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Histone-Targeted Gene Transfer of Bone Morphogenetic Protein-2 Enhances Mesenchymal Stem Cell Chondrogenic Differentiation

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Abstract

Skeletal tissue regeneration following traumatic injury involves a complex cascade of growth factor signals that direct the differentiation of mesenchymal stem cells (MSCs) within the fracture. The necessity for controlled and localized expression of these factors has highlighted the role gene therapy may play as a promising treatment option for bone repair. However, the design of nanocarrier systems that negotiate efficient intracellular trafficking and nuclear delivery represents a significant challenge. Recent investigations have highlighted the roles histone tail sequences play in directing nuclear delivery and activating DNA transcription. We previously established the ability to recapitulate these natural histone tail activities within non-viral nanocarriers, improving gene transfer and expression by enabling effective navigation to the nucleus via retrograde vesicular trafficking. Herein, we demonstrate that histone-targeting leads to ~4-fold enhancements in osteogenic bone morphogenetic protein-2 (BMP-2) expression by MSCs over 6 days, as compared with standard polymeric transfection reagents. This improved expression augmented chondrogenesis, an essential first step in fracture healing. Importantly, significant enhancements of cartilage-specific protein expression were triggered by histone-targeted gene transfer, as compared with the response to treatment with equivalent amounts of recombinant BMP-2 protein. In fact, a 100-fold increase in recombinant BMP-2 was required to achieve similar levels of chondrogenic gene and protein expression. The enhancements in differentiation achieved using histone-targeting were in part enabled by an increase in transcription factor expression, which functioned to drive MSC chondrogenesis. These novel findings demonstrate the utility of histone-targeted gene transfer strategies to enable substantial reductions in BMP-2 dosing for bone regenerative applications.

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Conflicts of Interest

The authors declare no competing conflicts of interest.

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Graphical Abstract

Keywords

histones; growth factor gene delivery; chondrogenesis; mesenchymal stem cells; bone regeneration

1. Introduction

Endochondral ossification during fracture healing involves the activation of proliferating mesenchymal stem cells (MSCs) through a series of complex signaling cascades to direct chondrogenic differentiation [1]. Chondrogenic bone induction is initiated by MSCs that invade the defect site and subsequently undergo enhanced proliferation, in preparation for differentiation and ultimately endochondral ossification to regenerate the damaged tissue [2]. Bone morphogenetic protein (BMP) expression, particularly BMP-2 expression [3], plays an essential role in directing early-stage MSC chondrogenesis. Chondrogenesis proceeds within the fracture gap, as chondrocytes mature, proliferate, and secrete an intermediate cartilage matrix. This soft cartilage phase forms the foundation on which mineralization and ossification occur, eventually leading to the reformation of mature bone.

Clinically, the direct delivery of exogenous BMPs have demonstrated promising therapeutic efficacy in accelerating fracture repair [4, 5]. Unfortunately, many complications are associated with this treatment approach, mainly due to the supraphysiological doses and off target effects as the applied protein diffuses into other areas [6]. Induction of *in situ*, localized, on-demand BMP-2 synthesis *via* gene delivery is a compelling alternative treatment strategy with a high potential for clinical success. However, in order for a gene therapy approach to be successfully applied to fracture healing, a robust and appropriately timed initiation of bone repair cascades must be achieved in the defect site [7–9].

Several gene transfer strategies have demonstrated promise in preclinical studies to deliver BMPs and other osteogenic growth factors. For example, viral approaches to deliver growth factors have shown potential to improve the healing response and enhance bone formation [8, 10–12]. Non-viral gene manipulations, including peptide and polymeric nanocarriers, also have shown a capacity to enhance MSC differentiation [13, 14] and/or promote bone formation *in vivo* [15, 16], and these carriers may offer potential benefits in terms of safety and controlled release as compared with their viral counterparts. Despite these accomplishments, existing approaches have on the whole lacked sufficient activity to progress through clinical translation [9, 17]. Specifically, while multiple proof-of-concept studies in rodent and murine models of bone regeneration have been successful, studies in

larger animal models have yielded only moderate success [18]. In addition, achieving effective nuclear delivery for enhanced growth factor expression represents a key challenge in MSC transfection, and non-viral gene transfer in general [19, 20]. These hurdles suggest a need for improved understanding of nanocarrier interactions with biological systems in order to design more effective gene transfer strategies for human application.

Knowledge of the enhanced mitotic behavior of MSCs during bone repair may offer insight into the design of nanocarrier delivery systems with improved efficacy. In particular, mitosis is known to play a key role in enhancing the transfection efficiency of non-viral nanocarriers [21–23], suggesting that the change in MSC proliferative state could be harnessed to locally accelerate growth factor gene transfer in coordination with repair activities. In fact, recent reports have illuminated exciting progress in the design of nanocarrier structures that can effectively navigate the intracellular space and gain access to the nucleus during mitosis [24, 25]. For example, both L-arginine [26] and glycopolymer [27] functionalities have been reported to harness caveolar trafficking and enhance nanocarrier accumulation in the perinuclear region. In addition, the use of transcription factors [28], chromatin interacting proteins [29], and nuclear localization sequences derived from SV40 large T-antigen [30, 31] have been shown to direct nanocarrier nuclear delivery in dividing cells by potentially engaging native nuclear import machinery.

