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Non-viral-mediated gene therapy approaches for bone repair

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Non-viral-mediated gene therapy approaches for bone repair

Structured Abstract

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Objectives – Bone repair strategies continue to be developed for alternatives to autografting, allogeneic implants of banked bone, and other bone substitutes. Efforts have included the delivery of potent growth and/or differentiation factors and the use of gene therapy. For bone regeneration, gene therapy is the delivery, uptake and expression of DNA that has been localized to a wound bed. The objective of the current study is to investigate methods to enhance non-viral-mediated means of gene uptake and expression for use in bone regeneration.

Methods – Several types of DNA-polymer complexes, either applied directly to baby hamster kidney (BHK) cells, or released from a porous, resorbable gene-activated matrix (GAM), were evaluated *in vitro* for their ability to transfect cells with a circular plasmid DNA construct expressing green fluorescent protein. Complexes included conjugates containing a lipophilic reagent, liposomes, poly-ethyl-oxazoline, and poly-ethyleneimine (PEI). Data were subjected to analysis of variance and Fisher's protected least significant difference for multiple comparisons with significance established at $p < 0.05$.

Results – Transfection efficiencies of the liposome and PEI complexes improved *in vitro* when released from resorbable GAMs. The lipophilic reagent FuGene 6 demonstrated abundant uptake and expression in the initial 1- and 2-day evaluation periods. In contrast, the DNA-liposome and PEI GAM complexes demonstrated a sustained release, uptake and expression by the BHK cells at the 2-, 4-, and 7-day, and 4- and 7-day evaluation intervals, respectively.

Conclusion – GAM technology appears to improve the functional stability and release duration of incorporated DNA-polymer complexes in the present *in vitro* studies. The ongoing objective of our research is to develop a localized

treatment to improve the uptake and expression of plasmid DNA by non-viral-mediated gene therapy.

Key words: collagen; gene-activated matrix; gene therapy; plasmid DNA, poly (*alpha*-hydroxy) acids; tissue engineering

Introduction

Modifications to cell functions during embryogenesis leads to a variety of craniofacial malformations. The cleft component of the most common malformation, cleft lip, and palate, are generally autografted with either cranial or iliac crest bone grafts to fill the maxillary alveolar defect. These grafts can restore enduring bony contour, allow tooth eruption and have a reported success rate approximating 80% (1). However, issues such as donor site morbidity, a finite supply of donor tissue, prolonged hospital stays, and increased costs have prompted the development of alternative treatment modalities. Bone regeneration with a gene therapy strategy is one of the clinically appealing alternatives. Localized delivery of DNA to a wound bed, provided the DNA can be protected, released over time, and taken-up by *in situ* resident wound healing cells to produce the therapeutic factor, would circumvent some of the regulatory issues surrounding *ex vivo* gene therapy.

Gene therapy was initially envisioned as the insertion of a functioning gene into cells of a host to replace a hereditary genetic abnormality, or to provide a new function in a cell, such as producing a growth factor or even killing cancer cells (2) The delivery of genetic material, i.e. the cDNA, can be delivered by an *ex vivo* approach, or directly into a target cell via *in situ* innoculation. The *ex vivo* method generally utilizes autologous cells that are removed from the body, generally transduced with viral vectors containing recombinant genes, and re-inserted into the chosen tissue. Many pre-clinical animal studies have been undertaken utilizing the *ex vivo* approach, and have been reviewed previously (3,4).

The *in situ* approach of somatic gene therapy introduces the cDNA directly into the targeted tissue. Some of these include the delivery of 'naked DNA' in a liquid buffer (5), or formulations in liposome carriers (6). In addition, DNA has been formulated in a number of polymer systems to attain sustained release, such as hydrogel suspensions (7), and polymer encapsulation for oral (8) and intra-arterial (9) delivery. DNA has also been incorporated into the structural matrix, the so-called gene activated matrix (GAM) formulation (10,11). The implantable GAM system is an important consideration for tissues like bone that prefer a scaffold for regenerating and has demonstrated the ability to provide an environment to protect the gene therapy vector for sustained release, uptake, and longer expression times (11,12). There are a variety of other non-viral-mediated gene transfer vector systems, including, but not limited to, electroporation, microinjection, particle bombardment, receptor-mediated endocytosis, immunopolyplexes, photochemical transfection, and nebulization of lipid/DNA complexes. These have been reviewed elsewhere (3,4).

