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Protein expression following non-viral delivery of plasmid DNA coding for basic FGF and BMP-2 in a rat ectopic model

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ABSTRACT

Non-viral delivery of genes involved in stimulation of bone formation has been pursued for clinical bone repair, but no effort has been made to assess protein expression levels after *in vivo* delivery. This is critical to better understand gene delivery efficiencies and to compare different modes of non-viral delivery. This study investigated expression levels of basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) after delivering expression vectors (plasmid DNA) with polymeric carriers in a rat subcutaneous implant model. The polymers used were a 2 kDa molecular weight polyethylenimine modified with linoleic acid (PEI-LA) and the 25 kDa PEI (PEI25) used for non-viral gene delivery in animal models. The PEI-LA mediated delivery of the plasmid DNAs in 293T cells led to ~3.5 and ~13 ng/10^6 cells/day secretion of bFGF and BMP-2 *in vitro*, respectively. Using the reporter protein, Green Fluorescence Protein (GFP), transfection in implants was readily detected by the presence of GFP-positive cells and a polymeric carrier was needed for this GFP expression. No bFGF and BMP-2 were detected in the scaffolds due to high background in detection assays and/or rapid diffusion of the secreted proteins from the implant site. However, using an *ex vivo* culture system, significant levels of BMP-2, but not bFGF, secretion were observed from the scaffolds. The BMP-2 secretion from PEI-LA delivered expression vector was equivalent and/or superior to PEI25 depending on the plasmid DNA implant dose. Gelatin scaffolds were able to sustain ~0.3 ng/sponge/day BMP-2 secretion as compared to collagen scaffolds (~0.1 ng/sponge/day). These values were equivalent to secretion rates reported with some viral delivery systems from independent studies.

1. Introduction

Fracture healing is a complex process governed by the expression of multiple growth factors that control cell recruitment, soft callus formation, angiogenesis, callus mineralization, and bone remodelling. Failure of these coordinated processes can lead to non-union, which requires additional intervention and surgical procedures. In non-unions incapable of healing, a stimulation *de novo* bone formation is required for clinical success. Synthetic scaffolds containing recombinant human growth factors, such as bone morphogenetic proteins (BMP), provide a bioactive material that can induce bone formation at repair sites [1]. Collagen-based scaffolds with BMP-2 and BMP-7 (also known as Osteogenic Protein-1) are currently clinically approved for a range of orthopaedic applications, including spinal fusion, oral/maxillofacial applications and orthopaedic trauma [2,3]. Due to short half-life of proteins *in situ*, however, excessive amounts of recombinant proteins are required to maintain concentrations within a therapeutic range for sufficiently long duration for stimulation of bone formation; for example, ~1.5 mg/cc of BMP-2 [2] and ~0.9 mg/cc of BMP-7 (estimate) [4] are needed to treat tibial trauma. This is despite that the fact that natural levels of the proteins are in the ng/mL to μg/mL range. Large doses of recombinant proteins and the resulting expense of treatment may limit widespread use of protein therapies, and may also contribute to inflammation and higher rates of complication compared to an autologous bone graft [5]. Gene delivery for local production of growth factor offers a solution to the limitations of the protein therapy. Direct administration of genes in a host is preferred due its convenience, possibility of immediate intervention with the trauma and the lower cost compared to costly cell culture-based therapies, where

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the desired genes are delivered via *ex vivo* modification of host cells. Viral delivery vectors have dominated the gene delivery approach for bone diseases, but they are generally more successful in immune-compromised animals with lower success rates in normal animals [6]. Clinical translation of viral vectors is also questionable due to safety concerns associated with viruses [7]. Non-viral vectors are, therefore, actively investigated for delivering therapeutic genes from plasmid DNA based expression systems in stimulating bone formation [8]. Plasmid DNA is attractive for driving expression of osteogenic proteins since it does not integrate into host genome and sustains transient gene expression that is sufficient (and desirable) in the case of bone regeneration.

Direct gene delivery for bone regeneration has been attempted by administration of plasmid DNA without the use of a DNA-binding carrier [9–12], and by electroporation without a carrier [13–15]. Such approaches are not likely to translate into a clinical setting due to low efficacy of transfection in the absence of a carrier or invasive treatment in the case of electroporation. In one study, BMP-4 plasmid complexed with 25 kDa polyethylenimine (PEI25) gave minimal bone formation in a rat skull defect, while naked BMP-4 plasmid showed no regeneration without a carrier [16]. It is estimated that >200 μg of PEI25 was used to be used in this study. PEI25 is exceptionally cytotoxic both *in vitro* [17] and *in vivo* [17,18], and considering that 16–32 μg PEI25 interfered with bone induction activity of BMP-2 [19], significant toxicity must have arisen and possibly impeded bone induction. The lack of histological analysis did not confirm if the bone deposition was indeed from the transfected cells. In a separate study, SuperFect™ (a cationic liposome) was employed to condense and deliver a BMP-2 plasmid in a hydroxyapatite scaffold in a rabbit skull defect [20]. After 3 weeks, implants with BMP-2 plasmid showed signs of new bone formation and, by 9 weeks, half of the defect was penetrated with new bone. Some bone formation was also observed when the BMP-2 plasmid/liposomes were administered to the site without a scaffold. HA fibers, however, showed radiopaque regions in μ-CT analysis, suggesting that the HA scaffold itself may induce calcification in the absence of gene expression [21]. Finally, Itaka et al. employed 1.3 μg of plasmid DNA and showed successful bone repair in a mouse skull defect [22]. A block polymer of PEG-aspartate-diethylenetriamine was used to deliver runt-related transcription factor 2 (Runx2) and activin receptor-like kinase 6 (caALK6) genes, both intracellular mediators involved in osteogenic differentiation. These are unique genes since they are not extracellularly acting proteins on stem cells, but rather intracellularly active proteins that will transform the transfected cells, rather than by acting on neighboring cells. Bone formation was observed histologically after 4 weeks covering ~50% of the defect.

While bone formation has been observed in some studies, there has been no assessment of recombinant protein expression *in situ*, a parameter that is crucial in the success of bone regeneration. With implantation of recombinant proteins, several studies have reported clear dose-response relationships [3,23], which helped to refine the devices for a robust bone induction. With non-viral gene delivery, no information is available about the local production rates of the therapeutic proteins. The confirmation of gene expression *in situ* is critical in order to validate the delivered therapeutic effect as well as to better predict the magnitude of the observed bone induction. It is also impossible to evaluate the *in vivo* utility of gene carriers without assessing gene expression directly; while some reagents are effective *in vitro* [24], their performance was found to be limited *in vivo* [22], and no information is available for the underlying reason(s) for this observation. Assessing the levels of secreted therapeutic proteins quantitatively will help advance the non-viral gene delivery approach.

This study was performed in order to assess expression of therapeutic proteins after non-viral delivery of the expression vectors with polymeric carriers. The genes delivered for this study were BMP-2 and basic Fibroblast Growth Factor (bFGF), coding for two proteins that were shown to stimulate bone formation on their own [25,26] and in combination with each other [27,28]. The polymeric carriers chosen were (i) PEI25, a gold standard for *in vitro* gene delivery and a carrier previously used for gene delivery in a skull defect, and (ii) a linoleic acid-substituted 2 kDa PEI (PEI-LA), which was developed in the authors' lab as a less toxic substitute for PEI25 [29]. By using a convenient and well-characterized animal model, i.e., rat subcutaneous implant model [19], we report protein expression levels following *in vivo* implantation of BMP-2 and bFGF expression vectors.