Investigations in our lab have employed histone tail peptides to increase both nanocarrier transport and nuclear deposition coincident with the cell division process. These approaches were inspired by the crucial role of the histone tail regions in native gene regulatory control [32], subcellular trafficking [33, 34], and post-mitotic nuclear deposition [35, 36]. When histone H3 tail sequences were co-assembled with polyethylenimine (PEI) and plasmid DNA to form nanoscale (ca. 120 nm) polyplex-based nanocarriers, the histone H3 tail was shown to significantly enhance transfection efficiency [37] and accelerate transcription [38] in mesenchymal and epithelial cell lines by altering cellular uptake and intracellular processing [39]. In particular, polyplexes containing the H3 tail engaged native histone effectors such as the H3K4 methyltransferase to enhance caveolae-mediated endocytosis and retrograde trafficking through the Golgi, ultimately leading to polyplex accumulation in the endoplasmic reticulum (ER) prior to mitosis [40]. The H3-targeted polyplexes gained improved access to the nucleus during post-mitotic redistribution of the ER by harnessing the native histone chaperone nuclear import machinery [41].

Building off of these fundamental insights, we questioned whether the improved subcellular trafficking and post-mitotic nuclear import properties associated with H3-targeted polyplexes could enhance gene delivery and expression of osteogenic growth factor BMP-2 in MSCs. We further hypothesized that the improved expression of nascent BMP-2 growth factor, correlated with the initiation of fracture healing by MSCs, would function to trigger more robust levels of MSC differentiation along cellular lineages essential to bone repair, not only when compared to untargeted non-viral polyplexes, but also when compared to topical application of exogenous growth factors. Collectively, our data show that H3-targeted polyplexes can achieve enhanced cartilaginous BMP-2 expression over untargeted polyplexes, with expression kinetics similar to the time period of the initial increase in BMP-2 expression during native skeletal repair [42]. Importantly, the levels of

chondrogenesis achieved following H3-targeted gene transfer significantly exceeded the levels achieved following either MSC treatment with an equivalent amount of exogenous BMP-2 protein, or BMP-2 gene delivery using untargeted polyplexes. These novel findings represent the first application of histone-targeted gene transfer for improved MSC delivery and differentiation for skeletal tissue repair and demonstrate the importance of developing improved nanocarrier design strategies to target the nucleus for the advancement of gene therapy in bone defect management.

2. Materials and Methods

2.1 Materials

H3 tail peptides comprised of the mammalian N-terminal H3 residues 1-25 (ARTKQTARKSTGGKAPRKQLATKAA-CONH₂) were purchased from Anaspec (Fremont, CA) at 95% purity. The scrambled H3 (sH3) peptide sequence was designed to incorporate residues 1-25 of the N-terminal H3 tail in a randomized sequence (LSAARPRTAKGARQTKRQKAKGTAK-CONH₂). The peptide was synthesized and purified as previously described [40]. The peptide was synthesized by solid phase peptide synthesis with a Protein Technologies Inc. Tribute series peptide synthesizer (Tuscon, AZ) and a rink amide ChemMatix resin from PCAS-Biomatrix Inc. (Quebec, Canada). Purification was performed by reverse-phase high performance liquid chromatography (RP-HPLC) on a UFLC 20 series instrument from Shimadzu Inc. (Columbia, MD) through a Viva C18 column from Restek (Lancaster, PA). The pCMV6-XL4 mammalian expression plasmid encoding BMP-2 was obtained from OriGene Technologies (Rockville, MD). The gWIZ mammalian expression plasmid encoding green fluorescent protein (GFP) was obtained from Genlantis (San Diego, CA). Both plasmids were amplified in DH5 α *Escherichia coli* in Lysogeny Broth, and purified with a QIAGEN Plasmid Maxi Kit (QIAGEN, Valencia, CA) following the manufacturer's protocols. Recombinant human TGF beta 1 protein was obtained from Abcam (ab50036; Cambridge, MA). Recombinant BMP-2 protein was obtained from GenScript (Z02913; Piscataway, NJ). Primers were synthesized by Eurofins MWG Operon (Huntsville, AL). Primary antibodies used for Western blot included: rabbit anti-MMP13 (54 kDa), Sox 9 (H-90) (56 kDa) and VEGF (45 kDa) and mouse anti- β -actin (45 kDa) (Santa Cruz Biotechnology, Dallas, TX); rabbit anti-COL-X (66k Da) (Abcam, Cambridge, MA); and mouse anti-RUNX2 (60 kDa) (Invitrogen); mouse anti-BMP2 (26 kDa) and goat anti-Col II (N-19) (177 kDa) (R&D Systems, Minneapolis, MN). Horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Cell culture reagents were purchased from Fisher Scientific (Pittsburgh, PA). Branched polyethylenimine (PEI) (25 kDa) and all other reagents were purchased at analytical grade from Sigma (St. Louis, MO).

2.2 Polyplex formation

H3-targeted PEI polyplexes, untargeted PEI polyplexes, and sH3 polyplexes were formed in 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) at a pH of 6 as previously described [37]. For all experiments, polyplexes were formed at an overall N:P ratio of 10. For the formation of H3-targeted (and sH3) PEI polyplexes, the peptide was added first, and an N:P ratio of 6/4 was used, with N = 6 from H3 (or sH3) and N = 4 from

PEI. This corresponds to ~90% (w/w) H3 (or sH3) and 10% PEI in the polycation solution for pDNA complexation. Polyplex structure and amount of peptide within polyplex were analyzed in our prior work by gel electrophoresis, zeta-potential, and RP-HPLC [37, 38].

2.3 Cell culture and transfection

Murine C3H10T1/2 MSCs were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured according to ATCC protocols at 37°C and 5% CO₂ in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For transfection, cells were plated in 12-well collagen-I coated plates at 10,000 cells/cm² in complete growth medium. 24 h later, cells were rinsed with phosphate-buffered saline (PBS) and incubated with polyplexes for 2 h in Opti-MEM. MSCs were transfected at a fixed dose of 1 µg pDNA/cm² well surface area. Cells were subsequently washed with PBS and fresh MEM was added to the cells until a specified time point.

