mineralization. This research points to a new clinically translatable strategy for achieving efficient bone regeneration.

Keywords

bone morphogenetic protein 2; fibroblast growth factor 2; human adipose derived mesenchymal stem cells; polyethylenimine; plasmid DNA; nanoplex; osteogenesis; BMP-2; FGF-2; pDNA; PEI; non-viral gene delivery

Introduction

More than half a million bone defect repairs occur annually in the United States and this number is expected to double by 2020.¹ The United States and other countries worldwide are facing an increasing demand for bone grafts. Clinically, autografts are still considered the gold standard treatment for bone repair and regeneration. However, harvesting the tissue from the patient results in a second surgical site with associated morbidity and longer recovery time.² This problem can be overcome by using other bone graft substitutes such as allografts or alloplasts that have the ability to promote cell migration, attachment, proliferation and differentiation. However, such substitutes lack osteo-induction properties and are not reliable.² In order to overcome these problems scientists have proposed tissue engineering as an alternative, which is a practice of combining scaffolds, cells, and biologically active molecules for the regeneration of functional tissues. Several studies have implemented stem cells as a tissue engineering strategy because stem cells have the potential for unlimited renewal and multipotent capacity.³

Multipotent mesenchymal stem cells have the potential to differentiate into multiple mesodermal tissue types including bone, cartilage and adipose tissue.⁴ In this study, human adipose derived mesenchymal stem cells (hADMSCs) were employed instead of bone marrow derived stem cells (BMSCs) because they are ubiquitous, can be easily retrieved, and require a less invasive procedure to harvest.⁵ Moreover, it has been suggested that aging has no untoward effects on the regenerative properties of these stem cells making it currently more relevant for aging U.S. population.⁶ Several studies have demonstrated the potential of hADMSCs to induce bone regeneration by enhancing osteogenic gene expression, alkaline phosphatase activity and mineralization.⁷ However, utilization of these undifferentiated multipotent hADMSCs for *in vivo* osteogenesis is unpredictable and unguaranteed, as they can differentiate into other tissues unrelated to bone.⁸ Hence, hADMSC pretreatment with osteogenic growth factors prior to *in vivo* implantation can be considered as a promising approach for transforming undifferentiated cells into osteoblast specific cells.

FGF-2, is a dominant mitogen and one among the few osteogenic growth factors⁹ capable of driving osteogenesis in stem cells. FGF-2 is expressed by the cells of osteoblastic lineage and accumulates in the bone extracellular matrix.¹⁰ Binding of FGF-2 to its receptor, FGFR1, results in signal transduction ultimately responsible for cell migration, proliferation and differentiation of a range of cell types.¹¹ The importance of FGF-2 in regulating bone formation was highlighted by a study demonstrating that *fgf2*^{-/-} mice had decreased osteoblast proliferation and an impaired capacity to form new bone.¹² In addition to FGF-2, BMPs, which belong to the transforming growth factor- β (TGF- β) super family, are involved in regulating bone formation.¹³ Similar to FGF-2, BMP-2 is also produced by osteoblastic cells and is stored in the extracellular matrix of bone.¹⁴ BMP-2 has the potential to promote the differentiation of mesenchymal stem cells into osteoblastic cells and promotes the maturation of osteoblasts by increasing the expression of runt-related transcription factor 2 (Runx-2), and a range of osteoblast marker genes (Figure 1A),¹⁵ by binding to type I and II serine/threonine kinase receptors and signaling through SMADs.^{16,17}

These two growth factors *i.e.*, FGF-2 and BMP-2 are considered to function in coordination through SMAD activation and the MAPK signaling pathway to synergistically enhance osteogenesis by promoting Runx-2 nuclear co-localization in hADMSCs which can further enhance osteocalcin activity (Figure 1B).¹⁸ Therefore, in this proof of principle study, we proposed to promote osteogenesis of hADMSCs *in vitro* by combinatorial plasmid encoding growth factor (FGF-2+BMP-2) delivery.

Delivering recombinant regenerative growth factors or morphogens such as BMPs and FGFs in protein form are considered to be promising treatment strategies for bone regeneration. However, relatively large amounts of proteins are required for significant bone regeneration. These high doses of proteins possess increased risk of toxicity and are expensive.¹⁹ Gene therapy is considered a logical alternative to protein therapy because it is more cost effective due to the inexpensive *ex vivo* production of plasmids, when compared to the recombinant protein production.¹⁹ The *in vivo* delivery of exogenous nucleic acids into cells can be performed via a number of methods, the most potent of which involves the use of viral vectors.²⁰ However, viral vectors can be immunogenic thereby limiting their utility in clinical applications. Several physical methods such as electroporation and microinjection are safer alternatives to viral delivery, however the transfection efficiency is typically relatively low.²⁰ In this study, polyethylenimine (PEI), a cationic polymer, was utilized to form nanoplexes with DNA encoding FGF-2 and BMP-2 by self-assembly through electrostatic interactions and has been previously shown to possess efficient transfection ability both *in vitro*²¹ and *in vivo*.²²

We demonstrated that the *pBMP-2* and *pFGF-2* combinatorial delivery through PEI significantly enhanced the production of bone morphogenetic protein-2 *in vitro*. In addition, the strategy of non-viral combinatorial plasmid delivery has enhanced the up-regulation of *Runx-2* and *osteocalcin* genes and thereby augmenting osteogenic potential. Furthermore, there was a significant improvement of calcium deposition and mineralization in the cells transfected with *pFGF-2* and *pBMP-2* via PEI.