2. Materials & methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) cell culture media, trypsin (0.05%, w/v), penicillin/streptomycin (10,000 U/mL/10,000 μg/mL), and DNAse/RNAse free water were purchased from Invitrogen (Grand Island NY). Fetal bovine serum was from PAA Laboratories Inc. (Etobicoke, ON) and Hank's Balanced Salt Solution (HBSS) was from BioWhittaker (Walkersville, MD). Absorbable gelatin (Gelfoam™) and collagen (Helistat™) sponges were from Pharmacia & Upjohn (Kalamazoo, MI) and Medtronic (Memphis, TN), respectively. The 2 kDa (PEI2) and 25 kDa PEI (PEI25) were from Sigma (St Louis, MO) and it was used without further purification. The human bFGF enzyme-linked immunosorbant assay (ELISA) was purchased from R & D Systems (Minneapolis, MN) and the BMP-2 ELISA was from Peprotech (Rocky Hill, NJ). Shandon cryomatrix was from Thermo Scientific (Pittsburgh, PA) and cryomolds were from Electron Microscope Services (Hatfield, PA). The pEGFP-N2 plasmid was purchased from BD Biosciences, while the gWIZ-GFP and gWIZ plasmids were purchased from Aldevron (Fargo, ND). The pIRES-AcGFP plasmid was purchased from Clonetech (Palo Alto, CA). The BMP2-pCMV6-XL4 plasmid was from Origene (Rockville, MD). T4 DNA Ligase and restriction enzymes NheI, SacII, BglII and EcoRI were purchased from New England Biolabs (Ipswich, MA). A 2 kDa PEI modified with linoleic acid (PEI-LA) was prepared as previously described [29]. The extent of lipid modification was 1.2 linoleic acids per PEI molecule for the polymer used in this study.

2.2. bFGF and BMP-2 plasmid construction

Plasmids expressing the growth factors bFGF or BMP-2 were constructed in house (Fig. 1). The pIRES-AcGFP vector contains an internal ribosome entry site (IRES) allowing simultaneous transcription of separate mRNAs for the growth factor (i.e., bFGF or BMP-2) and the *Aequorea coerulescens* GFP. The construction of the bFGF expressing plasmid was described elsewhere [30] and will not be repeated here. The resulting plasmid is 5764 base pairs and is referred to as bFGF-IRES-AcGFP. For the BMP-2 expression plasmid, the cDNA coding for growth factor was excised from the plasmid BMP2-pCMV6-XL4 with NheI and SacII restriction enzymes, and ligated with T4 DNA Ligase into NheI/SacII sites on the pIRES-AcGFP plasmid. BMP-2 gene insertion was confirmed by restriction mapping and sequencing. The resulting plasmid was sequenced at Molecular Biology Services Unit at the University of Alberta (Edmonton, AB) to confirm insertion of the BMP-2 gene. This plasmid is 7027 base pairs and referred to as BMP2-IRES-AcGFP. Analysis of bFGF or BMP-2 expression was determined by using bFGF or BMP-2 specific ELISAs, while the expression of GFP was assessed by flow cytometry.

2.3. Preparation of DNA/polymer complexes

The plasmid DNAs and polymers were dissolved in DNAse/RNAse free water at 0.4 mg/mL and 1 mg/mL, respectively. The DNA solutions were diluted in 150 mM NaCl, and then the desired polymer solutions (PEI25 or PEI2-LA) were added to the plasmid solutions. After gently vortexing, the solutions were allowed to incubate for 30 min at room temperature. Saline (150 mM NaCl) alone was used for no treatment groups, and complexes prepared with the blank gWIZ plasmid were used as treatment controls. The polymer/plasmid weight ratio was controlled during complex formation and specified in the appropriate experiments below. The weight ratio was 5/2 for PEI25 and 10/2 for PEI-LA. These optimized ratios were chosen based on previous studies, and showed complete polymer binding to plasmid [29], with excess polymer remaining in complex solution. For *in vitro* studies, plasmid concentration in culture media is provided as microgram of plasmid per millilitre of tissue culture media, whereas the amount of plasmid added per sponge is provided for *in vivo* studies.

2.4. In vitro transfection studies

The functionality and protein secretion rates from the constructed plasmids were evaluated in the immortal 293T cell line *in vitro*. For assessment of GFP

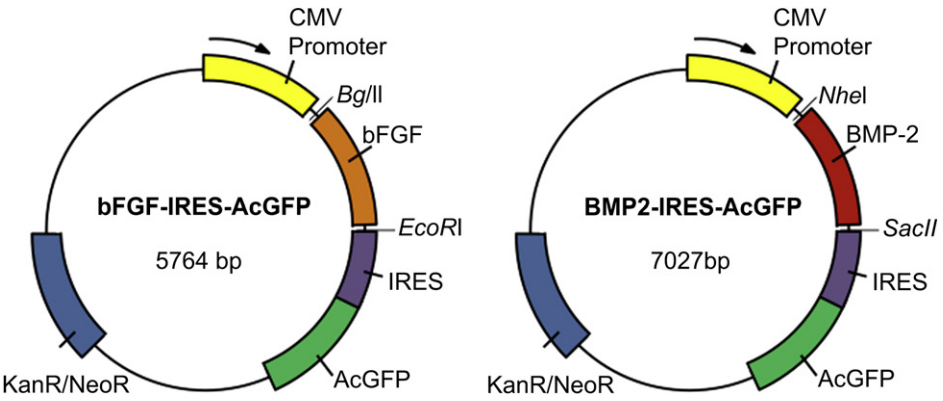

Fig. 1. Maps bFGF-IRES-AcGFP and BMP2-IRES-AcGFP plasmids used in this study. The bFGF and BMP-2 coding regions were ligated into the pIRES-AcGFP plasmid to construct the bFGF-IRES-AcGFP and BMP2-IRES-AcGFP plasmids, respectively.

expression, cells were seeded in 24-well plates either onto the tissue culture wells (2D monolayer culture) or onto absorbable gelatin sponges (0.75 cm × 0.75 cm; 3D culture) in media containing 10% FBS and 1% penicillin/streptomycin. PEI-LA/gWIZ-GFP (10/2 w/w ratio) or control PEI-LA/gWIZ (10/2 w/w ratio) complexes were added to the cells either during seeding or the day after seeding. The final DNA concentration was 2 μg/mL in the media. After 24 h exposure, the complex-containing media was removed and replaced with fresh media. The GFP expression of cultures was measured *in situ* with a fluorescent plate reader and the cells were imaged with an FSX 100 Olympus fluorescence microscope. On Day 6, cells were trypsinized and fixed in 3.7% formalin for flow cytometry analysis as previously described [29]. The cells not treated with DNA or polymers were also analyzed by flow cytometry and used to set a background level to designate 1% GFP-positive population.

To investigate protein secretion from bFGF and BMP-2 plasmids, bFGF-IRES-AcGFP and BMP2-IRES-AcGFP plasmids were used to form complexes with PEI-LA (10/2 w/w). The blank gWIZ plasmid was used as a negative control. The complexes were added to the cells for 24 h, after which the medium was replaced with fresh medium. After three days of protein accumulation, media were collected and frozen at −20 °C until further use. The cells were then washed with HBSS, trypsinized and viable cells were counted using Trypan Blue exclusion and a haemocytometer. Collected media was assessed for bFGF or BMP-2 secretion by using ELISA protocols according to the manufacturer suggestions (details not provided). The protein secretion rates were normalized with the cell counts and duration of secretion to provide protein secretion in ng protein/10^6 cells/day.

2.5. In vivo assessment of transgene expression

2.5.1. Animals and implantation procedure

Four to six-weeks old female Sprague–Dawley rats were purchased from Biosciences (Edmonton, Alberta) and kept in standard laboratory conditions (23 °C; 12 h of light/dark cycle). Rats were kept 2–3 per cage with free access to water and a commercial rat chow. All procedures involving the rats were pre-approved by the Animal Welfare Committee at the University of Alberta (Edmonton, Alberta). Rats were anaesthetised with isofluorane and small bi-lateral ventral incisions were made with blunt-ended surgical scissors to create subcutaneous pouches. One scaffold was inserted into each pouch, and the pouches were closed with wound clips. Each rat received two scaffolds, duplicates of the same type. For scaffold preparation, absorbable gelatin or collagen sponges (1 cm × 1 cm) were soaked with complexes for 15 min before implantation. The polymer/DNA complexes had been incubated for 15 min before addition to sponges so that the total complex incubation time was 30 min. At pre-determined time points (see Figure Legends), the rats were sacrificed by CO_2 asphyxiation to recover the scaffolds. Scaffolds were retrieved and used for (i) histological processing, (ii) lysis or (iii) *ex vivo* culture for GFP and recombinant bFGF and BMP-2 expression.

