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FGF2 Gene Activated Matrices Promote Proliferation of Bone Marrow Stromal Cells

Sheetal D'Mello*, Satheesh Elangovan[†], and Aliasger K. Salem^{*,†}

*Division of Pharmaceutics and Translational Therapeutics, College of Pharmacy, University of Iowa, IA, USA

[†]Department of Periodontics, College of Dentistry, University of Iowa, IA, USA

Abstract

Background—In this study, we report on the results from the development and early *in vitro* testing of a gene activated matrix encoding basic human fibroblast growth factor 2 (FGF2) in bone marrow stromal cells (BMSCs).

Methods—Polyethylenimine (PEI), a cationic polymer, was utilized as a gene delivery vector and collagen scaffolds were used as the carrier to deliver the PEI-pDNA nano-sized complexes (nanoplexes) encoding the FGF2 protein. Initially, the BMSCs were transfected *in vitro* with the PEI-pFGF2 nanoplexes, prepared at a N/P ratio of 10, with cells alone and naked DNA as controls. This was followed by transfection experiments using collagen scaffold containing complexes, with the scaffold alone as a control. The transfection efficacy of the nanoplexes was assessed using ELISA for the determination of FGF2 protein expressed by the transfected cells. The functionality of transfection was assessed by evaluating cellular recruitment, attachment, and proliferation of BMSCs on the scaffold using imaging techniques.

Results—BMSCs transfected with the PEI-pFGF2 nanoplexes (either alone or within the scaffold) led to higher expression of FGF2, compared to controls. Scanning electron microscopy and confocal imaging confirmed the recruitment and attachment of BMSCs to scaffolds containing the PEI-pFGF2 nanoplexes. Confocal microscopy showed a significantly higher number of proliferating cells within PEI-pFGF2 nanoplex-loaded scaffolds than with empty scaffolds.

Conclusions—This first *in vitro* evaluation in BMSCs provides evidence that gene activated matrices (GAMs) encoding the FGF2 protein may have strong translational potential for clinical applications that require enhanced osseous and periodontal tissue regeneration.

Corresponding Authors: Satheesh Elangovan BDS., D.Sc., DMSc, 801 Newton Road, S464, Department of Periodontics, University of Iowa College of Dentistry, Iowa City, IA 52242, USA. satheesh-elangovan@uiowa.edu, Tel # 319 335 7543, Fax # 319 335 7239, Aliasger K. Salem, PhD, 15 S. Grand Avenue, S228 PHAR, University of Iowa College of Pharmacy, Iowa City, IA 52242 USA. aliasger-salem@uiowa.edu.

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We have no conflicts of interest to declare.

Keywords

Bone regeneration; plasmids; gene therapy; polyethylenimine; fibroblast growth factors; tissue engineering

Introduction

Periodontal and bone regeneration are highly orchestrated dynamic events.^{1, 2} Advances in biotechnology over the last two decades have led to the identification of key molecules and processes intricately involved in such dynamic events.³ Growth factors are one such group of key molecules that, upon binding to target receptors, lead to chemotaxis, proliferation and/or differentiation of target cells.^{3, 4} In dentistry, after a series of human clinical trials, recombinant human growth factors, such as platelet derived growth factor (PDGF), have been cleared by the United States Food and Drug Administration for specific clinical indications.⁵ Similarly, another growth factor candidate that has the potential to be translated into clinical settings is human fibroblast growth factor -2 (FGF2).³

FGF, a protein originally isolated from cow pituitary gland was shown to induce significant fibroblast proliferation.⁶ Based on its isoelectric point, FGF can be both acidic and basic in nature and several genes encode this family of growth factors resulting in several subfamilies of this molecule.³ Nearly all FGFs work by binding to tyrosine kinase type receptors and from gene deletion studies it is clear that some FGF subfamilies are involved in the development of several key structures including the skeleton and the central nervous system.^{7, 8} Of the FGFs, FGF2 was quickly recognized to have angiogenic properties and the capacity to induce mesenchymal stem cell self-renewal and proliferation, making it a strong candidate for regenerative therapeutics.^{9, 10} FGF2 was shown to be an effective molecule in biomimetic systems promoting both bone and periodontal regeneration.^{11, 12, 13}