Results & Discussion

Formation of PEI-pDNA nanoplexes

In this study, we investigated the effect of combinatorial delivery of *pFGF-2* and *pBMP-2* plasmids complexed with PEI to stimulate osteogenesis in hADMSCs. The PEI/*pFGF-2*, PEI/*pBMP-2* and PEI/(*pFGF-2*+*pBMP-2*) nanoplexes were prepared as described in the methods section. During the preparation of nanoplexes, the amount of pDNA remained constant at 50 µg, whilst the PEI amount was varied to obtain different amine:phosphate (N/P) ratios of 1, 5, 10, 15 and 20. The threshold N/P ratio at which the PEI amount used can stably complex the pDNA was determined using gel electrophoresis (Figure 2). Following nanoplex formation, aggregates and a naked pDNA (without PEI) control were electrophoresed on an agarose gel. Naked pDNA and PEI/pDNA nanoplexes prepared at an N/P ratio of 1 migrated into the gel. Whereas PEI/pDNA nanoplexes prepared at N/P ratios of 5 did not release pDNA into the gel and therefore it can be deduced that they formed stable nanoplexes.

Characterization of the size and surface charge of PEI/pDNA nanoplexes

Particle size and surface charge are important parameters to be considered in achieving efficient cellular uptake. Particles with a size less than 150 nm and with a neutral or positive surface charge can result in efficient cellular internalization.²³ Subsequent to internalization, the positive charge of the nanoplexes can lead to a proton sponge effect resulting in the osmotic swelling and physical rupture of the endolysosomes, thus releasing the nanoplexes into the cytoplasm.²⁴ The PEI/*pFGF-2*, PEI/*pBMP-2* and PEI/(*pFGF-2+pBMP-2*) nanoplexes were prepared as described in the methods section and the size and surface charge were determined using dynamic light scattering and electrophoretic light scattering, respectively. The size of PEI/pDNA nanoplexes ranged from 130 - 180 nm with a polydispersity index (PDI) ranging between 0.1 - 0.3 (Table 1). In previous studies with PEI/pDNA nanoplexes, we have shown that a size difference of 50 nm does not significantly change transfection efficiency.²⁵ The net surface charge of the nanoplexes was found to be positive and ranged between 19 - 24 mV (Table 1).

N/P ratio and the amount of PEI/pDNA nanoplexes significantly affect cell viability

N/P ratio and the dose of pDNA are the major contributing factors for transfection efficiency. To achieve maximal transfection efficiency, a balance between the amount of transfection and the cell viability is desired.²⁶ The cytotoxicity of PEI/pDNA nanoplexes towards hADMSCs was studied using an MTS assay as a readout for cell viability. N/P ratios of 5 and 10 were chosen since these ratios have been previously shown to be optimal for transfection of hADMSCs, and less toxic compared to the N/P ratios of 15 and 20.²⁷ The cytotoxicity was tested at N/P ratios of 5 and 10, using 1 µg and 5 µg of pDNA.

Figure 3 demonstrates that hADMSC cell viabilities ranged between 80 and 95% when transfected with nanoplexes with an N/P ratio of 5 (with 5 µg or 1 µg pDNA) or an N/P ratio of 10 (with 1 µg pDNA). Cells treated with nanoplexes prepared at an N/P ratio of 10 (with 5 µg pDNA) possessed lower cell viabilities (mean: 53%) compared to the other treatments and this is likely due to the toxicity of higher concentrations of the positively charged PEI.²⁸ These results are consistent with our previous studies which have shown that, increasing the N/P ratio and the amount of PEI increases cytotoxicity.²⁵ Thus, the nanoplexes prepared at N/P ratios of 15 and 20 are considered to be more cytotoxic than N/P-10.