Gene therapy vectors have been described as either a disabled, replication incompetent virus or a DNA structure (e.g. plasmid DNA) used as a vehicle to transfer genes into cells (2). Vectors provide the means to deliver cDNA into the appropriate cells, either *ex vivo* or *in situ*, and render a cell capable of expressing the transgene product. Several vector systems are available, including replication-deficient recombinant viruses, as well as non-viral-mediated DNA molecules/complexes. Non-viral-based systems offer advantages such as proven stability under a variety of conditions, utilization in a number of delivery systems, and are less immunogenic than the viral vectors (13,14). The major drawback of these systems is that gene transfer is very inefficient, requiring large quantities of materials, sustained release or repetitive administration to achieve clinical success.

The present studies were undertaken to evaluate several non-viral-mediated DNA complexes containing circular plasmid DNA within a mammalian expression vector containing the reporter gene, green fluorescent protein (GFP). Transfection efficiencies, i.e. those cells that contain and express the plasmid DNA expressing GFP, were calculated to determine preferred candidates for subsequent studies in a rat cranial defect model and a juvenile canine alveolar cleft model.

Materials and methods

Materials

All reagents were purchased from Sigma Chemical (St. Louis, MO) or InVitrogen/GIBCO (Grand island, NY) unless otherwise specified. Falcon tissue culture-treated 12 well plates were obtained from Fisher Inc.

eGFP plasmid DNA

Recombinant PCR methodology was used to generate an enhanced green fluorescent protein fusion gene. Thereafter, a 478 bp IgSP-eGFP fusion PCR product and the cloning vector pCEF1*alpha* (15) were digested with *Nhe*I and *Not*I restriction enzymes and subsequently purified from 1% agarose gels using the SpinPrep Gel DNA Kit (Novagen Inc., Madison, WI, USA). The gel-purified insert (4 μl) and vector (1 μl) were added to a sterile 1.5-ml Eppendorf tube containing 5 μl of Clonables 2X Ligation Premix (Novagen). The ligation mixture was incubated at room temperature for 5 min prior to being transformed into NovaBlue Singles Competent Cells (Novagen), according the manufacturer's recommended transformation protocol. Small-scale plasmid preparations were made using SpinPrep Plasmid Kit (Novagen) and subjected to *Nhe*I and *Not*I diagnostic restriction digestions as well as the nucleotide sequence reactions. The resulting plasmid was purified by Mobius 1000 Plasmid Kits (Novagen) for subsequent transfection into mammalian cells (Fig. 1). Aliquots of the final preparation were nicked at appropriate sites and run on 1% agarose gels for quality assurance. This expression cassette has been utilized in our laboratory to express BMP-2, BMP-4, IGF-1, and VEGF.

DNA-polymer complexes and GAM fabrication

For the initial experiment, five types of DNA-polymer complexes and a control well of plasmid with the cells only were prepared. The GFP plasmid combined with the FuGene 6 lipophilic transfection reagent (Roche Diagnostics, Indianapolis, IN) and the GFP plasmid combined with 20 mM DOTAP:cholesterol (1:1) liposome (Sigma) was mixed immediately before application on the cells according to manufacturer's specifications. Three types of gene-activated matrices (GAMs) were fabricated by mixing 5 μl of 0.5 μg/μl GFP plasmid DNA with 5 μl of the FuGene 6 reagent, a second 5 μl of 0.5 μg/μl GFP plasmid DNA with 5 μl of the FuGene 6 reagent, and a third mix of of 0.5 μg/μl