Non-viral gene delivery is an exciting therapeutic approach that can overcome the problems of limited sustained protein delivery and safety concerns often associated with protein therapy and viral gene therapy, respectively.¹⁴ Moreover, plasmid DNAs (pDNA) are relatively inexpensive and can be customized according to requirements.¹⁵ Our group recently demonstrated the *in vivo* efficacy of such a non-viral gene delivery system encoding PDGF for bone regeneration.¹⁶ In this study, our objective is to utilize a similar strategy to develop and test (*in vitro*) a non-viral gene delivery system encoding human FGF2 for potential oral applications. This is the first demonstration of functionality of transfection of PEI pDNA (human FGF-2) in BMSCs.

Materials and Methods

Preparation of pDNA encoding the human fibroblast growth factor 2 (basic) (FGF2) protein: pFGF2

Plasmid DNA (4.7 Kb) encoding FGF2 protein (pFGF2) driven by a CMV promoter/enhancer was obtained (Origene Technologies, Inc, Rockville, MD). The chemically competent DH5 α TM bacterial strain (*Escherichia coli* species) was transformed with pDNA to amplify the plasmid. The pDNA in the transformed cultures was then expanded in *E. coli*

in Lennox L Broth (LB Broth) overnight at 37°C in an incubator shaker at 300 rpm. Plasmid DNA was extracted using the endotoxin-free plasmid maxiprep kit (Sigma-Aldrich®, St. Louis, MO) and was analyzed for purity using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE), by measuring the ratio of absorbance (A₂₆₀ nm/A₂₈₀ nm). The concentration of pDNA solution was determined by absorbance at 260 nm.

Fabrication of PEI-pDNA nanoplexes

Nanoplexes were prepared by adding 500 µl polyethylenimine (PEI) solution (branched PEI, mol. wt. 25 kDa, Sigma-Aldrich®, St. Louis, MO) drop wise to 500 µl pDNA (pFGF2) solution containing 50 µg pDNA and mixed by vortexing for 20 s. The mixture was incubated at room temperature for 30 min to allow nanoplex formation between the positively charged PEI (amine groups) and the negatively charged pDNA (phosphate groups).^{17, 18} Nanoplexes were fabricated using N (nitrogen) to P (phosphate) ratios (molar ratio of amine groups of PEI to phosphate groups in pDNA backbone) of 10. Final volume of the nanoplexes used in the transfection and cytotoxicity experiments was 20 µl and contained 1 µg of pDNA.

Cell culture

Human bone marrow stromal cells (BMSCs) (American Type Culture Collection, ATCC®, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Atlanta Biologicals®, Lawrenceville, GA) and 50 µg/ml gentamycin in a humidified incubator at 37°C. Cells were grown as a monolayer and subcultured (subcultivation ratio of 1:9 using Trypsin-EDTA (0.25%, 1X solution) (Invitrogen™, Grand Island, NY) upon reaching 80–90 % confluence. Cell lines were started from frozen stocks and the medium was changed every 2–3 days. Cell passage numbers used in the experiments were between 4 and 8.

In vitro evaluation of transfection of BMSCs with PEI-pFGF2 nanoplexes

Cells were plated at a seeding density of 80000 cells/well in 24-well plates. The next day, at ~ 80 % cell confluence, the cell culture medium was changed to serum-free medium and the PEI-pFGF2 nanoplexes were gently vortexed and added drop wise into the wells. Each well was treated with 20 µl nanoplexes containing 1 µg pFGF2 and incubated with cells for 4 h. At the end of the treatment period, cells were washed with 1X with phosphate buffered saline (PBS) (Invitrogen™, Grand Island, NY) followed by addition of fresh complete medium. After a total incubation time of 48 h, the cell culture supernatants were then harvested by centrifugation. We also determined levels of FGF2 expression by the cells seeded onto the nanoplex-loaded collagen scaffolds using ELISA specific for the FGF2 protein. The PEI-pFGF2 nanoplexes fabricated at a N/P ratio of 10 were injected into previously cut 5 mm × 2 mm discs of absorbable type-I bovine collagen scaffolds (Zimmer Dental Inc., Carlsbad, CA) and lyophilized for subsequent experiments. Empty scaffolds and nanoplex-loaded scaffolds incorporating 50 µg pFGF2 were placed into the wells of a 48-well plate. For ELISA, 100 µl of cell culture medium containing 390000 cells were seeded onto a single scaffold per well. After incubation for 4.5 h, 400 µl of medium was added to cover the scaffold completely and further incubated for 3 days. At the end of the experiment, the growth medium was collected for FGF2 analysis. The amounts of FGF2 in cell culture