In vitro transfection efficiency is affected by the N/P ratio and the amount of nanoplexes added

The transfection efficiencies of PEI/pDNA nanoplexes prepared at N/P ratios of 5 and 10 and containing 1 µg or 5 µg pDNA (encoding EGFP) were assessed using flow cytometry (Figure 4A). The percent of GFP+ve (transfected) hADMSCs treated with nanoplexes prepared with an N/P ratio of 5 and containing 5 µg or 1 µg of pDNA was 30% or 22% respectively. For nanoplexes prepared at an N/P ratio of 10, the percent GFP+ve cells dropped below 20%, irrespective of the amount of pDNA used. The results obtained with nanoplexes prepared at an N/P ratio of 10 are in line with findings from other studies.^{27, 29} However, in our hands, the transfection efficiency (30%) of nanoplexes prepared with a N/P ratio of 5 (+ 5 µg pDNA) was substantially greater than that obtained by Ahn et al (13%) most probably due to the difference in the MW of PEI used (25 kDa PEI was used in our

study versus 10 kDa PEI by Ahn et al).²⁷ The impact of PEI molecular weights on transfection ability has been previously documented.³⁰ Figure 4B demonstrates the mode of analysis of dot-plots generated by flow cytometry and using FlowJo software. Based on the findings from our transfection and cytotoxicity studies, we proceeded with using nanoplexes prepared with an N/P ratio of 5 (+ 5 µg pDNA) for subsequent experiments.

***pFGF-2* and *pBMP-2* combination enhances the production of FGF-2 and BMP-2 proteins**

FGF-2 and BMP-2 are among the few proteins that have the potential to induce osteogenesis. Fibroblast growth factor-2 plays a critical role in the early stages of bone healing by inducing osteoblast proliferation, whereas BMP-2 contributes significantly to the mineralization phase.³¹ FGF-2 and BMP-2 when given in appropriate doses are known to function in coordination through SMAD activation and the p38/44/42 MAPK signaling pathway to synergistically enhance osteogenesis by promoting Runx-2 nuclear co-localization in hADMSCs which can further enhance osteocalcin activity (Figure 1B).¹⁸ Furthermore, there is a strong evidence that BMP-2 and FGF-2 are involved in reciprocal regulation in osteoblasts.³² Hence in this study we demonstrated the impact of combining the plasmids encoding the growth factors together on osteogenesis.

We demonstrated the potential of transfecting hADMSCs with PEI/(*pFGF-2*+*pBMP-2*) to enhance BMP-2 and FGF-2 protein production, as measured by ELISA (Figure 5A). There was an almost 10-fold increase ($p<0.0001$) in the BMP-2 expression by cells transfected with PEI/(*pFGF-2*+*pBMP-2*), compared to untreated cells. Surprisingly, the cells transfected with PEI/*pBMP-2* only demonstrated a small but non-significant enhancement in BMP-2 production with respect to untreated cells. An almost 5-fold increase ($p<0.0001$) in the BMP-2 production was observed with cells transfected with the PEI/(*pFGF-2*+*pBMP-2*) compared to the PEI/*pBMP-2* transfected cells. Cells treated with PEI/(*pFGF-2*+*pBMP-2*) demonstrated an almost 4-fold enhancement ($p<0.0001$) in BMP-2 protein expression compared to PEI/*pFGF-2* transfected cells. These results are in accordance with the literature where exogenous FGF-2 supplementation to BMSCs synergistically upregulated BMP-2 gene expression through FGF/FGF receptor signaling. Figure 5B demonstrates that, compared to the synergistic enhancement in BMP-2 expression by transfection with PEI/(*pFGF-2*+*pBMP-2*), the effect on FGF-2 expression was negligible. Of importance in this result is that the presence of BMP-2 did not inhibit FGF-2 protein production.

Several studies have demonstrated that lower concentrations of FGF-2 compared to BMP-2 resulted in a significant enhancement of osteogenesis, whereas higher FGF-2 amounts compared to BMP-2 often inhibited osteogenesis.³³ One group reported that FGF-2 synergistically enhanced BMP-2-induced osteogenesis in an *in vivo* rat model of bone repair.^{33b} The observed enhancement in calcification and ALP activity was only evident when low doses of FGF-2 (25 ng) were used in combination with BMP-2. Another group, also using an *in vivo* rat model for bone regeneration, reported that the combination of BMP-2 and FGF-2, where 2 µg of FGF-2 was used, inhibited osteogenesis.^{33a} In this study, hADMSCs transfected with PEI/(*pFGF-2*+*pBMP-2*) nanoplexes produced 200 pg/mL and 50 pg/mL of BMP-2 and FGF-2 proteins respectively and this is considered to be very promising, as the concentrations of the proteins produced are comparable to the

concentrations reported in literature at which significant enhancement in osteogenesis was observed.