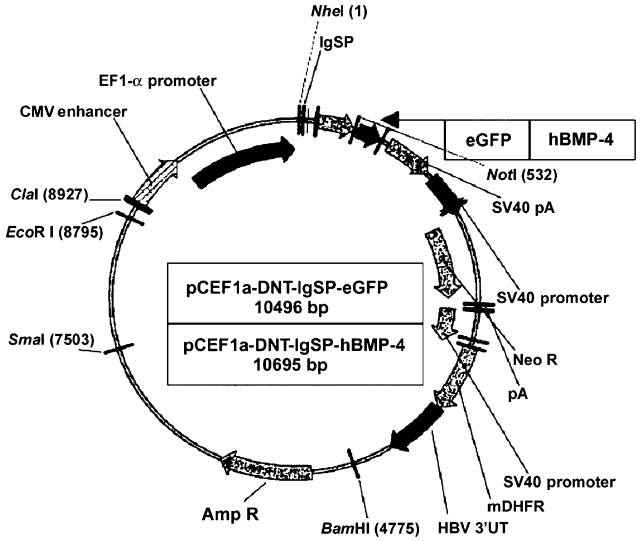

Fig. 1. The pCEF1α-DNT-IgSP-eGFP plasmid expression vector. A CMV enhancer element aids in the up-regulation of the elongation factor 1 *alpha* (EF1-α) promoter. The circular plasmid DNA contains an ampicillin resistant gene for bacterial expansion, and can provide resistance to neomycin analogs for eukaryotic selection. The mouse dihydrofollate reductase gene (mDHFR) provides an additional avenue for cell selection by providing resistance to methotrexate and amplifying gene copy numbers.

GFP plasmid DNA with 5 μl of the 0 mM DOTAP: cholesterol (1:1) liposome reagent. Of the three types, one had an additional 5 μl PBS buffer included (for the pGFP control), while the second and third types were mixed with 10 μl of a 2.5 μg/μl Type-I collagen. These mixtures (20 μl each) were infiltrated into 5 mm porous poly (D,L-lactide) disks fabricated as previously described (16). The lactide disks with their infiltrates were frozen and lyophilized. Prior to immersion into the cells, the disk lyophiles were quickly immersed in 70% filter sterilized isopropyl alcohol, rinsed twice in phosphate buffered saline, and placed in the wells containing BHK cells.

For the second experiment, five types of GAMs were fabricated as described above for the plasmid GFP group, the plasmid GFP/Fugene 6, and the plasmid GFP/liposomes. Two additional groups were fabricated by mixing 5 μl of 0.5 μg/μl GFP plasmid DNA with 5 μl of a 1 μg/μl poly(2-ethyl-2-oxazoline) (MW 5000, Polysciences Inc., Warrington, PA, USA) and 5 μl of 0.5 μg/μl GFP plasmid DNA mixed with 5 μl of a 1 g/μl polyethyleneimine (PEI) (linear MW 25 000, Polysciences). Each type of DNA-polymer complex was mixed with 10 μl of a 2.5 μg/μl Type-I collagen and these mixtures (20 μl each) were infiltrated into 5 mm porous poly (D,L-lactide) disks as described above. The infiltrated

disks were frozen, lyophilized, and prepared for the *in vitro* studies as described above.

Cell culture and analyses

The baby hamster kidney (BHK-21) cell line was utilized for the present studies. Prior to the exposure of the DNA-polymer complexes, controls with the cells, and the GAM lyophiles, 50 000 BHK cells were seeded into each well of the 12 well plates containing 1 ml of *alpha* MEM supplemented with 10% FBS, antibiotics, and maintained in a 37 °C tissue culture incubator. The following day, when the cells attached to the wells demonstrated a 40–60% confluence, the DNA-polymer complexes, and the GAMs (with the lactide polymer scaffolds) were placed in the wells. For the initial experiment, representative wells were visualized and digital images were captured for each group after a 3-day incubation period. Thereafter, the cells were removed by trypsinization, washed, and percent transfection efficiencies determined by a FACScan instrument (FACStar, Becton–Dickinson Inc., Franklin Lakes, NJ, USA). Data are presented as percent of cells expressing GFP vs. total cell number with ±SD from samples in triplicate. The second experiment utilized the same procedures as the initial experiment, with the exception that additional 12 well plates were seeded with BHK cells and the media and GAMs were transferred to new wells at days 1, 2, 4, and 7 to evaluate transfection efficiencies at these intervals. The wells were trypsinized and prepared for FACStar analyses at days 2, 3, 5, and 8.

Statistics

Data presented as mean ± standard deviation with at least triplicate samples analyzed per time and treatment group. Quantitative assessments of the transfection efficiencies as determined by the FACStar instrumentation were analyzed by multiple analysis of variance (ANOVA) and Fisher's protected least significant difference test for multiple comparsions to determie differences among treatments and between time periods. Statistical significance was established at $p < 0.05$.