supernatants were quantified using a FGF2 ELISA kit (Quantikine®, R & D Systems®, Minneapolis, MN). The data is represented as mean $\pm$ SD for each treatment carried out in triplicate.

Attachment of BMSCs on collagen scaffolds loaded with PEI-pFGF2 nanoplexes

The interaction of the cells within the nanoplex-loaded scaffolds was evaluated *in vitro* using scanning electron microscopy (SEM). The cells were seeded either on the surface of the scaffolds (top-to-bottom cell recruitment approach, variant 1) or directly on the 48-well plate surface followed by the addition of the scaffolds (bottom-to-top cell recruitment approach, variant 2). In variant 1, empty scaffolds and nanoplex-loaded scaffolds incorporating 50 μ g pFGF2 were placed into the wells of a 48-well plate. For SEM analysis of cell attachment, 300000 cells in 100 μ l cell culture medium were seeded onto a single scaffold in each well. After incubation for 4.5 h, 400 μ l medium was added to cover the scaffold completely. In variant 2, 300000 cells in 500 μ l medium were seeded into each well, and after 5 h, the empty scaffolds and the nanoplex-loaded scaffolds were placed into the wells. Following further incubation for 3 days, the scaffold discs were washed and fixed overnight in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for SEM (Hitachi Model S-4800, Japan).

SEM sample preparation

Standard methods for SEM were employed. Briefly, after aldehyde fixation, the scaffolds were post-fixed for 1 h at room temperature with a 1% solution of osmium tetroxide in 0.1 M sodium cacodylate buffer. The samples were dehydrated through a series of ethanol washes up to 100% ethanol before being subjected to critical point drying using CO₂ for 2 h. The samples were then mounted onto aluminum stubs, sputter-coated and examined with a scanning electron microscope.

Proliferation of BMSCs on collagen scaffolds loaded with PEI-pFGF2 nanoplexes

The proliferation of cells within scaffolds incorporating nanoplexes was evaluated *in vitro* using confocal microscopy. Empty scaffolds and nanoplex-loaded scaffolds incorporating 50 μ g pFGF2 were placed into the wells of a 48-well plate. For cell proliferation analysis, 390000 cells in 100 μ l of cell culture medium were seeded onto each scaffold. After incubation for 4.5 h, 800 μ l of medium was added to cover the scaffold completely and kept in culture for a total of 3 days. At the end of the experiment, the scaffold discs were washed and fixed overnight in zinc formalin for confocal microscopy.

Immunocytochemical staining

The fixed samples were cryoembedded and cryosectioned at 10 μ m thickness along the vertical plane of the scaffolds. Cryosections were collected on Superfrost Plus Slides (Fisher Scientific®, Fairlawn, NJ) and were post-fixed before incubating for 10 min with 0.05 % solution of Triton X-100 (Sigma-Aldrich®, St. Louis, MO). After endogenous biotin block, the background staining was blocked by treating the samples with 5% normal goat serum diluted in PBS for 1 h. This was followed by incubating the samples overnight at 4°C with 5 μ g/ml mouse anti-Ki67 IgG1k (Clone MIB-1) primary antibody (Dako, Carpinteria, CA).

The samples were then treated with biotinylated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. Finally, the sections were incubated for 1 h in the dark with streptavidin-Alexa Fluor 568 fluorescent conjugate (Invitrogen™, Grand Island, NY). Negative controls were treated as described above, however, the specific primary antibody was replaced by a normal mouse IgG isotype match at the same final concentration (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The washes between each step were performed with 1X PBS. The sections were then mounted under coverglasses with Vectashield® containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs Inc. Burlingame, CA) and observed for the fluorescently-labeled antigen using a confocal laser scanning microscope (Carl Zeiss 710, Germany) equipped with Zen 2009 imaging software. The images were processed using ImageJ® open access software (National Institutes of Health, MD).

