



Chemically modified RNA activated matrices enhance bone regeneration



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ABSTRACT

There exists a dire need for improved therapeutics to achieve predictable bone regeneration. Gene therapy using non-viral vectors that are safe and efficient at transfecting target cells is a promising approach to overcoming the drawbacks of protein delivery of growth factors. Here, we investigated the transfection efficiency, cytotoxicity, osteogenic potential and *in vivo* bone regenerative capacity of chemically modified ribonucleic acid (cmRNA) (encoding BMP-2) complexed with polyethylenimine (PEI) and made comparisons with PEI complexed with conventional plasmid DNA (encoding BMP-2). The polyplexes were fabricated at an amine (N) to phosphate (P) ratio of 10 and characterized for transfection efficiency using human bone marrow stromal cells (BMSCs). The osteogenic potential of BMSCs treated with these polyplexes was validated by determining the expression of bone-specific genes, *osteocalcin* and *alkaline phosphatase* as well as through the detection of bone matrix deposition. Using a calvarial bone defect model in rats, it was shown that PEI-cmRNA (encoding BMP-2)-activated matrices promoted significantly enhanced bone regeneration compared to PEI-plasmid DNA (BMP-2)-activated matrices. Our proof of concept study suggests that scaffolds loaded with non-viral vectors harboring cmRNA encoding osteogenic proteins may be a powerful tool for stimulating bone regeneration with significant potential for clinical translation.

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1. Introduction

The significant need for improved therapeutics promoting fracture healing and bone regeneration has led to the introduction and rapid expansion of biomimetic materials in medicine and dentistry [1–8]. One such advancement is the introduction of growth factors or morphogens, such as bone morphogenetic protein-2 (BMP-2) [9–13]. BMP-2 delivered as a human recombinant protein on an absorbable collagen sponge (INFUSE® Bone Graft, Medtronic Spinal and Biologics, Memphis, TN) was shown to be effective in the treatment of patients with degenerative disc disease, bone fractures, as well as oral and maxillofacial osseous defects [14,15]. However, there are a number of drawbacks to using recombinant BMP-2 for both approved and off-label indications [16,17]. In spite of its efficacy, the high cost associated with recombinant

protein therapy, as well as the supraphysiological dosage required to compensate for the short half-lives of these proteins *in vivo* [18], raises serious concerns and strongly underscore the need for alternative approaches. One promising alternative is gene therapy based therapeutics. Gene therapies performed using viral vectors have demonstrated successful delivery of single or multiple transgenes for effective bone regeneration [19,20]. Non-viral gene delivery vectors are relatively safe compared to viral vectors but have lower transfection efficiencies [21,22]. The safety concerns and low transfection efficiencies associated with viral and non-viral gene therapies, respectively, are potential barriers for their clinical translation. Therefore, there is a great demand in both medicine and dentistry to develop novel regenerative strategies that are safe, cost-effective and that could potentially overcome the barriers associated with current protein and DNA based approaches.

Here we propose a novel delivery system with the potential to overcome most of the barriers of protein as well as DNA based therapeutics. Employing inexpensive yet safe biomaterials to embed and release [23] chemically modified ribonucleic acid (cmRNA) in a controlled manner addresses the high cost and safety concerns that exist with recombinant protein and viral based gene therapeutic approaches. By eliminating the need for nuclear trafficking [24] (the ultimate barrier for successful

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transfection in non-dividing cells), cmRNA delivery would potentially address the lower transfection efficiencies associated with non-viral gene delivery systems [25,26] and, since this strategy employs non-viral vectors, it alleviates the immunogenic concern that exists with viral vectors as well [27]. Other advantages include a simpler purification process and greater safety, as mRNA does not integrate into the genome [28]. Furthermore, the *in vivo* approach rather than *ex vivo* transfection will further reduce the treatment cost significantly [29]. Previous murine studies demonstrated the safety and efficacy of cmRNA-based therapeutics to treat lethal lung disease or to prevent allergic asthma *in vivo* [27]. The major limitation of using mRNA is the associated immunogenicity, mediated primarily through toll-like receptors (TLR) - 3, 7 and 8 [30,31]. However, modifying the nucleotides significantly contributed to a reduction in immunogenicity while retaining its function [32]. To the best of our knowledge, this is the first study demonstrating the tissue regenerative potential of cmRNA-based therapeutics. Specifically, synthesized and well-characterized polyplexes of cmRNA and polyethylenimine (PEI) were embedded into collagen matrices which, upon implantation into rat calvarial defects, resulted in enhanced bone regeneration.

2. Materials and methods

2.1. Materials

Branched PEI (mol. wt. 25 kDa), the GenElute™ HP endotoxin-free plasmid maxiprep kit and sodium thiosulfate were obtained from Sigma-Aldrich® (St. Louis, MO). The BMP-2 ELISA kit was purchased from Quantikine® (R & D Systems®, Minneapolis, MN). Plasmid DNA (6.9 Kb) encoding BMP-2 protein driven by cytomegalovirus promoter/enhancer was obtained from Origene Technologies, Inc. (Rockville, MD). The RNA-easy kit was purchased from Qiagen Inc. The TaqMan Reverse Transcription Reagents and 18S-rRNA were purchased from Applied Biosystems (Foster City, CA). Absorbable type-I bovine collagen was obtained from Zimmer Dental Inc. (Carlsbad, CA). Human bone marrow stromal cells (BMSCs) were purchased from American Type Culture Collection (ATCC®, Manassas, VA). Dulbecco's modified eagle medium (DMEM), trypsin-EDTA (0.25%, 1 × solution) and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco® (Invitrogen™, Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals® (Lawrenceville, GA). Gentamycin sulfate (50 mg/ml) was purchased from Mediatech Inc. (Manassas, VA). All other chemicals and solvents used were of reagent grade.