2.4 Flow cytometry and transfection efficiency

Cells were plated in 12-well plates and transfected with polyplexes containing the gWIZ-GFP expression plasmid as stated above. After 24 h, cells were imaged with a Leica 6000 fluorescence microscope (Wetzlar, Germany). GFP expression was quantified on a FACS Caliber Flow Cytometer (San Jose, CA). For cytometry analyses, cells were collected after imaging and prepared for analysis by standard trypsin-mediated protocols. Briefly, cells were rinsed with PBS, incubated with trypsin, pelleted, re-suspended in 300 µl PBS, filtered through a 35 µm nylon mesh to remove aggregates, and stored at 4°C until analysis. Scatter plots were gated for quantification purposes, first by identifying dead cells by changes in light scattering intensity. Dead cells were excluded from analyses of transfection efficiency. Subsequently, the live cell population of the untransfected cell population was gated, and any fluorescence seen from these samples was defined as autofluorescence. Identical live cell gates were applied to the transfected samples and to the fluorescence from these samples. Overall transfection efficiency was taken as the percentage of cells that displayed a level of fluorescence outside of the autofluorescence gate. The mean fluorescence intensity (MFI) of this percentage of (transfected) cells was also calculated. Scatter plots were analyzed for both untransfected cells and cells transfected with identical polyplexes containing a gWIZ-luciferase plasmid (Genlantis, San Diego, CA). This vector served as a non-GFP expressing control for accounting for any shifts in live cell autofluorescence due to polycation presence. A total of 10,000 live cells were analyzed for each cell sample.

2.5 Cell viability and live surface area coverage

Live and dead cells were visualized by fluorescence microscopy as described above, following staining with Calcein-AM and propidium iodide. 24 h post transfection, cells were washed 2X with PBS and incubated in Opti-MEM containing 0.1 % (v/v) Calcein-AM and propidium iodide for 50 min at 37°C. The percent of viable cells was quantified by counting the number of live and dead cells using ImageJ analysis software [43]. Live cell surface area coverage was also quantified using ImageJ analysis software by counting the total number of live cells and scaling to the surface area of the well. Samples were analyzed relative to untransfected controls.

2.6 BMP-2 expression immunoassay

For analysis of BMP-2 expression following polyplex transfection, MSCs were plated and transfected as described above with polyplexes containing the BMP-2 expression plasmid. Every 24 h, 110 μ L of the supernatant was collected, and the expression level of BMP-2 analyzed using a Quantikine BMP-2 ELISA Kit (R&D systems; Minneapolis, MN) according to the manufacturer's instructions.

2.7 Evaluation of chondrogenic differentiation: Alcian blue staining

Following transfection with BMP-2 pDNA containing polyplexes, cells were maintained in serum free MEM media containing 0.2 mM ascorbic acid, 10^{-7} M dexamethasone, 10 ng/mL TGF- β 1, and a 1:100 dilution of ITS+ Premix solution (Corning; Corning, NY). Untransfected recombinant BMP-2 (rhBMP-2) protein control cultures were further supplemented with either 1 ng/mL or 100 ng/mL rhBMP-2. Media was half-refreshed every 3–4 days until the specified time point. Glycosaminoglycan (GAG) deposition following chondrogenic differentiation was evaluated via Alcian blue staining at days 7 and 14 post-transfection or treatment with rhBMP-2. A 1% Alcian blue solution (pH 2.5) was prepared in 3% acetic acid and filtered. Cells were washed 2X with room temperature PBS and fixed in 4% paraformaldehyde for 15 min. Cells were then rinsed 2X with ddH₂O and incubated with the Alcian blue solution in the dark for 45 min at room temperature. Cells were rinsed 3X with ddH₂O to neutralize acidity and imaged on a Zeiss LSM fluorescence microscope fitted with a AxioCam ERc 5s color camera (Jena, Germany).

2.8 Chondrogenic mRNA expression analysis

Gene expression levels of chondrogenic markers were evaluated on days 7 and 14 post-transfection using real-time quantitative PCR (RT-qPCR). Cells were maintained in the culture medium described above, and total RNA was isolated by TRIzol[®] Reagent (ThermoFisher Scientific; Waltham, MA) following the manufacturer's instructions. The iTaq[™] Universal SYBR[®] Green One-Step Kit (Bio-Rad; Hercules, CA) and primers specific to aggrecan (Acan), collagen Type-IIA (COL IIA), matrix metalloproteinase (MMP 13), and collagen Type-X (COL X) (Table S1) were used to prepare samples in triplicate as stated in the manufacturer's protocol. Primers were designed using Primer-BLAST [44]. Both cDNA synthesis and qPCR were conducted on a Bio-Rad CFX96 instrument under the following conditions: 10 min at 50°C; 1 min at 95°C; and 40 cycles of 10 s at 95°C and 30 s at 60°C. Following amplification, a melt curve analysis was performed from 65 to 95°C at 0.5°C increment steps every 5 s to analyze the purity of the product generated. The CT method was used for fold-change analyses [45], using Actin as the endogenous control. All test sample data were analyzed relative to untreated cell data.