Data presentation and statistical analysis

Numerical data were reported as mean $\pm$ standard deviation (S.D.) from at least three replicate samples from experiments performed and analyzed separately. The graphs were generated using Prism 5.0 (GraphPad Software Inc., San Diego, CA). The data were compared by ANOVA followed with a Tukey post-test analysis and by unpaired t-tests. The differences between the groups were considered to be statistically significant when the *P* value was < 0.05 . Statistical analyses were performed using Prism 5.0 software.

Results

Preparation of pDNA encoding the human fibroblast growth factor 2 (basic) (FGF2) protein: pFGF2

The purity of extracted pDNA as determined by the ratio of absorbance (A260 nm/A280 nm) was found to be within the range of 1.8 – 2.0 (as recommended by the manufacturer's protocol). The concentration of pDNA solution was determined by UV absorbance at 260 nm and was further concentrated as needed.

In vitro evaluation of transfection of BMSCs with PEI-pFGF2 nanoplexes

We evaluated the gene delivery efficacy of the PEI-pFGF2 nanoplexes through expression of FGF2 protein in BMSCs. Untreated cells and cells treated with naked plasmids were employed as controls. Detection and quantification of FGF2 protein formation by ELISA confirmed the transfection potential of the PEI-pDNA nanoplexes in the target cell line. After the transfection of cells with nanoplexes (containing 1 μ g pFGF2), FGF2 protein levels in cell culture medium supernatants were significantly higher than in cells treated with the naked pFGF2 (Fig. 1A). These results verified the ability of PEI-pDNA nanoplexes to efficiently transfect BMSCs with therapeutic genes. ELISA was also carried out to determine the transfection potential of three-dimensional collagen scaffolds incorporating the nanoplexes containing 50 μ g pFGF2. Cells seeded on these scaffolds were transfected by the pDNA which then eventually expressed the FGF2 protein. After 3 days of incubation, when the cell culture supernatants were analyzed for the presence of the protein, the concentration of FGF2 in the supernatants derived from nanoplex-treated scaffolds (1058.49

pg/ml) was approximately four-fold greater than in supernatants harvested from empty (control) scaffolds (264.48 pg/ml) (Fig. 1B).

Attachment and proliferation of BMSCs on collagen scaffolds loaded with PEI-pFGF2 nanoplexes

The migration into, and the attachment of BMSCs onto, the porous scaffolds containing the nanoplexes (50 µg pFGF2) was observed by SEM. For this experiment, the cells were both seeded on the surface of the scaffolds (top-to-bottom cell recruitment approach, variant 1) as well as placed beneath the scaffolds (bottom-to-top cell recruitment approach, variant 2). After seeding the cells onto the scaffolds (variant 1), the scaffolds were imaged immediately at a time point of 0 h and then again at 3 h (Figs. 2A–B). At 0 h, the individual cells, mostly spherical in morphology, were seen loosely lying in contact with the walls of the pores of the scaffolds. Later, after 3 h, microscopic analysis revealed the cells to be adhering to the porous scaffold walls via cellular extensions (Fig. 2B). The cells were more elongated in shape with some cells stretching and spreading across the pore wall. These cells appeared in the process of regaining their original fibroblastic morphology.

Cross sectional examination after 3 days of further incubation revealed all the cells to be anchored to the scaffolds and these cells appeared healthy, possessing an inherent spindle-shape and branched cytoplasm (Fig. 2C). In a different version of this experiment (variant 2), cells were first seeded into the 48-well plate, allowed to adhere for 5 h, followed by addition of nanoplex-loaded scaffolds. After a total of 3 days in culture, the scaffolds were processed for the presence of cells. The microscopy images showed pockets of cells adhering at places on the base of the scaffolds which were in contact with the cells during the incubation period (Fig. 2D). The cells were also seen attached to the inner walls of the porous scaffolds. Cells were found to be located in the outer regions (surface) of the scaffolds as well, confirming migration of cells through the entire scaffold thickness (Fig. 2E).