2.5.2. In vivo GFP expression

The scaffolds were analyzed for GFP expression by using flow cytometry, a fluorescent plate reader and histology. Complexes were prepared with the polymers PEI25 and PEI-LA, and the plasmid pEGFP-N2 and control gWiz, at the polymer:DNA ratios of 5:2 and 10:2, respectively (w/w), and loaded onto absorbable collagen or gelatin sponges with a total amount of 100 or 50 μg of plasmid DNA, respectively. From each group, 6 full scaffolds were washed in HBSS, trypsinized and the recovered cells were fixed in 3.7% formalin. Cells were analyzed for GFP expression using flow cytometry. The remaining three scaffolds were cut into equal pieces. One portion of the scaffold was put into a black 96-well plate containing HBSS. The fluorescence of the plate was read with an excitation and emission wavelengths of 485 and 527 nm respectively. Scaffolds for histology were placed in tissue section holders and embedded in Shandon Cryomatrix. The scaffolds were frozen at −20 °C and then sliced to obtain tissue sections. Slices were stained with a commercial reagent containing a nucleus stain and imaged with an FSX 100 fluorescent microscope (Olympus).

2.5.3. bFGF and BMP-2 expression

Recombinant growth factor production was evaluated either by immediate lysis of the scaffolds after recovery or following an *ex vivo* culture period. Complexes for implantation were made with either PEI25 or PEI-LA and plasmid DNA at a ratio of 5:2 and 10:2 respectively (w/w). Absorbable gelatin or collagen sponges (1 cm × 1 cm) were loaded with complexes containing 10 μg or 50 μg of plasmid DNA (see Figure legends). Following a 1, 2 or 5 week implantation, scaffolds were harvested and cut into small pieces in a lysis buffer (pH 8) containing 50 mM Tris-HCl, 150 mM NaCl, 1% Tween 20, 1% protease inhibitor. Samples were vortexed to enable complete cell lysis. The supernatant was evaluated with bFGF or BMP-2 ELISA. To determine *ex vivo* protein production, scaffolds containing were harvested in a sterile environment after a 1 week implantation and transferred to 24-well plates containing 1 mL of DMEM with 10% FBS and 1% P/S. Sponges were cultured *ex vivo* for a total of 5 days and media was changed on day 3. The supernatant was evaluated with bFGF or BMP-2 ELISA. Alternatively, complexes containing 10 μg of BMP2-IRES-AcGFP plasmid were implanted in gelatin scaffolds for 1, 2 or 3 weeks before a 5 day *ex vivo* culture to determine GFP expression with a fluorescent plate reader and BMP-2 expression with an ELISA.

2.6. Statistical analysis

The data was summarized with mean of the measured parameters with error bars representing one standard deviation. Results were analyzed with an analysis of variance (ANOVA) followed by the Dunnett multiple comparison post-hoc test. For *in vivo* studies that had non-Gaussian distribution, the Kruskal–Wallis test, the non-parametric ANOVA, was employed followed by Dunn's multiple comparison tests. The level of significance was set at $p < 0.05$.

3. Results

3.1. Comparison of GFP expression in monolayer and sponge cultures in vitro

We first evaluated the ability of complexes to transfect cells either in 2D monolayer cultures or 3D sponge cultures, which is more representative of *in vivo* transfection. The GFP expression by the transfected 293T cells is summarized in Fig. 2. Low levels of fluorescence were found in the No Treatment and control PEI-LA/gWIZ groups irrespective of whether the cells were grown on tissue culture plastic or in Gelfoam sponges (Fig. 2A). Generally, cells grown in gelatin sponges had higher autofluorescence values than the cells on a monolayer (1.4–1.8 vs. 0.1–0.2 au, respectively) for control groups, possibly reflecting the presence of residual media in sponges during the fluorescence measurements. A significantly elevated GFP fluorescence was observed in all groups treated with PEI-LA/gWIZ-GFP complexes. Adding the complexes

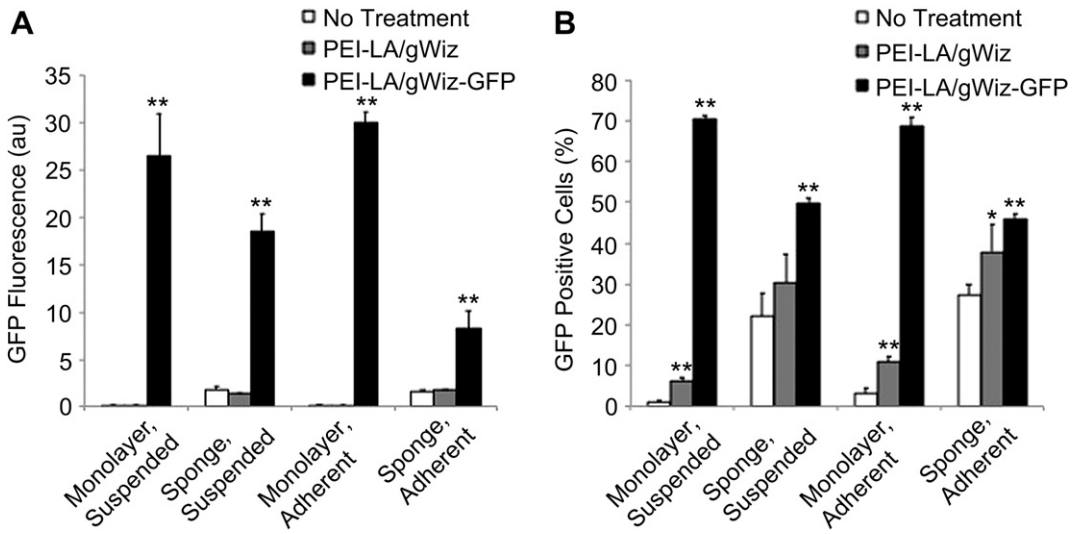

Fig. 2. GFP expression following delivery of PEI-LA complexes. Cells were grown on tissue culture plates as a monolayer or in gelatin sponges. Complexes were added either during seeding (suspended) or one day after cell seeding and attachment (adherent). 293T cells were evaluated for GFP expression 6 days after exposure to the PEI-LA/gWiz-GFP complexes. The GFP fluorescence was assessed by either a fluorescent plate reader to obtain total GFP fluorescence (A) or flow cytometry to obtain percentage of GFP-positive cells (B). $^{*}p < 0.05$, $^{**}p < 0.01$.

during cell seeding or 24 h following cell seeding was equally effective for GFP expression. For the latter group, GFP expression was also evident when the cells were examined with a fluorescent microscope (Fig. 3). No Treatment and control PEI-LA/gWIZ treated groups showed no fluorescence under the microscope (not shown).

The data from the flow cytometric analysis is summarized in Fig. 2B. For cells grown as a monolayer, exposure to control PEI-LA/gWIZ complexes did not lead to a change in GFP expression. However, cells treated with PEI-LA/gWIZ-GFP complexes gave ~70% GFP-positive population with no clear difference between cells exposed to complexes while seeding or after 24 h of attachment. Subtracting the background of control PEI-LA/gWIZ group, 64 ± 1% of cells were GFP-positive when complexes were added to suspended cells while adherent cells gave 58 ± 2% GFP-positive cell population. For cells grown in sponges, large increases (22–27%) in GFP-positive cells were observed in No Treatment groups. This