2.9 Chondrogenic protein expression analysis

Expression levels of chondrogenic ECM and transcription factor proteins were evaluated on days 1, 2, 7, and 14 post-transfection by Western blot analyses as previously described [46]. In brief, cells were lysed in Mammalian Protein Extraction Reagent (MPER, Thermo Fisher, Waltham, MA), and protein concentrations were measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories Inc.). Approximately 40 μ g of protein were loaded onto each lane of

a sodium dodecyl sulfate (SDS) polyacrylamide gel, and after electrophoresis the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked by incubation in Tris buffered saline (TBS) with 0.05% Tween 20 (Thermo Fisher, Waltham, MA) and 5% Membrane Blocking Agent (GE Healthcare, Buckinghamshire, UK) for 1 h while shaking. The membranes were then incubated with their respective primary antibodies (1:500 dilution) in 2% ECL membrane blocking agent (RPN2125V-GE Healthcare UK limited) diluted in TBS with 0.05% Tween 20 overnight at 4°C. The primary antibody solution was removed and the blots were washed 3X in TBS with 0.05% Tween 20. Subsequently, the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies were applied to the blots, and these blots were incubated for 1 h at room temperature, washed intensively in TBS with 0.05% Tween 20, and reacted with ECL Advanced Detection Reagent System (Amersham, Pittsburgh, PA) for 5 min at 25°C. Detection of the membranes was done using a FujiFilm Intelligent Darkbox (FujiFilm Co., Tokyo, JP). The band intensity of each target protein was quantified using ImageJ analysis software [43].

2.10 Statistical Analyses

Results for all plots are shown as the mean $\pm$ standard deviation of data obtained from at least 3 independent samples. Statistical analyses were performed using Student's *t*-test or one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Histone-targeted transfection efficiency and cellular viability

We first sought to assess the overall transfection efficiency of H3-targeted polyplexes in MSCs by delivering a GFP reporter plasmid and analyzing expression with fluorescence microscopy and flow cytometry (Figure 1). Different H3-targeted polyplex formulations were tested in order to maximize transfection efficiency in MSCs (Supplementary Figure S1). The optimized H3-targeted polyplexes transfected approximately 45% of MSCs in culture, as shown in Fig. 1B. In contrast, untargeted polyplexes (scrambled H3 (sH3) and PEI polyplexes) yielded significantly lower transfection efficiencies, with only ~25% of cells transfected. Complementary to these results, the mean fluorescence intensities of the transfected cell populations were assessed. As shown in Fig. 1C, cells transfected with H3-targeted polyplexes displayed nearly 2-fold and 4-fold higher mean fluorescence levels than sH3 and PEI polyplexes, respectively. These results suggest a more effective utilization of H3-targeted polyplexes for transfection in MSCs.

Phase contrast images from our GFP transfection experiments (Fig. 1A) also suggested significant differences in associated cytotoxicity between H3-targeted polyplexes and untargeted PEI polyplexes, with many fewer cells visible in PEI polyplex-treated cells 24 h post-transfection. To quantify the potential reductions in cytotoxicity resulting from the polyplex treatments, cellular viability analyses were performed using live/dead staining with Calcein AM and propidium iodide (Figure 2). Fluorescent images indicated a significant reduction in cytotoxicity following transfections with H3-targeted and sH3 polyplexes (Fig.

2A), consistent with the literature [37, 40]. Quantification of the live/dead staining revealed a 25% loss in cellular viability following transfection with untargeted PEI polyplexes (Fig. 2B). To further demonstrate the cytotoxicity associated with PEI polyplexes, the number of live MSCs present following transfection was also assessed. As shown in Fig. 2B, transfection with PEI polyplexes resulted in an 80% reduction in live cell surface coverage, relative to untransfected controls. In contrast, live cell surface coverage in MSCs transfected with H3-targeted or sH3 polyplexes remained unaffected.

3.2 BMP-2 expression

Given the significant enhancements in transfection efficiency and biocompatibility achieved with H3-targeted polyplexes in MSCs, H3-targeted delivery of DNA encoding the osteogenic growth factor BMP-2 was examined. Native BMP-2, in its mature form, is secreted into the extracellular space during the initial stages of fracture healing as MSCs enter the fracture site and begin to undergo chondrogenesis [42, 47, 48]. To measure mature BMP-2 expression in the cell culture supernatant, an enzyme-linked immunosorbent assay (ELISA) was employed following transfection with a BMP-2 reporter plasmid. As shown in Figure 3, BMP-2 was expressed in all transfected cell samples. In contrast, untransfected MSCs did not display any significant BMP-2 expression (data not shown). Maximum accumulation of BMP-2 in the cell culture supernatant was observed between day 3 and day 4 post-transfection following all polyplex treatments. Cells transfected with H3-targeted polyplexes achieved a maximum accumulation of approximately 0.9 ng/mL of BMP-2, which decreased to approximately 0.5 ng/mL by day 6 post-transfection. In comparison, cells transfected with both sH3 and PEI polyplexes displayed ~4-fold lower levels of maximum secreted BMP-2 accumulation, and the levels of BMP-2 in these cell samples decreased to approximately 0.05 ng/mL by day 6 post-transfection. These results demonstrated the ability of the H3-targeted polyplexes to not only improve growth factor expression over untargeted polyplexes, but also maintain higher levels of protein in the extracellular space over an extended period of time that was commensurate with the time frame of maximal BMP-2 activity during native repair.

3.3 Chondrogenic differentiation potential

After verifying the ability of H3-targeted polyplexes to improve osteogenic growth factor expression over the course of 6 days, we next sought to determine whether this enhanced and prolonged expression could ultimately augment MSC differentiation and proliferation along chondrogenic cellular lineages. We hypothesized that its increased levels in the supernatant following H3-targeted transfection would result in more robust levels of chondrogenesis when compared to cells transfected with control polyplexes (sH3 and PEI). In addition, we hypothesized that localized, cell-mediated production of BMP-2 by MSCs would induce higher levels of chondrogenesis than the levels in MSCs that received a dose of exogenously applied recombinant BMP-2 protein (rhBMP-2). For these comparative studies, we treated cells with a single dose of rhBMP-2 at a concentration of 1 ng/mL, similar to the maximum concentration of BMP-2 that accumulated in the cell culture supernatant following H3-targeted transfection (Fig. 3). Additionally, to evaluate the maximum activity that would result from a large supply of growth factor, we compared each of these treatments to the result in MSCs subjected to a sustained dose of rhBMP-2 at a

concentration of 100 ng/mL. These dosing schemes also are known to induce chondrogenic differentiation in MSCs [49, 50], and thus were designed to serve as a positive control. A schematic depicting the BMP-2 gene and protein dosing timelines in MSCs is compared with a general timeline of MSC chondrogenic differentiation in Figure 4. All cells were cultured on collagen type I-coated plates to simulate the native conditions that drive MSC aggregation and in preparation for chondrogenic differentiation.