The nanoplex-loaded scaffolds were subsequently investigated for their ability to promote *in vitro* cell proliferation, by detecting and quantifying proliferating cells in the matrix using indirect immunocytochemistry assays. Immunofluorescence staining was employed to assess cell proliferation by the indirect fluorescent labeling of the nuclear protein, Ki67, a cellular marker for proliferation. Using a mouse Ki67-specific primary antibody, the presence of Ki67 in actively dividing cells was detected. As a negative control, a non-binding isotype matched antibody was used and did not show any red fluorescence (data not shown). After incubation of nanoplex-loaded scaffolds with cells for 3 days, the scaffolds were harvested, stained and observed under a confocal microscope to detect proliferating cells (red fluorescence). The cell nuclei were stained by DAPI (blue fluorescence). The number of proliferating (Ki67+) cells was determined by counting within fields of view under the microscope (20X magnification). We observed a significantly higher number of proliferating BMSCs on scaffolds containing PEI-pFGF2 nanoplexes compared to empty scaffolds (Fig. 3A). The number of proliferating cells in the nanoplex-loaded scaffolds was approximately 3.5-fold higher than the number in empty scaffolds (Fig. 3B).

Discussion

In this study, we investigated a pDNA-based expression system for delivering *FGF2* therapeutic gene (coding for FGF2 protein) to BMSCs using non-viral PEI-pDNA nanoplexes embedded in collagen scaffolds. In this study, we utilized branched PEI that offers effective protonability, buffering capacity, high cationic charge density and tight condensation of pDNA molecules. These characteristics of branched PEI contributes towards its high transfection efficiency.^{19–22} Of the different molecular weights of branched PEI available, 25 kDa is frequently employed for achieving maximum transfection. The PEI-pDNA nanoplexes fabricated using lower molecular weight (< 25 kDa) PEI are characterized by rapid dissociation and lower transfection efficiencies.^{23, 24} In our previous studies, we systematically identified the N/P ratio or stoichiometry at which PEI-pDNA nanoplexes yielded optimal transfection efficiencies in human embryonic palatal mesenchymal cells (HEPM) as well as BMSCs without inducing significant cytotoxicity.^{16, 25} Nanoplexes with different N/P ratios were synthesized and characterized for their corresponding effects on the precipitate size, surface charge, stability, gene transfer efficiency and cellular toxicity. Our results showed the nanoplexes to be approximately 100 nm in size with a positive surface charge of ~ 35 mV. We found the PEI-pDNA nanoplexes prepared at N/P ratio 10 to demonstrate a balance between relatively high transgene expression and low cytotoxicity in both cell types.^{16, 25} As a result, the PEI-pDNA nanoplexes used in the subsequent studies were formulated at a N/P ratio of 10. We also compared the gene transfer efficiency of PEI with calcium phosphate, another widely used non-viral vector, in the HEPM cell line. The transfection efficiency was nearly 12-fold higher with PEI compared to calcium phosphate.²⁵

Among the various gene therapy approaches for bone tissue engineering, direct delivery of osteogenic plasmid genes (as nanoplexes) immobilized in biomimetic conductive porous three-dimensional scaffold matrices is considered to be effective and clinically translatable.^{26–28} This system has been shown to be efficient in generating sustained gene expression and continuous protein production by locally transfected cells that subsequently stimulate osteogenesis and bone repair. Other advantages include, lower cost when compared to cell culture-based therapies (e.g. gene delivery via *ex vivo* modification of host cells) and safer clinical use (avoiding systemic toxicity due to dose dumping in protein therapy).²⁹ Gene-activated matrices (GAMs) containing physically entrapped (adsorbed) pDNA/gene, encoding a suitable growth factor, can provide adequate bone regenerative capacity through localized transient gene therapy.^{14, 30} The pictorial illustration of our proposed gene activated matrices and the mechanism by which they will aid in growth factor synthesis is shown in Figure 4. . The cells take up the nanoplexes which eventually traffic to the nucleus, where the plasmid DNA transiently remains as extra-chromosomal material resulting in elevated synthesis of the encoded protein, thereby temporarily converting native cells into biologically relevant sources of the functional protein.