2.2. Preparation of cmRNA encoding BMP-2

To generate templates for *in vitro* transcription, BMP-2 cDNA was cut out of its original vector and subcloned into a PolyA-120 containing T7 pVAX1 (Life Technologies, Madison, WI). Plasmids were linearized with XbaI, following which, its purity was verified and quantified spectrophotometrically. Using MEGAscript T7 Transcription Kits (Life Technologies, Madison, WI) mRNA of BMP-2 was synthesized and capped with the anti-reverse cap analog (ARCA; 7-methyl (3'-O-methyl) GpppGm7G (5')ppp(5')G). To achieve mRNA modification, the following modified ribonucleic acid triphosphates were added to the reaction at a ratio of 25%: 2-thiouridine-5'-triphosphate and 5-methylcytidine-5'-triphosphate (s2U(0.25)m5C(0.25)) as well as pseudouridine-5'-triphosphate and 5-methylcytidine-5'-triphosphate (Ψ(1.0)m5C(1.0)) at a ratio of 100%. Synthesized mRNA was purified and analyzed for size and purity. Once the cmRNA of BMP-2 was synthesized, the degree of immune response to cmRNA was evaluated. Unmodified mRNA and cmRNA of BMP-2 were injected into the peritoneum of BALB/c mice and serum levels of IFN-α (R&D systems, Minneapolis, MN) were measured by ELISA, 24 h post-injection.

2.3. Preparation of pDNA encoding BMP-2

The chemically competent DH5α™ 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 GenElute™ HP endotoxin-free plasmid maxiprep kit and was analyzed for purity using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermoscientific, Wilmington, DE) by measuring the ratio of absorbance (A₂₆₀/A₂₈₀ nm). The concentration of pDNA solution was determined by absorbance at 260 nm.

2.4. Fabrication of PEI-pDNA and PEI-cmRNA polyplexes

PEI-pDNA polyplexes were prepared by adding 50 μL PEI solution to 50 μL pDNA (BMP-2) solution containing 25 μg pDNA and mixed by vortexing for 30 s. The mixture was incubated at room temperature for 30 min to allow complex formation between the positively charged PEI (amine groups) and the negatively charged pDNA (phosphate groups). To achieve optimal transfection efficacies, polyplexes were fabricated using N (nitrogen) to P (phosphate) ratios (molar ratio of amine groups of PEI to phosphate groups in pDNA backbone) of 10 [22]. Similarly, PEI-cmRNA polyplexes at N/P of 10 were synthesized by mixing 50 μL of PEI solution to 50 μL cmRNA encoding BMP-2 containing various amounts of cmRNA for 30 s. For *in vitro* transfection experiments, we utilized PEI-cmRNA polyplexes containing final amounts of 0.2, 0.72 or 1.2 μg of cmRNA (BMP-2). For *in vivo* testing we prepared polyplexes containing final amounts of 25 μg of cmRNA (BMP-2) that was then added to the collagen scaffolds, prior to implantation.

2.5. In vitro evaluation of cytotoxicity of PEI-pDNA and PEI-cmRNA polyplexes at a N/P ratio of 10 in BMSCs

Cytotoxicity of PEI-pDNA and PEI-cmRNA polyplexes on BMSCs, at an N/P ratio of 10 was evaluated using an MTS cell growth assay (Cell Titer 96 AQueous One Solution cell proliferation assay, Promega Corporation). Cells were seeded at a density of 10,000 cells/well in clear polystyrene, flat bottomed, 96-well tissue culture grade plates (Costar®, Corning Inc.) and allowed to attach overnight. The next day, at a cell confluence ~80%, the cell culture medium was changed to serum-free medium and the treatments were gently mixed and added drop-wise into the wells. Each well was treated with 20 μL of polyplexes containing 1 μg of pDNA or cmRNA. Untreated BMSCs were used as controls. Cells treated with PEI alone served as additional controls. To mimic the conditions used in the transfection experiments, the polyplexes were incubated with the cells for 4 h. At the end of the incubation period, the cells were washed with 1 × PBS and fresh complete medium was added. After a total incubation time of 48 h, cells were washed with 1 × PBS and fresh complete medium was added to the cells followed by addition of 20 μL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell growth assay reagent. The plates were then incubated at 37 °C in a humidified 5% CO₂ atmosphere for 3 h. The amount of soluble formazan produced by reduction of MTS reagent by viable cells was measured spectrophotometrically using SpectraMax® Plus384 (Molecular Devices, Sunnyvale, CA) at 490 nm. The cell viability was expressed by the following equation: cell viability (%) = (absorbance intensity of treated cells/absorbance intensity of untreated cells (control)) × 100. Values are expressed as mean ± SD and each treatment was performed in quadruplicate.

2.6. In vitro evaluation of transfection of BMSCs with PEI-pDNA and PEI-cmRNA polyplexes

The bone marrow stromal cells (BMSCs) were plated in 24-well plates at a seeding density of 8 × 10⁴ cells/well 24 h prior to treatments.

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