3.4 Chondrogenic extracellular matrix formation

To qualitatively confirm that active chondrogenesis was occurring in MSCs treated with the different BMP-2 gene or protein applications, extracellular proteoglycan deposition was analyzed. Proteoglycans such as aggrecan, decorin, and annexin are produced and secreted by actively proliferating chondrocytes [51], and these proteoglycans help form the intermediate cartilage matrix necessary for mineralization and endochondral ossification to form mature bone [2]. Proteoglycans are heavily modified with glycosaminoglycans (GAGs), which are easily stained with cationic dyes. To that end, Alcian blue staining was used to identify intermediary cartilage matrix formation on days 7, 14, and 21 post-treatment (Figure 5). GAG deposition is indicated if the area surrounding the cells is stained dark blue. For untreated MSCs, no extracellular staining was observed at any time point, and the only staining observable was punctate staining of chromatin within the nuclei of cells. Similarly, MSCs transfected with sH3 and PEI control polyplexes, as well as MSCs treated with a single dose of rhBMP-2 (1 ng/mL), did not exhibit any significant levels of GAG deposition over the course of 3 weeks. In contrast, extracellular GAG deposition following H3-targeted polyplex transfection was observed by day 7, and this staining continued to increase over the 3 week time period, indicating the presence of mature and active chondrocytes. Interestingly, GAG deposition levels on days 7 and 14 following H3-targeted transfection were similar to the levels obtained following repeated treatments with 100 ng/mL rhBMP-2. By day 21, excessive cellular proliferation and nutrient depletion from continuous cell culture in the presence of excess rhBMP-2 prevented effective Alcian blue staining analysis. Overall, these results suggested that H3-targeted gene therapy could trigger more robust levels of chondrogenesis in MSCs when compared to either untargeted gene polyplexes or MSCs treated with equivalent amounts of rhBMP-2. Notably, 100-fold excess rhBMP-2, as compared with the maximum BMP-2 expression levels in H3 transfected cells, was required to achieve similar GAG deposition.

3.5 Chondrogenic mRNA expression

To further confirm the ability of H3-targeted gene transfer to augment chondrogenic differentiation in MSCs, we quantitatively monitored changes in both mRNA and protein expression of specific chondrogenic markers over the course of 2 weeks. We examined the expression of extracellular matrix (ECM) protein markers indicative of either early-stage chondrogenesis (collagen type IIA [COL IIA] and aggrecan [Acan]) or late-stage chondrogenesis (collagen type X [COL X] and matrix-metalloproteinase 13 [MMP 13]). The mRNA expression levels for each of these chondrogenic markers were analyzed on days 7 and 14 post-transfection/treatment (Figure 6) using RT-qPCR with primers specific to COL IIA, Acan, MMP 13, and COL X. mRNA expression in all treated samples was analyzed relative to the mRNA levels expressed by untreated MSCs. Day 7 RT-qPCR analysis of

early-stage chondrogenic markers indicated an approximate 3-fold enhancement in COL IIA mRNA (Fig. 6A) and a 50-fold enhancement in Acan mRNA (Fig. 6B) in MSCs transfected with H3-targeted polyplexes. In contrast, MSCs transfected with untargeted control polyplexes or MSCs treated with equivalent amounts of rhBMP-2 displayed no significant increases in COL IIA or Acan mRNA expression relative to untreated controls. Additionally, both COL IIA and Acan mRNA expression levels had increased by day 14 following H3-targeted transfection to approximately 5-fold and 500-fold the levels in untreated MSCs, respectively, while MSCs treated with untargeted polyplexes and 1 ng/mL rhBMP-2 remained unchanged. Interestingly, by day 14 both COL IIA and Acan mRNA expression levels in cells treated with H3-targeted polyplexes exceeded those levels in MSCs treated with 100-fold excess rhBMP-2.

Similar trends in mRNA expression were observed when analyzing late-stage chondrogenic markers. By day 14, the mRNA expression levels of both MMP 13 (Fig. 6C) and COL X (Fig. 6D) in MSCs transfected with H3-targeted polyplexes had increased significantly, by approximately 20-fold and 200-fold, respectively, relative to the untreated controls. In comparison, mRNA expression levels from control samples treated with 1 ng/mL rhBMP-2 or untargeted polyplexes remained unchanged. Interestingly, by day 14, MSCs transfected with H3-targeted polyplexes exhibited higher levels of COL X mRNA expression relative to MSCs treated with 100-fold excess rhBMP-2, but lower levels of MMP 13 mRNA expression. Expression of both MMP 13 mRNA and COL X mRNA was evident as early as day 7 post-transfection with H3-targeted polyplexes or post-treatment with 100 ng/mL rhBMP-2.