In our earlier study, we reported significant bone regeneration resulting from delivering genes (as PEI-pDNA nanoplexes) encoding PDGF-B using the non-viral PEI vector and collagen scaffold carrier.¹⁶ The current study explored the possibility of delivering pFGF2 using the same carrier system. We observed the impact of transfection on induced cell

migration and cell proliferation with relevance to its potential for *in vivo* periodontal and bone regeneration applications. It should be noted that this study utilizes PEI and pDNA in significantly lower amounts compared to other studies evaluating GAMs for the same purpose.^{31, 32}

The morphology of the scaffold as observed by SEM was characterized by a highly porous interconnecting network with pore sizes ranging from 100–200 μm , that have been shown to be ideal for bone regeneration.³³ In tissue engineering, the three-dimensional porous scaffold provides a favorable environment for bone repair. Following implantation *in vivo*, it creates and maintains space within the defect region and being inert and biodegradable, is ultimately replaced as the new tissue is regenerated by osteogenic cells.^{34, 35} Type I collagen constitutes the main protein component of natural extracellular matrices and plays an important role in the process of tissue repair.^{36, 37} Type I collagen matrices utilized in our studies served as a platform for cell adhesion and migration, and promoted the growth of cells. For a guided bone-growth approach in bone regeneration, collagen scaffolds were shown to possess sufficient physical and mechanical properties to provide physical support and retain their original geometry after *in vivo* implantation, which is necessary for filling-in specific critical-sized defects.^{27, 38, 39} Together with the embedded PEI-pFGF2 nanoplexes, the GAM would facilitate the migration and recruitment of bone forming cells and other appropriate cell types from the nearby healthy tissues to the wound site once implanted *in vivo*. This structural design provides sites for cell attachment and promotes cell viability, proliferation, and differentiation. Since the pores are larger in diameter than the cells, the scaffold allows for the cells to migrate and move adequately within the scaffold space.⁴⁰ Eventually, a space-filling functional bone tissue is formed within the damaged area where the original shape and size is maintained by the structural geometry of the fabricated GAM.⁴¹ In the present study, we were able to demonstrate the effectiveness of the porous collagen scaffolds containing the PEI-pFGF2 nanoplexes in promoting the recruitment of cells and facilitating important cellular functions. Cells were placed either at the surface or the base of the scaffolds and cellular migration was assessed. This is a novel and stringent method to test cellular recruitment. In both the top-to-bottom as well as the bottom-to-top cell migration approaches, cross sectional analysis revealed BMSCs to be anchored to the deep pore surfaces of the scaffold. In the bottom-to-top approach, cells which were initially plated were observed in groups attached to the base of the scaffold. The initially plated cells were also found in the outer surface regions of the scaffold (after 3 days) as a result of their movement through the pores within the scaffold. These data point to the successful transfection of pFGF2 and expression of FGF2, resulting in enhanced cellular recruitment and proliferation within the scaffold which was confirmed by the cell proliferation assay. Once the transfection efficiency of the nanoplexes was determined, we followed up by testing the efficacy of the nanoplex-scaffold construct (GAM system) itself. When the cells were added onto the scaffolds, significantly enhanced cell transfection and protein production was attained with the GAMs compared to empty scaffolds, as determined by ELISA. Hence, the migration and movement of cells into the porous scaffold matrix can be explained by the production of FGF2 by the nanoplex-transfected cells.