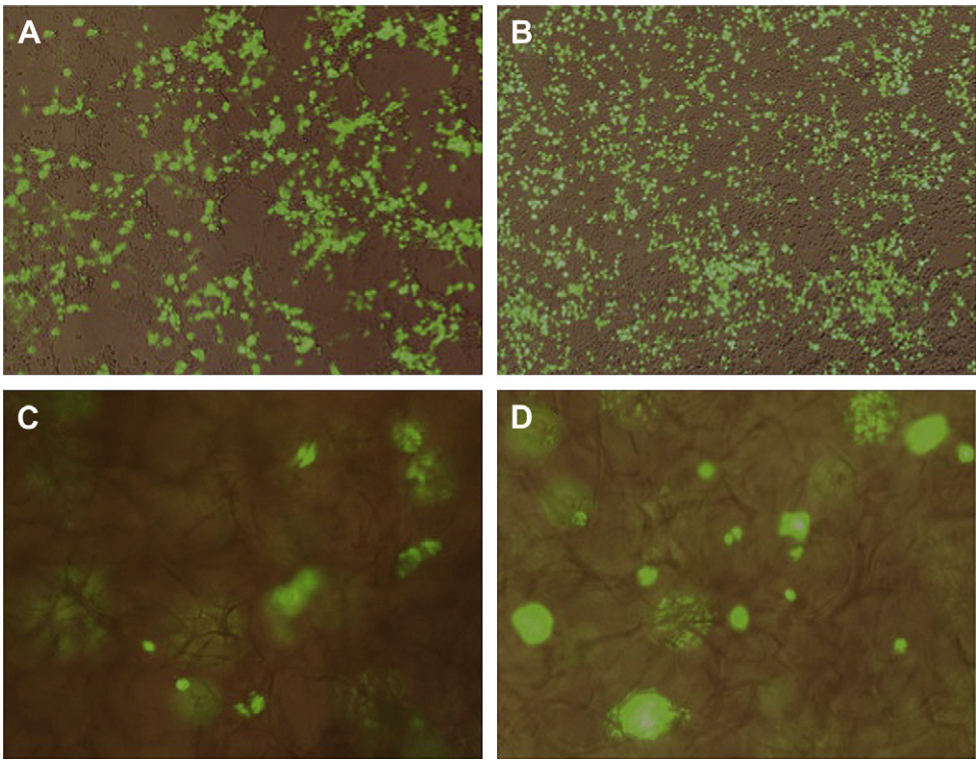

Fig. 3. Microscopic images of cells following delivery of PEI-LA complexes. Cells were grown as a monolater on tissue culture plates (A, B) or in gelatin sponges (C, D). PEI-LA/gWiz-GFP complexes were added to cells during seeding (A, C) or 24 h after cell seeding (B, D). Microscopic images were taken 6 days after incubation with the complexes.

increase was likely a consequence of the excessive trypsinization required to recover the cells from the sponges. Subtracting the background of PEI-LA/gWIZ group from the PEI-LA/gWIZ-GFP group, cells from the sponges were ~19% and ~8% GFP positive for suspended and adherent groups, respectively.

3.2. Growth factor secretion from monolayer and sponge cultures in vitro

To confirm growth factor expression, 293T cells were exposed to PEI-LA/bFGF-IRES-AcGFP complexes to determine bFGF production rates. As before, cells were grown as a monolayer and in sponges, and the complexes were added to suspended or adherent cells (Fig. 4A). Suspended cells grown in a monolayer produced ~2.5 ng of bFGF/well. Suspended and adherent sponge cultures along with monolayer adherent cultures all produced ~3.5 ng bFGF/well. No bFGF was detected in monolayer cultures when cells were untreated or treated with blank PEI-LA/gWIZ complexes. Small amount of bFGF (<0.3 ng/well) were detected in controls from cells in sponges, which was hypothesized to be due to cross-reactivity of scaffold components with the bFGF ELISA. Complete extraction of the cells from the sponge cultures was not possible, so that bFGF secretion rates could not be normalized to cell numbers. In a subsequent study, protein secretion rates from monolayer culture were investigated from cells treated with PEI-LA/bFGF-IRES-AcGFP and PEI-LA/BMP2-IRES-AcGFP complexes. Relatively large amounts of BMP-2 and bFGF secretion rates were evident: ~13 ng BMP-2 and ~3.5 ng bFGF per 10^6 cells/day (Fig. 4B). No BMP-2 or bFGF was detected in the No Treatment and PEI-LA/gWIZ control groups.

3.3. GFP transfection in scaffolds after implantation

Transgene expression *in vivo* was first investigated by delivering the GFP-expression vector pEGFP-N2, soaked in collagen (Fig. 5A) and gelatin sponges (Fig. 5B). Collagen scaffolds were first tested using the PEI25 carrier. The sponges showed extensive host cell infiltration around the scaffolds, whose outline was visible by the faint background fluorescence of the collagen implant. With PEI25/pEGFP-N2 complexes, localized regions of strongly fluorescent GFP-positive cells were observed unlike the sponges with blank PEI25/gWIZ complexes.

Gelatin scaffolds was then evaluated for GFP expression by implanting the plasmid DNA either naked (i.e. without a carrier) or in complexes (Fig. 5B). The bFGF-IRES-AcGFP plasmid was also delivered in addition to the pEGFP-N2 plasmid to ensure that the obtained results were not specific to one type of expression vector. Similar to collagen sponges, no GFP expression was obtained in the case of naked (i.e., without a carrier) pEGFP-N2 or bFGF-IRES-AcGFP delivery. No GFP expression was observed with PEI25/pEGFP-N2 or PEI25/bFGF-IRES-AcGFP complexes either. GFP expression was, however, observed in patches when PEI-LA was used to deliver pEGFP-N2 and bFGF-IRES-AcGFP plasmids. There was no apparent difference in GFP expression between the two plasmids in this set of implants. The PEI25 and PEI-LA complexes containing gWIZ complexes did not give any GFP expression (data not shown), as expected.

3.4. bFGF and BMP-2 secretion from implanted scaffolds

3.4.1. In vivo protein secretion

To evaluate bFGF and BMP-2 secretion *in vivo*, gelatin sponges were implanted with gWIZ, bFGF-IRES-AcGFP and BMP-2-IRES-AcGFP complexes prepared with PEI25 and PEI-LA. An obvious difference between the implants containing the bFGF and BMP-2 expression vectors and the control gWIZ vector was the difference in tissue organization around the scaffolds and new blood vessel formation at the time of explantation (Appendix 1). Gelfoam sponges containing PEI25/gWIZ and PEI-LA/gWIZ complexes showed little integration with the surrounding tissue with no evidence of visual angiogenesis (Appendix 1A, B). Little or no effort was required to extricate these sponges from their subcutaneous sites. However, sponges with polymer complexes containing bFGF-IRES-AcGFP and BMP2-IRES-AcGFP were well integrated with the surrounding tissue (Appendix 1C–F). These implants were surrounded by soft tissue to such an extent that surgical scissors were required to fully excise the implanted sponges. Additionally, hematomas were observed within these sponges.

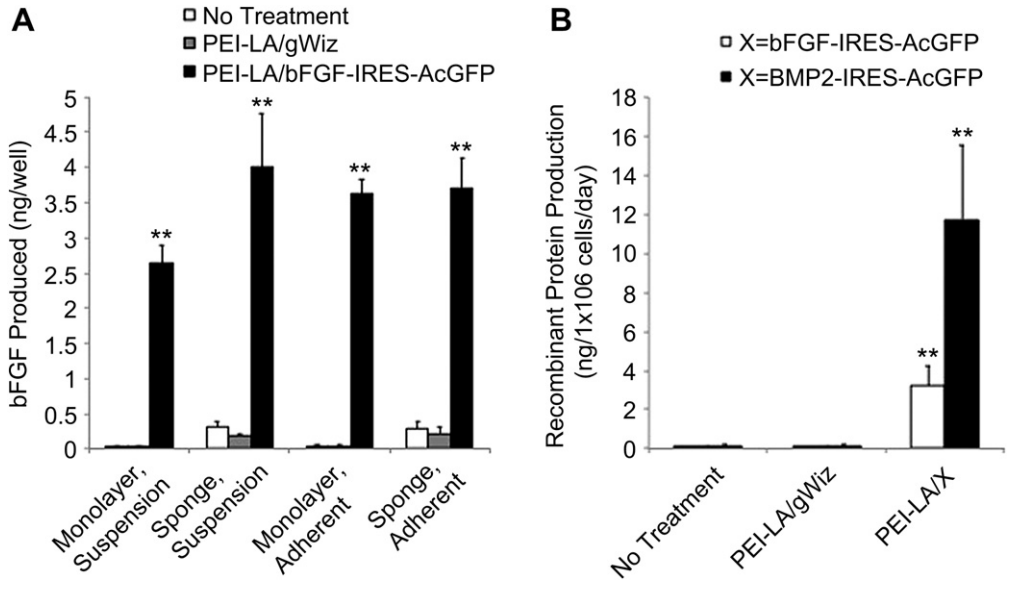

Fig. 4. Recombinant growth factor production in 293T cells. (A) PEI-LA/bFGF-IRES-AcGFP and PEI-LA/gWiz complexes were added to 293T cells grown as a monolayer and in gelatin sponges. Complexes were added either during seeding (suspended) or one day after cell seeding and attachment (adherent). Media was assayed for bFGF production 3 days later and results are summarized as ng protein secreted per well. (B) Specific protein secretion rates from monolayer cultures for bFGF and BMP-2 expression vectors. Media was assayed for protein production 3 days later and results are summarized as ng protein secreted per 10^6 cells per day. $^{**}p < 0.01$.