Transfection of hADMSCs with PEI/(pFGF-2+pBMP-2) enhances *Runx-2* and *osteocalcin* gene expression

The role of *Runx-2* in osteoblast differentiation was shown to depend on its nuclear accumulation, and co-localization with certain SMADs, a phenomenon controlled by the coordinated signaling of FGF-2 and BMP-2.³⁴ A study further confirmed the involvement of FGF-2 in influencing the function of BMP-2 where *fgf2*^{-/-} mice had impaired *Runx-2*/SMAD nuclear accumulation, however, when these *fgf2*^{-/-} mice osteoblasts were transfected with a retroviral *fgf2* construct there was enhanced *Runx-2*/SMAD expression and nuclear localization.^{32a} Although BMP-2 signals mainly through SMAD pathways, it can also signal via the p38/44/42 MAPK signaling pathway, the major signaling pathway of FGF-2.³⁵ The activation of the MAPK signaling pathway leads to the up-regulation of *osteocalcin* gene expression by phosphorylating *Runx-2*.³⁶

We therefore investigated the osteogenic gene expression of PEI complexed with pFGF-2 and/or pBMP-2 on hADMSCs. To achieve this, real time PCR was utilized to assess the levels of transcription of *Runx-2* and *osteocalcin*, which are early and late markers of osteogenesis, respectively. In this study *Runx-2* and *osteocalcin* gene expression were analyzed at optimal time-points of day 3 and day 7 post transfection respectively.³⁷ It was observed that hADMSCs transfected with PEI/pBMP-2 alone or PEI/pFGF-2 alone, resulted in a statistically insignificant 2-fold increase in *Runx-2* mRNA by day 3 post transfection compared to untreated control cells (Figure 6A). Meanwhile, cells transfected with the PEI/(pFGF-2+pBMP-2) resulted in a 4-fold increase of *Runx-2* mRNA compared to untreated cells. This enhancement in *Runx-2* expression is possibly due to the coordination between FGF-2 and BMP-2 signaling to control the nuclear accumulation of *Runx-2* through SMAD and MAPK signaling pathways.^{34a} *Runx-2* is known to bind to other proteins to form a multi-component complex, which can then interact with the promoter regions of osteoblastic genes. This interaction can stimulate genes that encode for osteocalcin, alkaline phosphatase, osteopontin, collagen I and other proteins that are important in subsequent stages of osteoblast differentiation.³⁸

Osteocalcin is a bone-specific protein expressed in the late stages of osteoblast maturation and is considered a reliable indicator of osteoblast differentiation.³⁹ It has the potential to upregulate bone matrix synthesis and mineralization. On day 7 post-transfection of hADMSCs with PEI/pFGF-2 alone or PEI/pBMP-2 alone, *osteocalcin* mRNA levels were, respectively, 5-fold and 3-fold higher than untreated control cells. Upon transfection with PEI/(pFGF-2+pBMP-2), a 12-fold increase in *osteocalcin* mRNA expression was observed, compared to untreated cells (Figure 6B). It is therefore likely that the enhanced expression of *Runx-2* mRNA in the early pre-osteoblastic stage translated into enhanced *Runx-2* protein expression which in turn led to enhanced production of *osteocalcin* mRNA in the later stages of osteoblast maturation. This data demonstrates the potential of non-viral transfection mediated FGF-2 and BMP-2 production to enhance *Runx-2* and *osteocalcin* expression leading to enhanced osteogenesis.

Mineralization of hADMSCs demonstrated by Alizarin Red staining and atomic absorption spectroscopy

Several studies have confirmed the role of Runx-2 in regulating osteoblastic gene expression during osteogenic differentiation. A study by Ducy et al demonstrated that mice lacking Runx-2 had impaired bone mineralization.⁴⁰ Furthermore, the ability of Runx-2 to enhance osteoblast commitment and to inhibit late adipocyte maturation of human marrow stromal precursor cells strengthens its importance in osteoblastogenesis.⁴¹ Phosphorylation of Runx-2 protein through MAPK signaling pathway results in the enhancement of *osteocalcin* gene expression. Osteocalcin protein plays a key role in the bone matrix synthesis and mineralization. Thus, the measurement of calcium deposition and mineralization of hADMSCs is an ultimate indication of the effectiveness of FGF-2 and BMP-2 encoded plasmid co-delivery in transforming hADMSCs to osteoblasts.

Alizarin Red is a stain used to detect calcified bone matrix and is therefore an indicator of osteoblast maturation.⁴² We qualitatively assessed extracellular matrix calcification of variously transfected hADMSCs cultured for 14 days (Figure 7A) and 30 days (Figure 7B) using an Alizarin Red staining technique. Visual observation at both time-points indicated that cells transfected with PEI/(*pFGF-2+pBMP-2*) stained (red) more intensely than untreated cells, and cells transfected with PEI/*pFGF-2* alone or PEI/*pBMP-2* alone. A more intense staining in the combinatorial treatment was observed after 30 versus 14 days.

To obtain a quantitative assessment of calcium deposition, atomic absorption spectroscopy was utilized. Transfected hADMSCs were analyzed for calcium content on days 14 and 30 post transfection (Figure 8A). At 14 days, cells transfected with PEI/(*pFGF-2+pBMP-2*) and cells transfected with PEI/*pBMP-2* alone demonstrated a 4-fold increase in calcium deposition compared to untreated cells, whilst PEI/*pFGF-2* resulted in a 2-fold increase in calcium deposition compared to untreated cells. At the 30 day time point, transfection with PEI/(*pFGF-2+pBMP-2*) resulted in a significant 3-fold (**p*<0.1) increase in the calcium content compared to the untreated sample (Figure 8B). In contrast, cells transfected with *pFGF-2* alone and *pBMP-2* alone demonstrated insignificant increases in calcium content compared to the untreated cells. In summary, we have shown that the presence of non-viral gene delivery generated FGF-2 and BMP-2 together resulted in a significant improvement in mineralization compared to the individual growth factors on day 14 and day 30.