3.6 Chondrogenic protein expression

After verifying that the H3-targeted polyplexes were inducing increased mRNA expression levels of early- and late-stage chondrogenic markers, we next sought to analyze corresponding changes in protein expression using Western blotting (Figure 7). The protein expression levels of COL IIA, MMP 13, and COL X were evaluated in MSCs at day 7 and 14 following BMP-2 transfection or treatment with rhBMP-2, and these levels were calculated relative to the expression levels in untreated MSCs. As shown in Fig. 7A, COL IIA protein expression was significantly enhanced (~1.8-fold increase) at day 7 following H3-targeted transfection, and these COL IIA protein levels were equivalent to the levels in MSCs treated with 100-fold excess rhBMP-2. By day 14, COL IIA protein expression had decreased back to its basal level, consistent with the timing of COL IIA expression during native progression through chondrogenesis (Fig. 4). In contrast, MSCs treated with untargeted polyplexes or equivalent amounts of rhBMP-2 did not exhibit any significant enhancements in COL IIA expression at either day 7 or day 14. Investigation of MMP 13 protein expression showed a significant increase in cells transfected with H3-targeted polyplexes (Fig. 7B) from basal expression at day 7 to a 1.2-fold increase by day 14, equivalent to the expression level in MSCs treated with 100-fold excess rhBMP-2. Again, no significant enhancements in MMP 13 protein expression were observed in MSCs treated with untargeted polyplexes or equivalent amounts of rhBMP-2. As expected, the protein expression patterns of COL X (Fig. 7C) were similar to those of MMP 13. By day 14, COL X exhibited an approximate 1.6-fold increase in expression following H3-targeted

transfection or treatment with 100-fold excess rhBMP-2, relative to untreated MSCs. Interestingly, MSCs treated with a single dose of rhBMP-2, equivalent to the maximum accumulated BMP-2 following H3-targeted transfection, also exhibited a slight increase (1.2-fold) in COL X protein expression relative to untreated MSCs at day 14. However, this fold-increase in expression was significantly less than the increase induced by H3-targeted transfection. Collectively, these data further confirm the ability of H3-targeted gene transfer to trigger robust chondrogenesis in MSCs, by enhancing chondrocyte proliferation from early to late hypertrophic stages in preparation for matrix mineralization and ossification.

3.7 Mechanisms underlying H3-targeted chondrogenic enhancement

The ability of H3-targeted gene transfer to improve chondrogenesis in MSCs as compared with either untargeted polyplexes or equivalent amounts of recombinant protein suggested that a more global change in cellular behavior was occurring in response to H3-targeted BMP-2 expression. We hypothesized that the enhanced MSC-mediated expression of growth factor would result in both improved MSC proliferation/condensation in preparation for differentiation, as well as increased activation of major regulatory factors driving chondrogenesis. To test this, we examined changes in the protein expression of the transcription factors Sox9 and Runx2-related transcription factor 2 (Runx2) induced by either BMP-2 transfection or treatment with rhBMP-2 (Figure 8). Sox9 plays an essential role in activating MSC condensation prior to chondrogenesis [52, 53], and continues to activate genes essential to driving the initial stages of chondrogenic differentiation. Runx2 plays an essential role in driving matrix mineralization and helps to promote chondrocyte hypertrophy [2, 52]. As shown in Fig. 8A, an approximate 1.5-fold increase in Sox9 protein expression in MSCs was observed by day 7 in cells transfected with H3-targeted polyplexes, relative to untreated MSCs. This increase in Sox9 expression was equivalent to Sox9 expression in MSCs that had been treated with 100-fold excess rhBMP-2. In fact, Sox9 transcription factor activation was observed as early as 48 h post-transfection with H3-targeted polyplexes (Supplementary Figure S2). In contrast, Sox9 expression remained unchanged in MSCs following transfection with untargeted polyplexes or treatment with equivalent amounts of rhBMP-2. Similar protein expression trends were observed for Runx2 on day 14 in both H3-targeted transfected cells and 100 ng/mL rhBMP-2 treated cells (Fig. 8B), which displayed approximately 1.5- and 1.4-fold increases, respectively, in Runx2 protein relative to untreated MSCs. Collectively, these data suggest that growth factor expression following H3-targeted gene transfer functions to activate MSC cellular mechanisms that drive condensation and chondrogenesis more efficiently than either untargeted gene transfer or topically applied recombinant protein therapy.

4. Discussion

Non-viral gene therapy possesses enormous potential to improve fracture healing, as the rapidly dividing cells within the fracture site are an ideal target for gene transfection with enhanced efficiency. Local, transient expression of key growth factors by these cells can stimulate the healing response to enable significant reductions in exogenous growth factor stimulation required to initiate bone repair cascades. Furthermore, substrate-mediated gene delivery via localized injection at a potential defect site provides additional benefits as cells

(including MSCs) will naturally invade the matrix and uptake the gene particles for enhanced and localized growth factor expression. However, many non-viral gene polyplexes have a critical limitation of low transfection efficiency. Accordingly, this study sought to address this challenge by utilizing a materials design approach aimed towards improved gene transfer, and ultimately improved growth factor production, to accelerate MSC differentiation and thereby enable faster fracture healing. Our studies document the first examination of histone-targeted polyplexes for bone regenerative applications and demonstrate their utility in triggering more robust levels of chondrogenic differentiation, an essential first step in the bone repair process, as compared with exogenously applied recombinant growth factor proteins. An unprecedented finding was that nearly 100-fold excess recombinant growth factor was required to produce similar levels of chondrogenic mRNA and protein expression, illustrating the importance of well-controlled non-viral polyplex design for the advancement of bone defect management.

GFP transfection studies and cellular viability analyses demonstrated that H3-targeting could enable distinct improvements in gene transfer and reductions in cytotoxicity in MSCs compared with untargeted PEI polyplexes. Closer examination of cellular morphology post-transfection revealed the detrimental cytotoxic effects PEI polyplexes exhibited in MSCs (Fig. 1A). The phase contrast images show a significant reduction in total number of MSCs still adhered to the plate following transfection with PEI polyplexes. This substantial loss in cell surface coverage due to the PEI polymer was reflected in Fig. 2B, showing a 5-fold reduction in the number of live cells post-transfection with PEI polyplexes. Similar expression and viability trends were observed in studies conducted on human bone-marrow derived MSCs (data not shown), indicating a clear feasibility for longer-term applications of H3-targeted polyplexes in humans.