Results

The pCEF1α-DNT-IgSP-eGFP plasmid mammalian expression vector contains a cytomegalovirus (CMV) enhancer element to up-regulate the elongation factor 1 *alpha* (EF1-α) promoter. The circular plasmid DNA contains an ampicillin resistant gene for bacterial expansion, and also provides resistance to neomycin analogs for mammalian cell selection of transfectants. The mouse dihydrofollate reductase gene (mDHFR) provides an additional avenue for cell selection by providing resistance to methotrexate and provides a method to amplify gene copy numbers within stable transfectants. This system has been utilized to incorporate genes for the expression of morphogens and growth factors such as BMP-2, BMP-4, IGF-1, and VEGF.

The results from the initial experiment at day 3 demonstrated transfection efficiencies of 30.9 ± 4.9 and 22.4 ± 5.2% for the direct application of DNA-polymer complexes with Fugene 6 and liposomes, respectively (Fig. 2). BHK cell toxicities in the range of 5–8 and 30–35% was observed for the FuGene 6 and liposome groups, respectively. Direct application of the circular plasmid DNA containing GFP onto the BHK cells resulted in a transfection efficiency of 0.9 ± 0.2% (Fig. 2). A significant difference was observed between the FuGene 6 and liposome groups as compared to the BHK cell only group. The DNA-polymer complexes delivered from the porous poly (lactide) GAMs demonstrated transfection efficiencies of 1.9 ± 0.5,

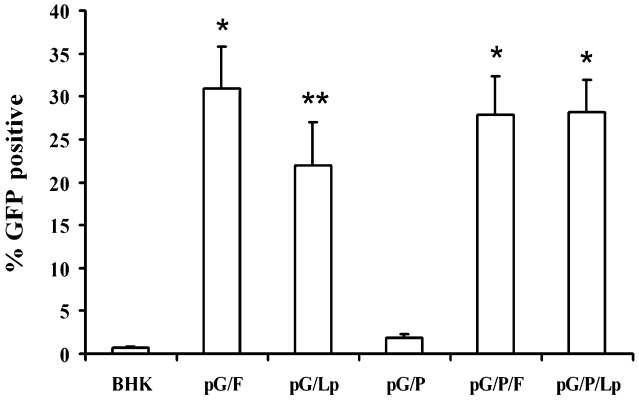

Fig. 2. Percent of the BHK cells expressing GFP as determined by the FACStar cell sorter. The cells were exposed to the DNA-polymer complexes, as well as control groups, and transfection efficiencies were determined 3 days later. Equivalence, with statistically significant (*$p < 0.05$) increases over the control BHK cells, and the plasmid DNA in the polymer GAM (pG/P), was observed in the plasmid GFP-FuGene 6 group (pG/F), the plasmid GFP-FuGene 6 polymer GAM group (pG/P/F), and the plasmid GFP-liposome polymer GAM group (pG/P/Lp). The plasmid GFP-liposome group (pG/Lp) was significantly higher than the controls (**$p < 0.05$), but less than the other three groups (*).

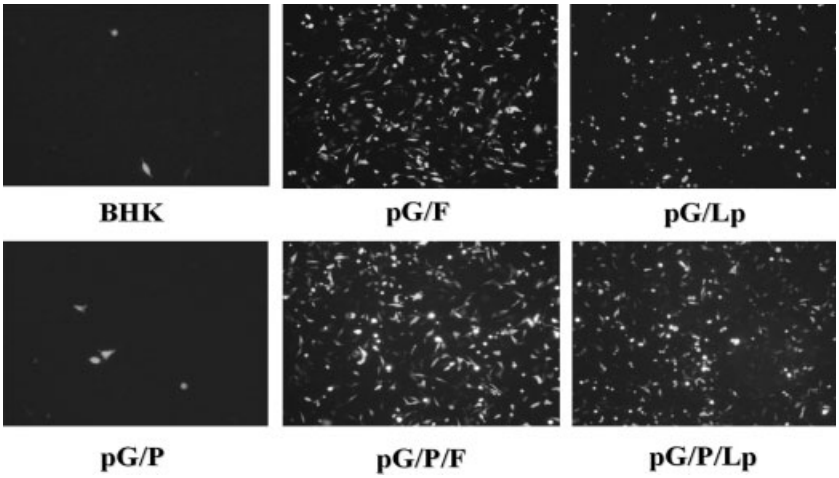

Fig. 3. Representative fluorescent micrographs of the various treatment groups and their expression of GFP at 3 days following exposure to the DNA-polymer complexes delivered 'naked' or via the gene-activated matrix (GAM) configuration. FuGene 6 and liposome groups demonstrated abundant GFP positive cells after the 3-day incubation period independent of the type of delivery (direct vs. GAM).