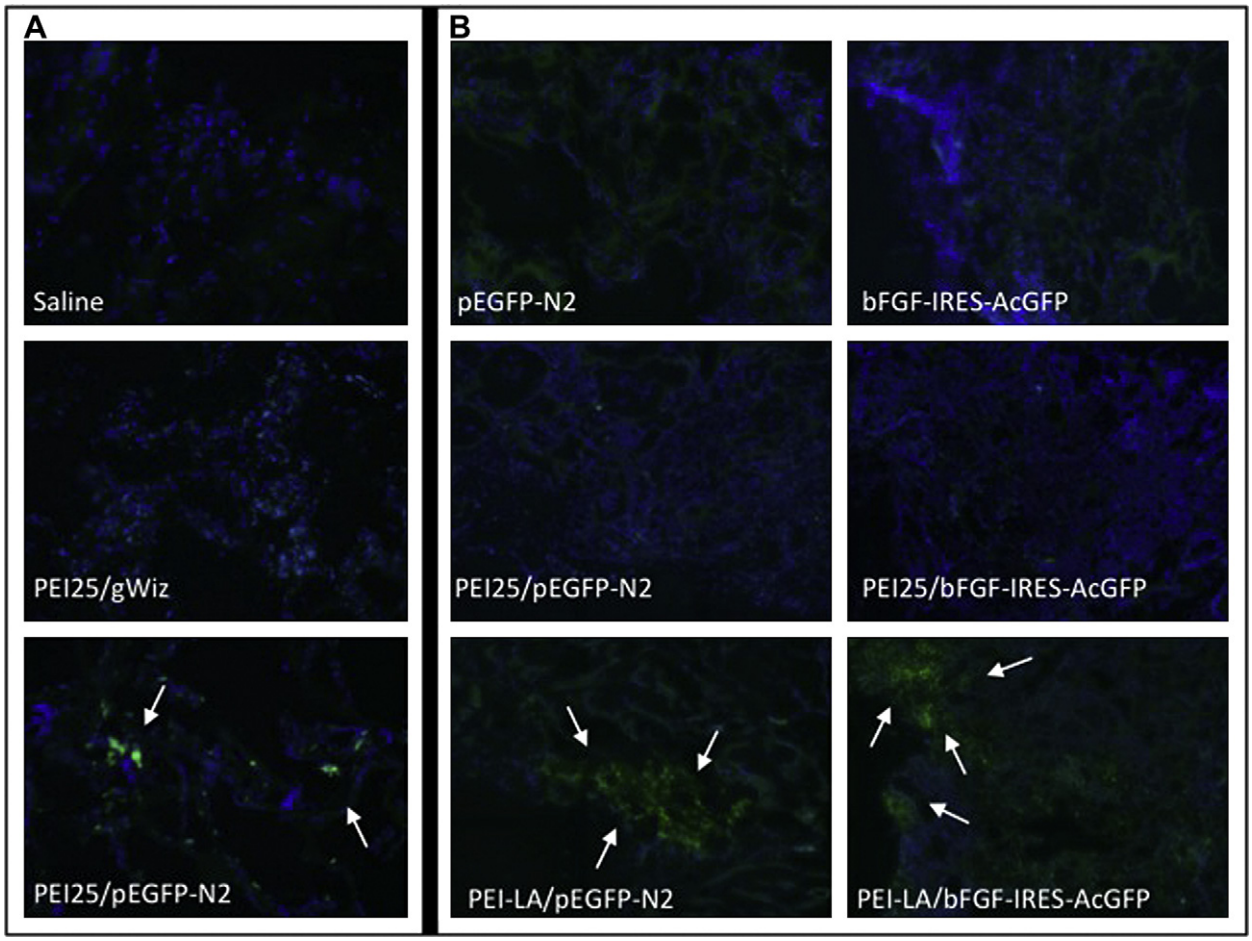

Fig. 5. Histology of polymer/pDNA-loaded sponges following recovery from subcutaneous implantation. Collagen scaffolds were implanted for 8 days (A), and gelatin scaffolds for 14 days (B). The plasmids or polymer/plasmid complexes delivered in each scaffold is indicated on each image. Arrows indicate GFP-positive regions. The plasmids in the absence of a carrier did not give any GFP-positive cells. With collagen sponges, PEI25 was effective for GFP expression, but not with gelatin sponges, where only PEI-LA was effective in supporting the trangene (GFP) expression. Note that the scaffolds themselves showed low levels of diffuse autofluorescence.

To determine whether recombinant growth factors were being expressed, explanted sponges were lysed and the lysates were analyzed by ELISAs. The bFGF amount in scaffolds implanted for 1, 2 or 5 weeks is shown in Fig. 6. The bFGF amounts were minimal (0–0.2 ng/implant) for implants containing bFGF-IRES-AcGFP and pEGFP-N2 plasmids alone (i.e., without a carrier) after a 1-week implantation (Fig. 6A). There were no changes in bFGF levels for PEI25/pEGFP-N2 and PEI-LA/pEGFP-N2. For the polymer/bFGF-IRES-AcGFP complexes, one implant in both PEI25 and PEI-LA groups (out of 6 implants) showed increased bFGF concentration (0.8 and 1.1 ng/implant, respectively), but the mean differences in these groups were not significantly different from the control group. The bFGF expression after 2 weeks of implantation is shown in Fig. 6B for PEI2-LA complexes. For these implants, complexes with BMP2-IRES-AcGFP plasmid were also implanted to ensure there was no ELISA cross-reactivity between the two recombinant proteins. After 2 weeks, the range of bFGF detected was 0–0.5 ng/implant in the control group, and there was no significant increase in bFGF secretion with PEI-LA/bFGF-IRES-AcGFP and PEI-LA/BMP2-IRES-AcGFP complexes. Similar results were observed for groups following a 5 week implantation with a range of 0.1–0.7 ng/implant for the three groups (Fig. 6C).

Similarly, BMP-2 expression was evaluated in scaffold (Fig. 7). No BMP-2 was detected in the No Treatment group after a 1 week of implantation (Fig. 7A). Up to 1.1 ng/implant was detected when the BMP2-IRES-AcGFP plasmid alone (without a carrier) was delivered after one week. No BMP-2 was detected when the BMP-2 plasmid was delivered with PEI25; however, there was up to 1.2 ng BMP-2/implant when the plasmid was delivered by PEI-LA (not significantly different from no carrier group). At 2 and 5 weeks, BMP-2 protein was detected following delivery of both PEI-LA/bFGF-IRES-AcGFP and PEI-LA/BMP2-IRES-AcGFP complexes (Fig. 7B, C). At 5 weeks, BMP-2 protein was detected in implants receiving PEI-LA/gWIZ, PEI-LA/bFGF-IRES-AcGFP, and PEI-LA/BMP2-IRES-AcGFP, which ranged from 0 to 3.5 ng/implant with no apparent differences among the study groups (Fig. 7C).