Examination of osteogenic BMP-2 growth factor expression indicated a substantial increase in the levels of BMP-2 secreted into the extracellular space following transfection with H3-targeted polyplexes. BMP-2 remained present in the cell culture supernatant for 6 days (Fig. 3) following a single transfection. This was a surprising result, given the fact that non-viral transfections are transient in nature. Many growth factors, including BMPs, are natively sequestered by components of the ECM, such as proteoglycans, elastins, and collagens, in order to regulate their activity [54]. BMP-2 itself is known to bind collagen [55, 56], which has been used as a means to enhance therapeutic activity of rhBMP-2 via prolonged release [57–59]. Sustained and localized release from ECM scaffolds can greatly enhance the half-life of rhBMP-2 from ~7 min (following systemic administration) to several days [58, 60]. As these experiments were conducted on collagen type I coated culture plates, it is possible that the collagen functioned to sequester the initially expressed BMP-2 to some extent, prolonging its release to the cell culture supernatant. In addition, our previous investigations using histone-targeted polyplexes demonstrated that the H3 tail functioned to enhance polyplex accumulation in the ER [40] and retain polyplexes in the nucleus of cells post-mitosis [23]. This prolonged accumulation/nuclear retention may have helped promote gradual gene expression over the course of several days. Together, these factors could have contributed to sustained BMP-2 expression over a period of 6 days.

The capacity for prolonged BMP-2 accumulation in the cell culture supernatant following H3-targeted transfection prompted us to examine the potential of the expressed BMP-2 to induce chondrogenic differentiation in MSCs. In particular, we sought to demonstrate the advantage of histone-targeted gene therapy by triggering increased chondrogenic differentiation following transfection than the levels achieved via a single equivalent dose of recombinant BMP-2 protein. Analyses of proteoglycan deposition (Fig. 5), mRNA expression (Fig. 6), and protein expression (Fig. 7) of key chondrogenic markers illustrated a clear progression from early- to late-stage chondrogenesis (Fig. 4), consistent with the literature [51], following H3-targeted gene transfer. Early-stage markers included COL IIA and Acan, both of which form the major structural components of the intermediary cartilage matrix secreted by active chondrocytes. Late-stage markers included COL X and MMP 13, both of which indicate fully mature and hypertrophic chondrocyte formation [2]. Acan also continues to be expressed at this later stage [51].

Furthermore, a 1.8-fold increase in vascular endothelial growth factor (VEGF) was observed by day 14 (Supplementary Figure S3), indicating that the mature chondrocytes were beginning to prepare the intermediate cartilage matrix for vascular invasion and ossification by osteoclasts, an essential step for the replacement of cartilage by bone [2, 61]. In contrast, MSCs treated with a single equivalent dose of rhBMP-2 (1 ng/mL) displayed no indications of chondrogenic differentiation, with the exception of a slight enhancement in COL X protein expression on day 14 post-treatment. This considerable discrepancy between gene and protein therapy was partially explained by the absence of any transcription factor activation following rhBMP-2 treatment (Fig. 8). Expression of Sox9 is essential to MSC progression through the early stages of chondrogenesis (Fig. 4) and is responsible for the activation of both COL IIA and Acan genes in proliferating chondrocytes [62]. Similarly, Runx2 is an important driver of terminal chondrogenic differentiation and has been shown to activate MMP 13 and COL X genes in hypertrophic chondrocytes [2, 52, 63]. Without the coordinated expression of these transcription factors, chondrogenic differentiation is not possible. In addition, by delivering the BMP-2 plasmid, we expect that we were able to achieve more natural expression profiles of the fully mature BMP-2 protein, with native post-translational modifications (PTMs) and more similar BMP-2 induction kinetics. BMPs require PTMs to enable their biological stability and activity [50, 64], and hence the absence of appropriate PTMs in topical rhBMP-2 may have contributed to the insignificant levels of chondrogenic differentiation achieved using equivalent rhBMP-2 doses in our investigations. Furthermore, it is likely that the increased cell-cell interactions established during the first few days of culture functioned to amplify both transcription factor and native BMP signaling, since MSC condensation and cellular communication are required for chondrogenic differentiation [51].

Due to the short systemic half-life of rhBMP-2 [65], sustained and localized delivery of excess recombinant growth factor is often required to initiate bone repair cascades in both *in vitro* and *in vivo* models of bone regeneration [4]. To simulate these approaches, we subjected MSCs to repeated doses of excess rhBMP-2 at concentrations known to initiate 10T1/2 MSC chondrogenic differentiation *in vitro* (100 ng/mL) [66, 67]. The amount of rhBMP-2 used in these studies was over 100-fold greater than the maximum accumulation of nascent BMP-2 achieved following a single H3-targeted transfection (Fig. 3). At these

high repeated doses, MSCs in this study were shown to successfully undergo chondrogenic differentiation. In comparing the experimental results from these repeated excess rhBMP-2 treatments to those achieved with a single H3-targeted gene transfection, it is interesting to note the similarities in overall expression levels of chondrogenic markers. Equivalent levels of ECM protein expression (Fig. 7), transcription factor expression (Fig. 8), and proteoglycan deposition (Fig. 5) were observed on days 7 and 14 following repeated excess rhBMP-2 treatment and H3-targeted gene transfer, demonstrating the utility of H3-targeting to enable substantial reductions in growth factor dosing for bone regenerative applications.