27.9 ± 4.5, and 28.1 ± 3.9% for the plasmid DNA containing GFP only, DNA-FuGene 6 complexes, and DNA-liposome complex groups, respectively. BHK cell toxicities in the range of 1–2, 3–4, and 10–15% was observed for the control, FuGene 6, and liposome groups, respectively. The fluorescent micrographs (Fig. 3) of representative wells at the 3-day interval captured prior to cell collection corroborated the transfection efficiency data obtained from the FACStar cell sorter analyses.

The results from Experiment 2 evaluated the impact of moving the DNA-polymer complexes delivered from the porous poly (lactide) GAMs to new wells after intervals of 1, 2, 4, and 7 days. The group exposed to the plasmid DNA containing GFP demonstrated low transfection efficiencies in the 0.8–1.2% range. The GAM containing the DNA-FuGene 6 complexes demonstrated the highest transfection efficiency at day 1 of 22.3 ± 4.1% (Fig. 4). Following the transfer into a new well, the data at the day 2 interval revealed a transfection efficiency of 12.5 ± 1.3%, with diminishing efficiencies demonstrated at days 4 and 7. The GAM containing the DNA-poly(2-ethyl-2-oxazoline) demonstrated relatively modest transfection efficiencies with the highest levels expressed at day 2 of 8.4 ± 2.2% (Fig. 4). The DNA-liposome GAM revealed a dramatic

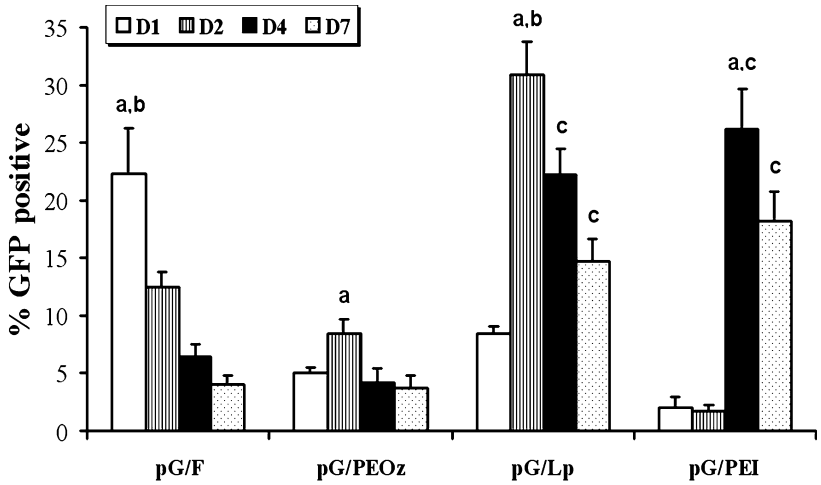

Fig. 4. Percent of the BHK cells expressing GFP as determined by the FACStar cell sorter. The cells were exposed to the DNA-polymer complexes, as well as control groups, and transfection efficiencies were determined following days 1, 2, 4, and 7. The group exposed to the plasmid DNA containing GFP demonstrated low transfection efficiencies in the 0.8–1.2% range (data not shown). The GAM containing the DNA-FuGene 6 complexes demonstrated the highest transfection efficiency at day 1. The DNA-liposome GAM (pG/Lp) revealed increases in transfection efficiencies from days 1 to 2, while the DNA-PEI GAM (pG/PEI) significant increase was from days 2 to 4 interval. The Lp and PEI groups displayed the highest transfection efficiencies at day 7. a = significantly higher than other time intervals within each group. b = significantly higher than other treatment groups at same interval. c = significantly higher than other groups, but equivalent between the pG/Lp and pG/PEI groups.