Our confocal microscopy results complemented our SEM microscopy and transfection results, where we show that cell proliferation was significantly greater in nanoplex-loaded scaffolds than in empty scaffolds. From our results above, we have determined that PEI-pFGF2 nanoplexes are able to transfect BMSCs efficiently allowing expression of FGF2. FGF2 signals through FGF receptors to regulate a broad spectrum of cellular functions, including cell proliferation, survival, migration, and differentiation of mesenchymal cells.^{42, 43} Thus we investigated the proliferative effect of the GAM system on cells migrating through the scaffold. The Ki-67 antigen is a prototypic cell cycle related nuclear protein that is preferentially expressed by proliferating cells during all phases of the active cell cycle (G₁, S, G₂ and M-phases), but is absent in resting cells (G₀-phase). Hence, antibodies specific for Ki-67 antigen are useful in direct monitoring of the proliferating fraction of normal and neoplastic cells.^{44–46} In our examinations, more migrating and proliferating cells were found in deeper regions of the sections of scaffold samples treated with the nanoplexes than in the empty scaffolds (data not shown). With the empty scaffolds, the cells were mostly localized on the scaffold surface where they had been initially seeded rather than in the deeper areas of the matrix. Confocal imaging confirmed the enhanced recruitment of cells into, and subsequent proliferation within the scaffolds containing PEI-pFGF2 nanoplexes compared to empty scaffolds. These results clearly, emphasize the role of FGF2 in chemotaxis and bone cell growth, and suggesting the potential role for nanoplex-loaded scaffolds in promoting osteogenesis and bone regeneration *in vivo*.^{47, 48}

Thus it is evident from the transfection and cell proliferation data that the nanoplexes were able to transfect the cells, which then synthesized the FGF2 protein thereby further enhancing cellular functions. This emphasizes the need for PEI-pFGF2 nanoplexes to be incorporated within the scaffold matrix to facilitate cell localization and the subsequent bone formation phases. Taken together, these results from this proof of concept *in vitro* study demonstrate the capacity of nanoplex-loaded scaffolds to promote cell recruitment, migration, and attachment, as well as cell proliferation, and now require validation in animal models and in humans to establish their potential use in clinical settings.

Conclusions

The *in vitro* experiments demonstrated the ability of PEI-pDNA nanoplexes prepared at a N/P ratio of 10 to efficiently transfect the *FGF2* gene into BMSCs. The *FGF2* gene-activated collagen scaffold matrices favored the migration, attachment and proliferation of BMSCs in culture. These findings from this proof of concept *in vitro* study suggest that non-viral *FGF2* gene-activated scaffolds can be an attractive gene delivery system which may be effective for bone regeneration *in vivo* with a significant potential for clinical translation. In summary, these results provide the rationale for future studies exploring the efficacy of this system in enhancing osseous and periodontal healing *in vivo*.

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Highlights

- Functional transfection of PEI-pDNA (FGF-2) in BMSCs was demonstrated.
- FGF-2 gene activated matrix promoted attachment and proliferation of BMSCs.
- This strategy has immense clinical translational potential.

Figure 1.

Expression of FGF2 protein by BMSCs after treatment with (A) PEI-pFGF2 nanoplexes prepared at a N/P ratio of 10 for 4 h and (B) scaffolds containing PEI-pFGF2 nanoplexes for 3 days (* $p < 0.01$, ** $p < 0.001$)

Figure 2.

SEM images of BMSCs cell attachment and migration assay using PEI-pFGF2 nanoplex-embedded scaffolds. In variant 1 of this assay, cells were seeded on the surface of the gene activated scaffold and migration of cells from top to bottom of the scaffold was assessed. Cells on the surface of the scaffold at 0 h (A) and 3 h (B) of incubation; The cells which migrated into the middle section of the scaffold were captured by SEM after cross-sectioning the scaffold (C). In variant 2, cells were plated first and then scaffold was placed on top of the cells for 3 days. Cells at the scaffold base (D) and those that migrated up through the scaffold reaching the surface (E) were shown.

Figure 3.

Influence of the scaffolds incorporating PEI-pFGF2 nanoplexes on cell proliferation: (A) confocal image demonstrating proliferating cells (DAPI and Ki67+ cells) on empty scaffolds (20X) and on PEI-pFGF2 nanoplex-loaded scaffolds (20X) and (B) measurement of proliferation of BMSCs seeded on empty scaffolds compared to nanoplex-loaded scaffolds at day 3 of incubation. For each group, we analyzed three different slides in 5 different fields of view. Scale bar, 20 µm

Figure 4. Schematic illustration of PEI based non-viral gene delivery. PEI-pDNA (FGF2) nanoplexes released from collagen scaffold enter the target cells, make their way into the nucleus subsequently resulting in the synthesis and release of FGF2 protein from transfected cells