3.4.2. *Ex vivo protein secretion*

Given the difficulty in detecting bFGF and BMP-2 proteins in scaffolds, we cultured the explanted scaffolds *ex vivo* for up to five days and assayed the media for protein secretion. It was hypothesized that (i) the secreted proteins did not have a chance to accumulate in implants, and/or (ii) supernatant from *ex vivo* culture would show less background than lysate from the scaffolds, so that the secreted proteins would be more easily detected in this way. A preliminary study was performed to determine whether explanted scaffolds would survive *ex vivo* culture long enough to produce recombinant growth factor. The implants received BMP2-IRES-AcGFP plasmid (10 μg/implant) without a carrier, or as complexes with PEI-LA and PEI25, and recovered after 1, 2 and 3 weeks. The GFP fluorescence in the implants receiving complexes was significantly higher than the implants receiving the plasmid

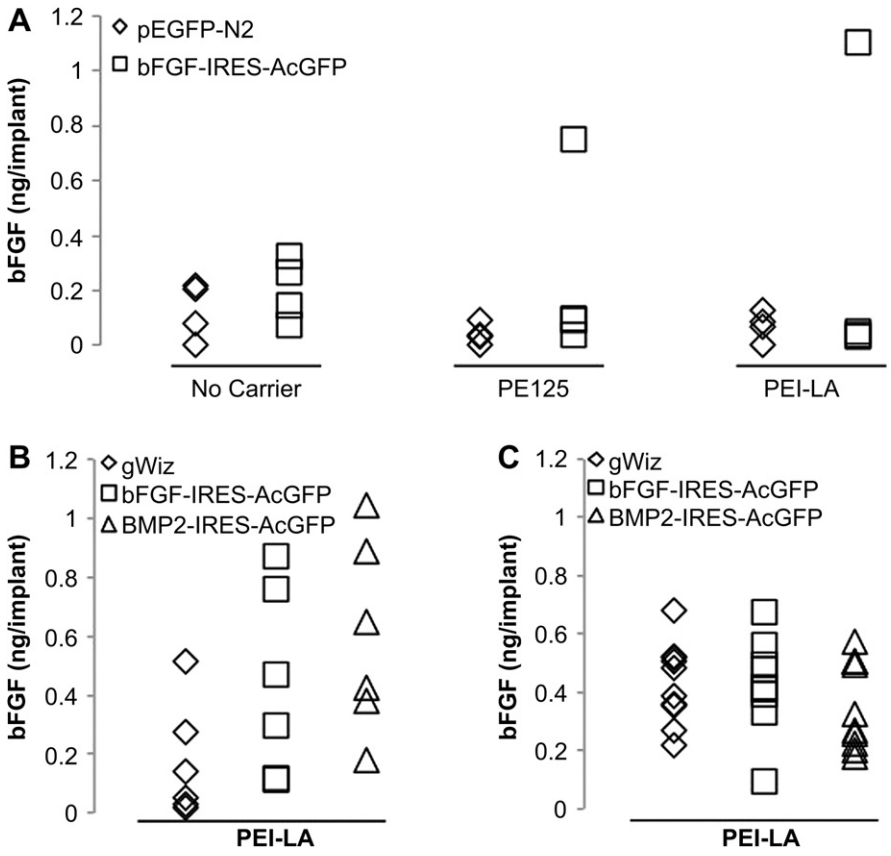

Fig. 6. *In situ* detection of recombinant bFGF in sponges following subcutaneous implantation. (A) For implants recovered after 1 week, gelatin sponges were loaded with plasmids pEGFP-N2 and bFGF-IRES-AcGFP without any carriers or with PEI25 and PEI-LA complexes. For implants recovered after 2 (B) and 5 weeks (C), gelatin sponges were loaded with PEI-LA complexes of gWIZ, bFGF-IRES-AcGFP and BMP2-IRES-AcGFP. The weight ratios of polymer/plasmid were 5/2 and 10/2 for PEI25 and PEI-LA, respectively. The lysates from the recovered implants were assayed for bFGF production.

without any carrier, especially for implants recovered after 2 and 3 weeks ($p < 0.01$; Fig. 8A). No significant BMP-2 secretion was detected following *ex vivo* culture (Fig. 8B), although several PEI-LA explants showed increased BMP-2 expression on week one and two.

A similar study was repeated by implanting the BMP-2 expression vector by using gelatin and collagen scaffolds, but a significantly higher dose (50 μg) of plasmid was used. The implants received either gWIZ or BMP2-IRES-AcGFP complexes of PEI-LA. Following 1 week of implantation, *ex vivo* BMP-2 secretion rates were summarized in Fig. 9. Compared to PEI-LA/gWIZ controls, sponges with PEI-LA/BMP2-IRES-AcGFP complexes showed increased BMP-2 secretion during day 0–3 post explantation (Fig. 9A; $p < 0.01$ and $p < 0.05$ for collagen and gelatin sponges, respectively). The net BMP-2 secretion rates on Day 0–3 from the collagen and gelatin sponges (i.e., difference between average secretion from PEI-LA/BMP2-IRES-AcGFP complexes minus average secretion from PEI-LA/gWIZ complexes) were ~0.12 and 0.28 ng/implant/day, respectively. Similar net secretion rates of 0.16 and 0.30 were obtained for collagen and gelatin implants, respectively, on Day 4–5 (Fig. 9B), although this difference was not significantly different at this time point.

A similar study was conducted for assessment of bFGF secretion as well, by using bFGF-IRES-AcGPF plasmid delivered without a carrier or as complexed with PEI-LA. No bFGF was detected from the delivery of naked bFGF-IRES-AcGFP plasmid while PEI-LA complexes gave a net bFGF secretion rate that was lower than the BMP-2 secretion rate (<0.08 ng/implant/day). The difference between the two groups was not significant ($p > 0.05$; not shown).

4. Discussion

Direct gene delivery offers an exciting strategy for bone regeneration and repair. *In vivo* gene delivery, however, requires confirmation of effective gene expression *in situ*, as the success of many carriers *in vitro* does not readily translate to success in an animal model. Recombinant protein expression *in situ* allows direct estimation of gene delivery efficiency, and provides a method for comparing the effectiveness of carriers intended for direct gene delivery. This study reports on the feasibility of assessing transgene expression and compares *in vivo* performance of two polymeric systems, namely PEI-LA and PEI25. The latter is the 'gold standard' for *in vitro* transfection studies, where it provides a cost-effective, albeit relatively cytotoxic [31], reagent for routine cell modification. PEI25 serves as a routine reference for *in vitro* transfection studies, and it is one of the two polymers used for non-viral gene delivery in bone regeneration (as stated in Introduction). Our previous studies showed that local concentration of PEI25 was critical when it was used in nanoparticulate formulations for BMP-2 protein delivery; measures to reduce its toxicity was needed to sustain a robust bone induction *in vivo*. The PEI-LA, on the other hand, was derived from a non-toxic PEI molecule (2 kDa PEI), which was lipid-substituted for better packaging of nucleic acids and improved interaction with cellular membranes necessary for cellular delivery. Using the reporter protein GFP, PEI-LA was shown to give superior transgene expression compared to PEI25 in primary bone marrow stromal cells from rats *in vitro* [32], but with less toxicity displayed on the highly sensitive primary cells.

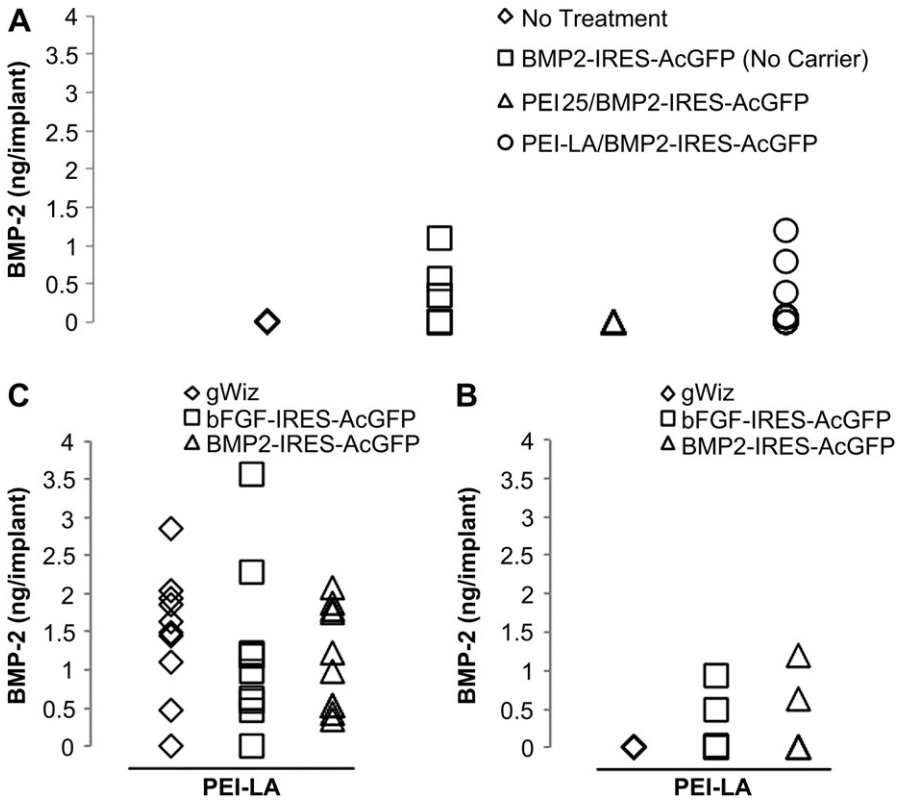

Fig. 7. *In situ* detection of recombinant BMP-2 in sponges following subcutaneous implantation. (A) For implants recovered after 1 week, gelatin sponges were loaded with saline (no treatment), BMP2-IRES-AcGFP without any carrier or BMP2-IRES-AcGFP complexed with PEI25 and PEI-LA. For implants recovered after 2 (B) and 5 weeks (C), gelatin sponges were loaded with PEI-LA complexes of gWIZ, bFGF-IRES-AcGFP and BMP2-IRES-AcGFP. The weight ratios of polymer/plasmid were 5/2 and 10/2 for PEI25 and PEI-LA, respectively. The lysates from the recovered implants were assayed for BMP-2 production.