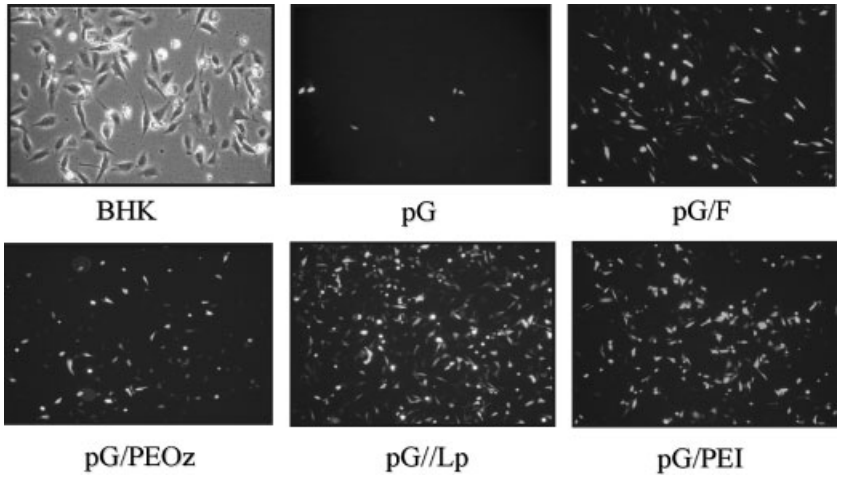

Fig. 5. Representative fluorescent micrographs of the various treatment groups and their expression of GFP at 4 days following exposure to the DNA-polymer complexes delivered with the gene-activated matrix (GAM) configuration. The BHK micrograph demonstrates the cells immediately before the addition of the GAM treatment groups. A greater number of fluorescent GFP positive cells are observed in the plasmid GFP-liposome polymer GAM group (pG/P/Lp) and the plasmid GFP-poly-ethyleneimine (pG/PEI) polymer GAM group.

increase in transfection efficiencies of 8.4 ± 0.9% at day 1 to 30.8 ± 3.1% at day 2 (Fig. 4). Similar to what was observed with the direct application of the DNA-liposomes from the initial experiment, cellular toxicity in the 20–25% range was observed in the day 1 samples. However, by day 2, the toxicity was noted to be significantly lower in the 8–14% range. This group also demonstrated sustained transfection efficiencies of 22.2 ± 2.2 and 14.7 ± 4.1% for the days 4 and 7 intervals, respectively. The DNA-PEI GAM revealed a dramatic increase in transfection efficiencies of 3 ± 1.6% at day 2 to 26 ± 4.8% at day 4. The day 7 interval revealed transfection efficiencies of 18.2 ± 2.6% (Fig. 4). The fluorescent micrographs also (Fig. 5) of representative wells at the 4 -day intervals captured a day before the cell collection corroborated the transfection efficiency data obtained from the FACStar cell sorter analyses.

Discussion

The present studies were undertaken to investigate methods to enhance non-viral-mediated means of gene uptake and expression *in vitro* for use in craniofacial bone regeneration. Several types of DNA-polymer complexes, either applied directly to BHK cells, or released from a porous, resorbable GAM, were evaluated *in vitro* for their ability to transfect cells with a circular plasmid vector DNA construct expressing green fluorescent protein (GFP). Complexes included conjugates containing a lipophilic reagent (FuGene 6), liposomes, poly-ethyl-oxazoline (PEO), and PEI. The results from the present studies indicated that the preferred candidates for future *in situ* delivery in animal models of craniofacial defects could consist of either a DNA-polymer complex containing the liposomes or the PEI fabricated in a GAM configuration.

An alternative to the delivery of high doses of potent morphogens/growth factors (e.g. rhBMP-2) at the osseous wound site could include regional gene therapies. As previously presented, one possibility is a regional, targeted gene therapy utilizing an *ex vivo* adenoviral gene transfer to generate BMP-2-producing bone-marrow cells (17,18). These studies determined critical-sized defects in rats could regenerate with adenoviral gene transfer of autogenous marrow cells delivered and positioned by allogeneic, inactivated demineralized bone matrix (18). However, the authors noted concerns about safety of the adenoviral vector, such as the immunological sequelae (both from the vector and the allogeneic delivery system), and the fate of BMP-transfected cells. Delivery of genetic material into the host wound site via the direct *in situ* delivery has been described with a number of viral vectors, and has similarly been achieved through a localized, direct delivery of non-viral-mediated circular plasmid expression vectors (10,11,19).