This study demonstrated the coordinative action of non-viral gene delivery generated FGF-2 and BMP-2 in enhancing osteogenesis. In addition, in our unique non-viral nanoplex approach to co-delivering FGF-2 and BMP-2, we have found that FGF-2 and BMP-2 are produced at concentrations that act synergistically to enhance osteogenesis. The enhanced osteogenic potential was demonstrated by the enhanced up regulation of *RUNX-2* and *osteocalcin* genes, which are the early and late markers of osteogenesis, respectively. There was an enhancement in calcium deposition (mineralization) by cells transfected with PEI nanoplexes containing *pFGF-2* and *pBMP-2* in combination. In conclusion, the co-delivery of *pFGF-2* and *pBMP-2* resulted in a significant enhancement in osteogenic marker expression, osteogenic protein synthesis and mineralization, thereby indicating the promotion of osteoblast differentiation. This study also reiterates the importance of co-

delivery of FGF-2 and BMP-2 in potentially eliminating the high dose requirement for BMP-2 when delivered alone and thus minimizing potential adverse effects such as abnormal bone formation, cancer cell growth and adverse immune system reactions often associated with such high doses.⁴³ Potential future directions include evaluating PEI/*pFGF-2+pBMP-2* transfected rat/mouse adipose cell sheets for bone regeneration *in vivo*. This research points to a new clinically translatable strategy for enhanced bone regeneration and for treatment of skeletal disorders.

Materials & Methods

Cell culture

Human ADMSCs (hADMSCs) were cultured as a monolayer in 75 cm² polystyrene cell culture flasks (Corning Incorporated, Corning, NY). The culture was maintained in mesenchymal stem cell basal medium supplemented with mesenchymal stem cell growth kit for adipose and umbilical-derived mesenchymal stem cells in low serum conditions (ATCC PCS-500-040) and 50 mg/mL gentamycin (Mediatech Inc., Manassas, VA, USA) in a humidified incubator at 37°C containing 5% CO₂ (Sanyo Scientific, Wood Dale, IL). Human ADMSCs were started from the frozen stocks and the medium was changed every 2 to 3 days. Cells were passaged when cultures reached 70 - 80% confluence, and were actively proliferating. This study was conducted using cells of passage numbers 3 to 5.

Isolation of plasmid DNA (pDNA) encoding EGFP-N1, FGF-2, and BMP-2

Plasmid DNA (4.7 Kb) coding for the enhanced green fluorescent protein (EGFP-N1) driven by CMV promoter/enhancer was obtained from Elim Biopharmaceuticals, Inc. (Hayward, CA). Plasmid DNA (4.9 Kb) encoding basic fibroblast growth factor 2 protein (FGF-2) driven by CMV promoter/enhancer was obtained from Origene Technologies, Inc. (Rockville, MD). Plasmid DNA (5.0 Kb) encoding bone morphogenetic protein 2 protein (BMP-2) driven by CMV promoter/enhancer was obtained from Origene Technologies, Inc. Plasmids were amplified using chemically competent DH5 α *Escherichia coli* transformed with the respective pDNA. The transformed *E.coli* bacteria were cultured overnight in Lennox L Broth (LB Broth) in an incubator shaker (300 rpm) and 37°C. The pDNA was isolated from the expanded bacterial culture using GenElute HP Endotoxin-free Plasmid Maxiprep Kit (Sigma-Aldrich; St-Louis, MO). The concentration and the quality of the isolated pDNA was analyzed by optical density at A260 nm and A280 nm using NanoDrop 2000 UV-Vis Spectrophotometer (Thermoscientific, Wilmington, DE). The size and quality of the isolated pDNA was demonstrated by electrophoresis in a 1.2% agarose gel. The isolated plasmid was determined to be pure, as the absorbance ratio was in the range of 1.8-2.0 (as recommended by the manufacturer's protocol).