The *in vitro* efficiency of PEI-LA was first investigated with 293T cells and by using the vectors specifically designed for growth factor expression. Standard transfection protocols reported in the literature typically expose monolayer cells to complexes in media on the day after seeding, whereas direct *in vivo* gene delivery involves host cells infiltrating the complex-loaded sponge and internalizing the complexes as they penetrate into the sponge. A variety of host cells are expected to invade the sponge at ectopic sites [33], but the specific phenotype conducive for complex uptake and expression remains elusive. To account for this important difference, the ability to transfect both adherent cells and suspended cells, which may better represent cell invasion into the scaffold, were investigated. Gene delivery with gelatin scaffolds was assessed to ensure that there was no unintended interaction between the complex and sponge components that might have prevented transfection. Extensive GFP expression was seen in 293T cells exposed to PEI-LA complexes in the sponge, which was also confirmed by the bFGF expression and secretion into the culture

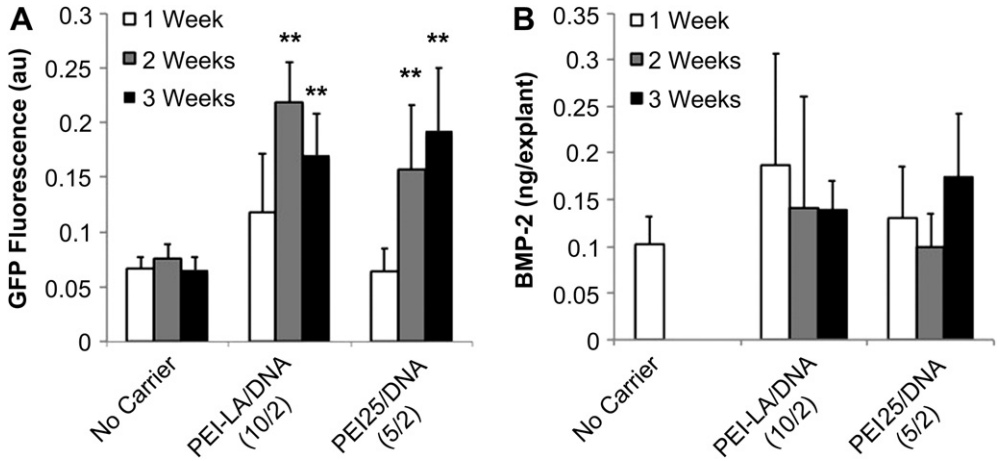

Fig. 8. Assessment of GFP (A) and BMP-2 (B) expression in gelatin sponges following *ex vivo* culture. Gelatin sponges were loaded with BMP2-IRES-AcGCP plasmid (10 μg) without a carrier or as complexes with PEI-LA (10/2 w/w) and PEI25 complexes (5/2 w/w). The implants were harvested after 1, 2 and 3 weeks. The GFP expression was assessed by recovering the cells in implants and measuring GFP fluorescence in a plate reader, which BMP-2 in the supernatants was detected by ELISA.

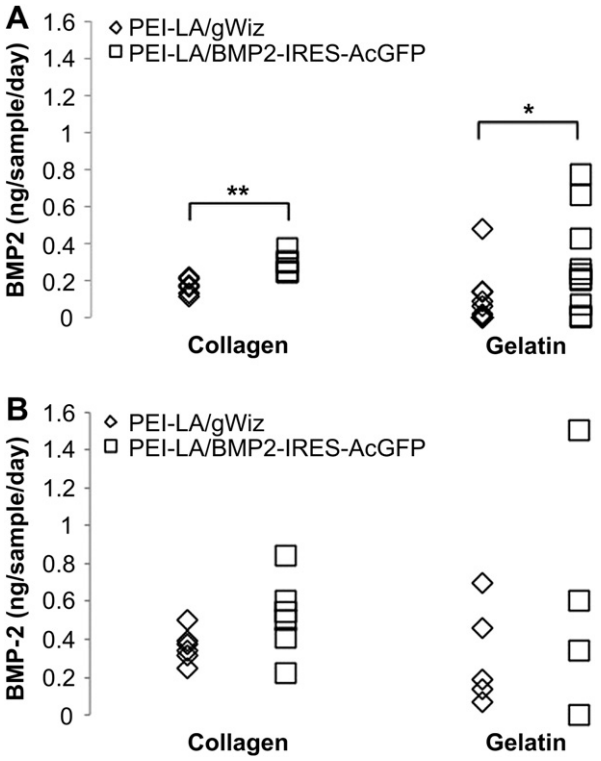

Fig. 9. Recombinant human BMP-2 secretion in collagen and gelatin sponges following *ex vivo* culture. Absorbable collagen or gelatin sponges were loaded with polymer/pDNA complexes by using gWIZ and BMP2-IRES-AcGFP plasmids and PEI-LA polymer. Following subcutaneous implantation for 1 week, sponges were cultured *ex vivo*, and the media assayed for BMP-2 secretion between day 0–3 (A) and day 4–5 (B). $^{*}p < 0.05$, $^{**}p < 0.01$.

supernatants. These results indicated that gene delivery was successful in sponges and there were no major differences between the ability of cells getting transfected as a monolayer or in three-dimensional sponge culture. A comparison between the bFGF and BMP-2 expression vectors showed that the BMP-2 vector gave better protein expression/secretion than the bFGF plasmid (~3-fold better), based on results with monolayer cultures. The difference between bFGF and BMP-2 expression may be due to differences in mRNA stability, as bFGF mRNA contain an antisense transcript used to regulate expression [34].

The recombinant protein production obtained with PEI-LA mediated delivery (3.5–13 ng/10^6 cells/day) was similar to *in vitro* reported rates with 293T cells using PEI25 and Lipofectamine2000™ (estimated at 30 ng/10^6 cells/day) [30], and using adenovirus vectors (15 ng/10^6 cells/day) [35]. Higher (~10-fold) expression was observed with an integrating retroviral transduction system on a murine chondrogenic cell line [36]. In primary fibroblasts transfected with PEI25 [37] or nucleofection [38], chondrocytes transfected with Fugene™ 6 [39], and bone marrow stromal cells transfected with Lipofectamine or PEI [30], similar production rates was obtained compared to fibroblasts transduced with an adenovirus of <1 ng/10^6 cells/day [40]. Others reported much higher protein secretion rates (10–100 fold) for retrovirus transduction of chondrocytes [36], and adenovirus transduction of mesenchymal stem cells [41] and gingival fibroblasts [42]. Therefore, non-viral carriers, including PEI-LA, seem to match some of the 'lower-performing' viral vectors for *in vitro* transfection, but exuberant secretion rates, which might be needed for some applications, seem to be obtained with only certain viral vectors.