One of the developments to deliver plasmid DNA directly to the repair cells involved in fracture repair was previously described as a gene activated matrix (i.e. GAM) (10,11,19). The GAM provides a porous architecture (scaffolding) to promote cell in growth and delivers the plasmid DNA that has been incorporated into the degradable matrix. The local granulation tissue fibroblasts, along with capillaries, migrate into the GAM, uptake and transiently express the local plasmid DNA. The transfected reactive cells secrete the plasmid-encoded factors of interest to stimulate and

augment bone regeneration. As previously described, some of the advantages of delivering pure DNA complexes are the virtually unlimited size of recombinant plasmid constructs, ready availability of low-cost straightforward methods for DNA production, ability to combine DNA with pharmaceutical delivery systems and carriers (20), and the proven safety of pure DNA (11).

Targeted administration of plasmid DNA from GAMs to regenerate bone in ostectomy gaps in rats has been reported (10). The GAMs consisted of lyophilized bovine tracheal collagen incorporating DNA plasmids encoding human parathyroid hormone peptide fragment 1–34 (hPTH1–34) and/or mouse BMP-4. While each plasmid elicited a favorable response of new bone filling the gaps, the GAM implants containing both plasmids resulted in an increase in the rate of new bone formation. Similar results were demonstrated in a canine pre-clinical tibial defect model (11). Bone induction was observed in a stable, reproducible, dose- and time-dependent manner, and reporter gene data demonstrated a 30–50% expression range, a vast improvement for *in situ* transfection efficiencies (11).

A study from our laboratory was undertaken to evaluate the bone induction ability of a BMP-4 plasmid DNA delivered from a GAM device to treat critical-sized cranial defects in adult rat calvaria (21). This study indicated that BMP-4 plasmid delivered from a GAM could stimulate new bone to fill critical-sized rat cranial defects that was superior to the other treatment groups. In a recent study using a juvenile canine model, we hypothesized that alveolar clefts treated in a similar manner with a plasmid BMP-4 GAM (gene therapy) would promote bone healing and canine tooth eruption equal to their autografted counterparts. In this new juvenile canine alveolar cleft model, the plasmid BMP-4 GAM recipients were equivalent to autografting and both were superior to the unrepaired cleft in initial bone regeneration at the 4-week interval. In addition, the plasmid BMP-4 GAM recipients were at least equivalent to autografting and superior to the unrepaired cleft in promoting tooth eruption. The plasmid DNA expression vector for both experiments was identical to that described in the present studies, with the exception that the gene encoding BMP-4 was inserted instead of that for green fluorescent protein.

In addition to improved transfection efficiency, plasmid DNA has a stable, flexible chemistry compatible with polymer-based drug delivery systems (19). In terms of safety-related issues, systemic toxicity from the DNA turnover should not be a concern (22), and quiescent, non-healing tissue should be impacted minimally by plasmid gene transfer (5). Lastly, plasmid DNA is economical and relatively simple to manufacture (23,24). However, care must be taken to avoid unmethylated CpG dinucleotides. Oligodeoxynucleosides containing the unmethylated CpG motif (CpG ODN) are immunostimulatory, can induce production of a wide variety of cytokines and activate B cells, monocytes, dendritic cells and NK cells, and switch on T helper 1 (Th1) immunity (25,26).

The localized delivery of pure DNA, which can be administered locally in a mammalian expression vector, is taken-up by the local reactive cells, and the transgene product is expressed by an epichromosomal action in the host cell. Having local cells secrete factors such as hPTH1-34, BMP-2, -4 or -7, or PDGF-B provides physiologic levels of these potent factors, which should overcome safety issues related to both the relative short half-lives of these molecules, as well as potential safety issues related to massive dosing presently required in the clinical setting (reviewed in Ref. 3). A recent clinical trial is examining a GAM formed from bovine Type-I collagen containing the gene for platelet-derived growth factor B, which has been previously approved for use in treating diabetic ulcers, contained within an adenoviral expression vector (27). The results from this initial trial should provide feasibility data to validate the GAM technology.

Restoring damaged or genetically inadequate bone to a form and function that is equivalent to its pre-injury state is the gold standard for bone regeneration. Although bone tissue demonstrates an ability to regenerate itself in certain conditions, if the injury exceeds a critical limit, regeneration will not occur. Without interventions, these damaged states may continue to exhibit inadequate form and function. A variety of physical techniques, as well as bone grafts and bone graft substitutes, have been developed to overcome complex, potential non-union fractures. Many of these treatments continue to exhibit limitations. An evolving treatment modality, gene therapy, offers an exciting avenue for bone regeneration. The present studies provide some of our strategies for developing preferred candidate GAM implants for a rational gene therapy approach to bone regeneration.

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