Fabrication of PEI/pDNA nanoplexes

Nanoplexes were fabricated using different molar ratios of PEI amine (N) to pDNA phosphate (P) groups by varying the amounts of PEI (branched; Mol wt. 25 kDa; Sigma-Aldrich) and maintaining the pDNA concentration constant (N/P ratios of 1, 5, 10, 15 & 20). Appropriate amounts of pDNA and PEI were diluted in 500 μ L of DNase/RNase free water separately. Nanoplexes were then prepared by adding 500 μ L of PEI solution to 500 μ L of

pDNA (*pEGFP-N1/pFGF-2/pBMP-2*) solution containing 50 µg of pDNA to achieve desired N/P charge ratio and vortexed immediately for 30 sec. A combination of plasmids that included *pFGF-2* and *pBMP-2* was prepared by mixing 500 µL (having 50 µg) of *pFGF-2* and 500 µL (having 50 µg) of *pBMP-2* to a final volume of 1 mL. This 1 mL solution was mixed well and 500 µL was taken into a separate vial to which 500 µL of appropriate PEI concentration was added and vortexed immediately for 30 sec to prepare nanoplexes of the desired N/P ratio. In the final mixture, there was 25 µg of *pFGF-2* and 25 µg of *pBMP-2*. The PEI/pDNA mixture was then allowed to interact at room temperature for 30 min before use. During this 30 min duration, the positively charged amine groups of PEI electrostatically interacts with the negatively charged phosphate groups of pDNA to form nanoplexes.

Determination of PEI/pDNA complexation using gel electrophoresis (gel retardation assay)

Gel electrophoresis was employed to determine the optimum N/P ratio at which the polycationic PEI polymer can condense and retain the pDNA within the nanoplexes. Samples were loaded into the wells of a 1% agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) with Blue Juice gel loading buffer in the presence of in 1X TAE buffer with 5 µg/ml ethidium bromide. Electrophoresis was carried out at 80 mA in 1X TAE running buffer and then separated pDNA bands were observed using a UV transilluminator.

Characterization of PEI/pDNA nanoplexes

Particle size and surface charge was determined using Zetasizer Nano-ZS (Malvern Instruments, Westborough, MA). Dynamic light scattering (4 mW He-Ne laser with a fixed wave length of 633 nm and 173° backscatter at 25 °C) was employed to analyze the particle size and size distribution in 10 mm diameter cells. Zeta potential (surface charge) was determined by electrophoresis using folded capillary cells by the principle of laser scattering technique. Triplicate measurements were made and the mean value was reported. The effective complexation ratio of nanoplexes with N/P ratios of 1 to 20 was determined using a gel retardation assay.

***In vitro* evaluation of cytotoxicity of PEI/pDNA (*pEGFP*) nanoplexes**

Cytotoxicity of PEI/*pEGFP* nanoplexes, at different N/P ratios, cultured with hADMSCs was studied using MTS cell growth assay reagent (Cell Titer 96 AQueous One Solution cell proliferation assay, Promega Corporation). Cells were seeded at a density of 5,000 cells/well in flat bottom 96 well cell culture plates (Costar, Corning Inc.). The cells were allowed to attach overnight and after 24 hrs the complete medium was replaced with serum free medium and the cells were treated with nanoplexes (prepared using serum free DMEM instead of DNase, RNase free water), having 1 µg or 5 µg of pDNA as described in the above section. Untreated cells and the cells treated with PEI alone and pDNA alone served as controls. After 4 hrs of transfection, cells were washed with 1X PBS and fresh complete medium was then added to the cells. A further 20 hrs later, the cells were treated with 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) cell growth assay reagent. The plates were then incubated at 37°C and 5% CO₂ for 4 hrs. The amount of soluble formazan produced by the reduction of MTS reagent by

viable cells was measured spectrophotometrically using SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

The cell viability was expressed by the following equation:

$$\text{Cell viability (\%)} = (\text{absorbance intensity of treated cells}) / (\text{absorbance intensity of untreated cells}) \times 100$$

Values are expressed as mean $\pm$ SEM for each treatment performed in triplicate.

Determination of transfection efficiency of PEI/*pEGFP-N1* nanoplexes in hADMSCs

hADMSCs were plated at 30,000 cells/well in 24-well plates (Costar, Corning Inc., NY) 24 hours prior to transfection. After 24 hours the PEI/*pEGFP-N1* nanoplexes were prepared at N/P ratios of 5 and 10 as described above. The formed nanoplexes were gently vortexed and added drop-wise into the 24 well plate in serum free conditions. Each well was treated with nanoplexes prepared at N/P ratios of 5 or 10 and containing 1 μ g or 5 μ g of *pEGFP-N1*. Untreated cells served as controls and cells treated with pDNA alone and PEI alone served as additional negative controls. After incubating the treatments and controls for 4 hours in the wells, the medium was replaced with fresh serum containing medium. After 48 hours of transfection process, the wells were washed with 1X PBS and trypsinized using 0.25% trypsin. The added trypsin was then neutralized using serum containing medium and this cell suspension was transferred to flow cytometer tubes. Cells were centrifuged at 230 xg for 5 min, the supernatant was aspirated and the cell pellet was resuspended in serum containing medium. Cell suspension was then directly run through a FACScanTM (Becton Dickinson) flow cytometer equipped with a 15 mW, 488 nm, and argon-ion laser and forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1) parameters were measured. FSC versus SSC dot-plot was generated to gate on cells and to exclude debris. Data from 5,000 events were collected and using FlowJo software the percent transfection was determined by determining the percent of cells (or events) that were positive for green (FL1) fluorescence relative to the untreated negative control population. Positive events detected in the untreated population were assumed to be artefacts and this percentage value was consistently subtracted from all treated samples so as to avoid overestimation of transfection.