It is interesting to note that the mRNA expression levels of COL IIA (Fig. 6A), Acan (Fig. 6B), and COL X (Fig. 6D) remained unchanged from day 7 to day 14 following excess rhBMP-2 treatment, despite the changes that were observed in protein expression of these same factors (Figs. 7A–C). Although there is not always a direct correlation between mRNA and protein expression levels, the unchanged mRNA expression may indicate a difference in cellular behavior in response to excess rhBMP-2 treatment. Studies of MSC chondrogenesis *in vitro* have shown that BMP treatment induces proliferation [51] and drives terminal chondrocyte hypertrophy [2, 68] in a dose dependent manner. Hypertrophic chondrocytes increase in cell volume, degrading the ECM around them, and ultimately undergo cell death in preparation for osteoclast invasion and mineralization [2]. Thus, overstimulation with excess rhBMP-2 likely enhanced the rate of cell death by increasing chondrogenic hypertrophy and promoting over-proliferation which led to cell crowding and depletion of necessary nutrients. These cell culturing effects became especially pronounced by day 21, at which point cellular overcrowding and cell death prohibited effective analysis of GAG deposition with Alcian blue following excess rhBMP-2 (100 ng/mL) treatment. In contrast, GAG deposition was significantly enhanced from day 14 to day 21 following H3-targeted polyplex treatment (Fig. 5), indicating the presence of healthy chondrocytes that were actively secreting and forming a mature cartilage matrix. In the context of fracture healing and endochondral ossification, healthy cartilage matrix formation is critical to ensure mature bone integrity. This further demonstration shows that a histone-targeted gene-based approach mimics the complex signaling cascade required for the natural progression of endochondral bone repair. Our detailed analyses of gene and protein expression on day 7 and 14 confirms the effectiveness of histone-targeted gene transfer to promote the initiation of chondrogenic differentiation; while the analysis of GAG deposition on day 21 provides a functional assay to demonstrate progression of normal chondrogenic behavior. Additionally, it ensures over-proliferation or cell death does not occur as a result of excess recombinant protein expression.

In contrast to MSCs transfected with H3-targeted polyplexes, untargeted PEI polyplexes and sH3 polyplexes did not trigger any significant levels of chondrogenic differentiation in MSCs. The cytotoxicity associated with the untargeted PEI polyplexes likely played a key role in hindering chondrogenesis, as evidenced by the decreased fold-changes in protein expression of certain ECM and transcription factor proteins. Fig. 5 shows evidence of GAG deposition following untargeted PEI polyplex transfection on days 14 and 21. In addition, PEI transfected cells also displayed a minor 1.3-fold increase in VEGF expression by day 14 (Fig. S2). As these MSCs recovered from PEI transfection they likely began to proliferate in isolated “colonies” rather than in a continuous monolayer. Since MSC condensation plays an

important role chondrogenesis, the close proximity of these MSCs likely triggered chondrogenic differentiation, resulting in low observed levels of GAG deposition and VEGF expression. Finally, the insignificant levels of chondrogenesis triggered by sH3 polyplexes strongly indicate that transfection alone cannot drive chondrogenic differentiation, but requires elevated expression of native BMP-2 growth factor protein.

It is important to consider how this histone-targeted gene transfer approach could be applied in the clinical context of tissue regeneration. Localized injection of polyplexes formulated into ECM-mimetic matrices or coated onto various biomaterials deposited into a defect site could be useful in both enhancing regeneration and promoting integration with native tissue. Our lab is working toward establishing the conditions for polyplex nanocarriers to be formulated into matrix solutions and maintain robust activity [69, 70]. So far, these investigations have demonstrated clear benefits in gene localization, activity, and stability when employing substrate-based delivery strategies. In the context of healing bone tissues, it will be important to ensure that scaffold formulations provide adequate polyplex retention and localization to enable desirable expression of BMP-2 and other osteogenic factors.

5. Conclusions

In this study, we observed that H3-targeted gene therapy functions to enhance transfection efficiency and reduce cytotoxicity in MSCs. H3-targeting resulted in a 4-fold enhancement in expression of the osteogenic growth factor BMP-2 compared to untargeted gene polyplexes, and H3-transfected cultures maintained significant accumulation of BMP-2 in the cell culture supernatant over the course of 6 days. As a result of this prolonged expression, H3-targeting improved chondrogenic differentiation potential in MSCs, an important first step in the fracture healing process, over untargeted polyplexes. More importantly, both qualitative and quantitative analyses of chondrogenic mRNA and protein expression demonstrated that a single transfection with H3-targeted polyplexes could trigger more robust levels of chondrogenic differentiation in MSCs when compared to treatments with a single equivalent dose of exogenously applied BMP-2 protein. In fact, a 100-fold increase in rhBMP-2 dosage was required to trigger similar levels of chondrogenic gene and protein expression. Analysis of transcription factor expression indicated that H3-targeted gene transfer functioned to activate the MSC cellular processes that drive proliferation and chondrogenesis more efficiently than topically applied recombinant protein. Collectively, these findings demonstrate the utility of using histone-targeted gene transfer to gain more effective control over growth factor expression and MSC differentiation, and ultimately contribute to the overall advancement of gene therapy in the context of bone defect management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

This contribution addresses significant limitations in non-viral gene transfer for bone regenerative applications by exploiting a novel histone-targeting approach for cell-triggered delivery that induces osteogenic BMP-2 expression coincident with the initiation of bone repair. During repair, proliferating mesenchymal stem cells (MSCs) respond to a complex series of growth factor signals that direct their differentiation along cellular lineages essential to mature bone formation. Although these MSCs are ideal targets for enhanced transfection during cellular mitosis, few non-viral delivery approaches exist to enable maximization of this effect. Accordingly, this contribution seeks to utilize our histone-targeted nanocarrier design strategy to stimulate BMP-2 gene transfer in dividing MSCs. This gene-based approach leads to significantly augmented MSC chondrogenesis, an essential first step in bone tissue repair.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.