The ultimate test of a delivery system, however, is its *in vivo* performance and viral vectors show a well-documented decrease in efficiency in component animal models due to immune system interference. The gene delivery was attenuated [43] or ineffective in immune-competent animals [44–47] with viral vectors. Immune suppression can restore efficiency [48,49], but such treatment makes clinical translation of these viral systems unlikely. On the other hand, non-viral carriers are not expected to be affected by the immune response to the same extent. Not all non-viral carriers are an appropriate choice for use *in vivo*, however, and they are greatly limited by their toxicities. Both PEI25 [17] and SuperFect™ [20] were limited by high toxicity. Similar to PEI25, no more than 40 μg SuperFect™ could be employed without concerns of toxicity, which limited the amount of plasmid administered to 10 μg [20]. Although increased bone formation was observed with SuperFect™, new bone was formed mostly on the underside of the implant without infiltrating into HA scaffolds in that study. Similar bone formation patterns were observed with PEI25 as well [16], where new bone was primarily found in the periphery of cranial defects. The pattern of tissue regeneration in these models may suggest that the recombinant BMPs are expressed at a low dose, and therefore acting as a chemotactic agent [50], instead of a morphogen [1]. While chemotactic effects would lead to tissue induction, higher doses would be needed for tissue calcification to fully heal critical sized defects [3].

Bone regeneration is affected by a myriad of factors including the species, immune status, defect model, type of implant scaffold, and the choice of therapeutic protein and, hence, comparisons among different gene delivery studies in animal models are difficult. Recombinant protein expression *in situ* is potentially a better parameter for delivery system development and optimization. Several studies have investigated non-viral gene delivery for bone regeneration but none have quantified recombinant protein production. We readily detected GFP expression with as little as 10 μg plasmid DNA delivered with the PEI-LA; however, expression of recombinant BMP-2 was not as clear at this plasmid dose. This may be due to differences in localization of the gene product: GFP is retained within the transfected cells, while the growth factors such as BMP-2 are mostly secreted and diffuse away from the implants. Although providing no therapeutic effect, GFP allows for facile detection of successful gene delivery. At this plasmid dose, the effectiveness of PEI-LA was equivalent to PEI25 for GFP expression (Fig. 8). At the higher dose of 50 μg, PEI-LA outperformed PEI25, based on histological assessment of GFP expression (Fig. 5). This may be a manifestation of the cytotoxicity of PEI25 at the higher dose.

Several studies have investigated delivery of naked plasmid DNA for bone regeneration but few have directly compared delivery of naked to polymer-mediated delivery. Higher amounts of plasmid were generally required for bone regeneration with naked plasmid. No bone formation was observed with 100 μg of BMP-4 plasmid delivered intramuscularly [15], whereas minimal bone formation was detected with 200 μg of BMP-4 plasmid in a cranial defect site [16] or 500 μg of BMP-7 plasmid in a collagen solution [12]. Bone formation was observed with 100 μg of VEGF plasmid in a radial critical defect model [11], 500 μg to 1 mg of BMP-4 and hPTH1-34 plasmids in a femur critical sized defect [9], and 40 mg of h-PTH1-34 plasmid in a tibia critical model defect [10]. Lower doses of plasmid have led to bone formation when combined with a polymeric carrier. Bone formation was observed when PEI25 delivered 200 μg of BMP-2 plasmid in cranial defect [16]. Similarly, 10 μg of BMP-2 plasmid delivered by SuperFect™ in cranial critical defect [20] or by calcium phosphate in subcutaneous model [21] led to bone formation. The smallest amount of plasmid DNA that led to bone formation was 1.3 μg of plasmid DNA coding for Runx2 and caALK6 that was delivered with a novel polymer in a rat cranial defect model [22]. Some of these studies included histological

assessment to confirm efficacy, although these studies provided no quantitative expression data and not all included a control plasmid to account for un-specific effects. Unmethylated plasmid DNA produced by bacteria can induce immune responses [51,52]. Methylated DNA can attenuate immune response [53] to extend transgene expression [54], but it can still induce immune activation and contribute to osteogenesis. Cytokines produced by monocytes can stimulate an osteogenic response from bone marrow stromal cells, including increased BMP-2 and Runx2 production [55]. Such immune regulated changes in osteogenesis highlight the need to confirm recombinant protein expression and compare bone formation against appropriate controls including non-expressing plasmids.

At the plasmid dose employed in this study, a polymeric carrier was required for transgene expression. Clear detection of recombinant proteins was successful only in an *ex vivo* culture model with the 50 μg plasmid implant dose; high background in control implants made detection of proteins in scaffold difficult when we attempted to extract the proteins from implants. It is possible that intracellular proteins released by the lysis buffer and/or extracellular proteins deposited in implants, including chromogenic components of vascular system (such as red blood cells), were the reasons for this background. Even in the *ex vivo* secretion model, significant increases in recombinant protein secretion were observed for only the BMP-2 (Fig. 9), and not the bFGF (not shown), which most likely reflected differences in the expression rates between the two plasmids, as observed *in vitro*. In addition to the recombinant protein, collagen and gelatin scaffolds were compared for delivery of gene complexes. Collagen sponges are used for delivery of BMP-2 for bone regeneration [2,3,19], whereas gelatin (i.e., denatured collagen) sponges are primarily used as a hemostatic agent. Gelatin sponges have been investigated to deliver proteins for bone regeneration [56,57], so that the use of gelatin was not likely an impediment for bone regeneration. Although the details of fabrication (e.g., crosslinking reactions) for these two sponges are not readily known, we wanted to use both sponges to make sure that the nature of a scaffold did not bias the obtained results. Higher BMP-2 expression (~0.3 ng/implant/day) was observed on gelatin sponges compared to collagen sponges (~0.1 ng/sample/day). The reasons for this difference is not obvious at this time, but differences in the recombinant protein expression relay the importance of selecting not only appropriate gene carriers, but also appropriate scaffolds. To compare the rate of PEI-LA-mediated gene expression, an independent study reported BMP-2 secretion rate of 0.1 ng BMP-2/implant/day [58] with *ex vivo* adenovirus transduction of fat pads, and was accompanied by robust bone regeneration and healing of a critical-sized femur defect. Similarly, 0.25 ng BMP-2/clot/day was produced by chondrocyte clots transduced *ex vivo* with a retrovirus for repair of an osteochondral defect [59]. Both of these studies were conducted at a bony site and they were able to demonstrate a stimulation of bone formation in the employed model. Based on release rates alone, it is likely that the non-viral approach reported here should be also suitable for these models.

We were not able to see any osteogenic transformation at the subcutaneous implant site employed for this study. BMP-2, but not bFGF, usually provides a robust bone induction at this site when a sufficiently high dose of protein (>1 μg/implant) is administered ectopically. With our system, we exposed to local site at most ~10 ng BMP-2 during 1–2 weeks of implantation period, which is below the dose needed to sustain an osteogenic effect at an ectopic site. The small amounts of BMP-2 produced with PEI-LA gene delivery, however, might be sufficient to produce bone formation in a bone defect model, which has a greater osteogenic capacity than an ectopic site [60]. It might be possible to add supplementary factors to attract a robust population of target cells (e.g., by using SDF-1 for BMP-2 responsive, CXCR4-positive stem cells [61],), where sub-optimal doses of osteogenic proteins was sufficient to induce effective osteoinduction. Future studies using orthopaedic sites will provide a better indication if the non-viral gene expression levels reported in this study will translate into new bone induction.

5. Conclusions

Recombinant protein expression was determined *in vitro* and, more importantly, *in vivo* using a rat subcutaneous implant model. Despite being expressed from the same plasmid vector, BMP-2 expression was found to be higher than the bFGF in 293T cells *in vitro* and after implantation *in vivo*. GFP was readily detected histologically *in vivo* with as little as 10 μg of plasmid, and was detected macroscopically for up to 3 weeks after implantation when delivered with PEI-LA. High background in controls prevented clear detection of secreted proteins in implants but, by using an *ex vivo* culture method, implants receiving the plasmid complexes with polymeric carriers were shown to secrete significant amount of recombinant proteins. Compared to *in vitro* studies, where ~2 μg/mL DNA concentrations were sufficient to provide readily detectable proteins secretion, larger amounts of plasmid DNA (50 μg/implant) were required to observe significant increases in BMP-2 secretion. Furthermore, scaffolds delivering complexes influenced recombinant protein production: more BMP-2 was produced from PEI-LA/plasmid complexes delivered on a gelatin scaffold than a collagen scaffold. Taken together, our studies indicate that PEI-LA was an effective *in vivo* gene delivery carrier and yields BMP-2 production rates similar to viral gene delivery reported in the literature. Given the perceived notion that viral vectors are generally more effective, it is particularly important to investigate recombinant protein production for non-viral carriers *in situ* for better assessment of their clinical potential.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.biomaterials.2012.01.031.

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