Determination of protein expression

Expression of proteins (hFGF-2 basic and hBMP-2) by transfected cells was determined using hFGF-2-basic and hBMP-2 ELISA kits (Quantkine, R&D Systems, Minneapolis, MN). The cells were plated at a density of 30,000 cells/well and treated in a similar fashion as described in the above section. On days 3 and 7 post-transfection, 500 μ L of the cell supernatant was collected into appropriately labeled eppendorf tubes and then subjected to ELISA. All the processes were performed in high binding clear, polystyrene, 96 well plates (R&D systems, Minneapolis, MN, USA) and in accordance with the manufacturer's protocol.

Determination of osteoblastic gene expressions

Osteoblastic gene (*Runx-2* and *osteocalcin*) expression was analyzed using real-time PCR. hADMSCs were plated at a density of 30,000 cells in a 24 well plate with 1 mL DMEM containing 20% FBS and transfected with PEI/*pFGF-2*, PEI/*pBMP-2* and PEI/*(pFGF-2+pBMP-2)* nanoplexes in the presence of serum free (0% FBS) DMEM. Nanoplexes of volume 100 μ L having 5 μ g of DNA were added to the cells. On day 3 and day 7 post-transfection, total cell RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), by following the manufacturers protocol. Cells were then homogenized (QIAshredder column, Qiagen) and using RNeasy column total RNA was eluted out. The quantity and the quality of the isolated RNA was measured from the absorbance at A260 nm and the ratio of A260/A280 respectively. Reverse transcription reactions were carried out on the extracted RNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) and the reverse transcription reactions were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, BioRad, Waltham, MA, USA). Initially the mixture was subjected to 25°C for 10 min, then incubated at 48°C for 30 min, and this mixture was heated at 95 °C for 5 min, and finally chilled to 4°C. TaqMan Ribosomal RNA Control Reagents Kit (Applied Biosystems, Foster City, CA, USA) was used to detect 18s ribosomal RNA as an endogenous control. TaqMan Universal PCR Master Mix (Applied Biosystems), primers and probes for *Runx-2* and *osteocalcin*, the endogenous 18S rRNA control and the cDNA were mixed in 96-well Optical Reaction Plates (Applied Biosystems) and the real time PCR reactions were performed in an Applied Biosystems 7300 Real Time PCR System, with thermal cycling parameters set at 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min.⁴⁴ Steady-state mRNA levels were normalized to 18s rRNA and calculated relative to untreated controls by “the relative quantitation using comparative C_T” in Multiplex Reactions (Perkin Elmer). The

C_T values were subjected to one-way ANOVA (n = 3) followed by Tukey’s post-test to a confidence level of p < 0.05.⁴⁴

The human sequence primers and probes used for real-time PCR were as follows:⁴⁵

Runx-2

Forward primer 5’–3’: CAACAAGACCCTGCCCCGT

Real-time probe 5’–3’: CTTCAAGGTGGTAGCCC

Reverse primer 5’–3’: TCCCATCTGGTACCTCTCCG

Osteocalcin

Forward primer 5’–3’: TAGTGAAGAGACCCAGGCGC

Real-time probe 5’–3’: TGTATCAATGGCTGGGAGCCCCAG

Reverse primer 5’–3’: CACAGTCCGGATTGAGCTCA

Qualitative calcium detection

Alizarin Red S is a 1,2-dihydroxyanthraquinone and can stain calcium. Calcium can form an Alizarin Red S-calcium complex through chelation resulting in a birefringent end product. Alizarin red (AR) stain (2%) was prepared by dissolving 2 g of alizarin red S in 100 ml of distilled water. The pH was adjusted to 4.1-4.3 using 10% ammonium hydroxide. For the staining of cells on day 14 and day 30 post-transfection, cells were washed with 1X PBS for 5 min and fixed using 10% formalin for 10 min. Then, the formalin was aspirated out and washed with Millipore water. Then 500 μ L of 2% alizarin red S was added to the wells for 10 min. After 10 min, the alizarin red was aspirated out and the plates were washed with distilled water until no additional AR continues to come off the wells. The wells were observed under microscope and images were captured.

Quantitative determination of calcium content

Atomic absorption spectroscopy (Perkin Elmer Model 2380) was employed for the quantitative analysis of calcium content in the cells. The cells at day 14 and day 30 of transfection were acid hydrolyzed for 24 h by adding 1 ml of 0.6 N HCl in PBS per well. After 24 h, 450 μ L of acid hydrolyzed samples was mixed with 550 μ L of 2.5% lanthanum oxide in 0.6 N HCl. Instrumental parameters were set at a wavelength of 422.7 nm, slit width of 0.7 nm, air-acetylene (55:15) as a recommended flame. Lanthanum oxide was used in order to avoid interference obtained from phosphates. The instrument was calibrated using commercial calcium standards. Calcium standards were prepared by dissolving 20 ppm calcium stock in required volumes of solution containing 0.6 N HCl in 1X PBS and 2% lanthanum oxide. The prepared solutions and cell suspensions were directly injected into the instrument, which can then give the calcium concentration readings.

Statistical Analysis

All the experiments were performed three times and each experiment contained triplicate samples. Statistical analysis of data was performed using statistical and graphing software GraphPad PRISM (GraphPad, San Diego, CA). One-way analysis of variance (ANOVA) was performed followed by Tukey's post-hoc test to compare all the pairs of treatments and determine the statistical significance. Values are expressed as mean $\pm$ SEM and *p*-values less than 0.05 were considered as statistically significant.

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A.

B.

Figure 1.

A) Schematic demonstrating the action of FGF-2 and BMP-2 at various stages of osteopromotion of human adipose stem cells. B) Schematic demonstrating the combinatorial action of FGF-2 and BMP-2 on mesenchymal stem cells through the RAS/RAF/MEK/MAPK pathway and a SMAD pathway, thus transforming mesenchymal stem cells to osteoblasts.

Figure 2.
Analysis of the complexation efficiency of PEI at different N/P ratios using gel electrophoresis

Figure 3. MTS assay using hADMSCs analyzing the cytotoxicity of PEI/pDNA complexes at N/P ratios of 5 and 10 and at 1 µg and 5 µg of pDNA. Significant differences between the treatments and untreated cells were assessed by one way ANOVA followed by Tukey's post-test (**p<0.01). Values are expressed as mean ± SEM.

A.

B.

Figure 4.

A) Histogram demonstrating the transfection efficiency of PEI/pEGFP nanoplexes at N/P ratios of 5 and 10 and at 1 μ g and 5 μ g of pDNA (n=3). B) Dot plot of flow cytometric results obtained after analyzing with FlowJo Software. B-iv) Represents the GFP expression of cells transfected with N/P-5 and 5 μ g of EGFP DNA, the EGFP expression is highlighted in a red polygon. Significant differences between the treatments and the untreated controls were assessed by one way ANOVA followed by Tukey's post-test (**p<0.001). Values are expressed as mean $\pm$ SEM

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A.

B.

Figure 5.

ELISA of supernatants from cultures of hADMSCs after 7 days of transfection with indicated treatment. A) BMP-2 protein detection (n=3) B) FGF-2 protein detection (n=3). Significant differences between the treatments and the untreated controls were assessed by one way ANOVA followed by Tukey's post-test (** $p < 0.001$; * $p < 0.1$). Values are expressed as mean $\pm$ SEM.

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A.

B.

Figure 6.

Real time PCR analysis of hADMSCs derived from cultures 3 and 7 days post-transfection with indicated treatments. A) RUNX-2 expression analysis after 3 days of transfection (n=3) B) Osteocalcin expression analysis after 7 days of transfection (n=3). Significant differences between the treatments and the untreated controls were assessed by one way ANOVA followed by Tukey's post-test (****p<0.0001; ***p<0.001; **p<0.01). Values are expressed as mean $\pm$ SEM.

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Figure 7. Alizarin Red staining of hADMSCs A) after 14 days and B) after 30 days of transfection. A/B-i) hADMSC untreated control; A/B-ii) PEI/pFGF-2 transfected hADMSCs; A/B-iii) PEI/pBMP-2 transfected hADMSCs; A/B-iv) PEI/(pFGF-2+pBMP-2) transfected hADMSCs.

A.

B.

Figure 8.

Determination of calcium levels in samples using atomic absorption spectroscopy on hADMSC cell suspension after 14 days and 30 days of transfection. A) Calcium ion concentrations in hADMSCs on day 14 post-transfection with indicated treatments (n=3) B) Calcium ion concentrations of hADMSCs on day 30 post-transfection with indicated treatments (n=3). Significant differences between the treatments and the untreated controls are assessed by one way ANOVA followed by Tukey's post-test (****p<0.0001; *p<0.1). Values are expressed as mean $\pm$ SEM.

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Table 1

Zeta size and zeta potential of PEI/pDNA nanoplexes containing *pFGF-2* and *pBMP-2* prepared either individually or in combination at N/P ratios 5 and 10.

Growth Factor	Size (nm) ± SEM	PDI ± SEM	Zeta Potential (mV) ± SEM
phFGF-2	179.4 ± 5.7	0.315 ± 0.047	23.8 ± 2.7
pBMP-2	181.0 ± 4.9	0.225 ± 0.034	19.5 ± 1.4
phFGF-2 + pBMP-2	139.7 ± 7.3	0.191 ± 0.057	24.1 ± 2.9